PROTEIN STRUCTURE AND DESIGN

Dale Oxender, Organizer

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Poster Sessions

DNA-Protein Complexes - I

N 001 ALTERING THE BINDING SPECIFICITY OF CRO REPRESSOR FOR OPERATOR, M. H. Caruthers, L. P. Bracco, L. Cummins, D. R. Dodds, S. J. Eisenbeis, R. Gayle, P. Gottlieb and A. Hubbard, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309

The recognition of cro repressor for cro operator has been studied by altering the amino acid sequence of cro repressor and the DNA sequence of cro operator. By substituting amino acid changes in the cro repressor domains where specific contacts between protein and DNA have been proposed (the α-3 helix and the carboxyl terminus), we have derived the following conclusions. (1) Amino acids located at positions 27, 28, 32 and 38 (gln, ser, lys and arg, respectively) are essential to the formation of a stable repressor-operator complex. Substitution by any other amino acid, including other potential hydrogen bonding amino acids, leads to complexes that are less stable than wild type cro. This was observed not to be the case at several other amino acid positions within or near the α-3 helix. (2) Insertion of cysteine for valine at position 25 generates a cro repressor having a ten fold higher binding affinity for cro operator (O_R3). (3) The cro carboxy terminus (amino acids 62-66) is important for stabilizing the repressor-operator complex. This was shown initially by sequential removal of these amino acids (-K-K-T-T-A-CO_2H) which generates a series of repressors with reduced affinity for operator. Further evidence supporting this conclusion was also obtained from repressors having additional lysines (-K-K-K-T-A and -K-K-K-K-A) with increased affinity and from repressors having rearranged sequences (-T-T-K-K-A and -T-K-T-K-A) with reduced affinity for O_R3. Further insights regarding the cro repressor-operator interaction were also obtained by changing a presumed hydrogen bonding interaction to one involving hydrophobic contacts. This was accomplished by substituting glutamine at position 27 with leucine or cysteine and changing the operator at the proposed contact site (reference 1) from A-T to the T-A transversion. Thus a contact involving a thymine methyl group and a hydrophobic amino acid side chain was substituted for a hydrogen bonding interaction between glutamine and adenine. Research supported by NIH (GM21120).

1. D. H. Ohlendorff, W. F. Anderson, R. G. Fisher, Y. Takeda, and B. W. Matthews, *Nature* **298**, 718-723 (1982).

DNA-Protein Complexes - II

N 002 STRUCTURE-FUNCTION STUDIES ON THE TRYPTOPHAN REPRESSOR, D.L. Oxender[1], T. Graddis[1], L. Klig[2] and C. Yanofsky[2], University of Michigan[1] and Stanford University[2]. The tryptophan repressor is responsible for negative control of the transcription of the tryptophan biosynthesis genes. The dimer of the aporepressor is activated by tryptophan and the complex binds to the operator region of the trp operon. The gene for the repressor has been cloned and sequenced and mutations representing a large variety of altered repressors have been identified by Yanofsky and co-workers. The repressor has been crystallized and the three-dimensional structure determined by Sigler and co-workers. The availability of mutants and structural information has permitted a study of structure-function relationships for the trp repressor. For this study we selected a group of mutants of the trp repressor which, based on the three-dimensional structural analysis, were predicted to be defective in binding tryptophan. Second-site revertants of these mutants were isolated following in vitro hydroxylamine mutagenesis. DNA sequence analysis identified the location of the second amino acid substitutions that were responsible for partial reversion of the mutant phenotypes. Second site revertants were obtained for five different mutant trp repressors. The major second site mutations found in this study were mutations that produced super-repressor phenotypes when present as the only mutation. For example, a change in the glutamate residues at amino acid 18 or 49 to lysine residues lead to second site revertants for five or three of the primary mutants, respectively. Some of the mutant trp repressor proteins were purified and the tryptophan binding activity was measured by equilibrium dialysis. There was a correlation between repressor activity and trp binding activity. The ability of some of these mutant repressors to form a heterodimer with the wild type repressor has been demonstrated in vitro using gel electrophoresis. Heterodimer formation presumably accounts for the negative complimentation for these mutants observed in vivo. These studies have contributed to our understanding of the relationship of the structure of trp repressor to its function.
(Supported by the American Cancer Society)

N 003 *trp* REPRESSOR: MOLECULAR DETAILS OF ACTIVATION AND OPERATOR BINDING, A. Joachimiak, R. W. Schevitz, R-g. Zhang, C. L. Lawson, Z. Otwinowski, R. Q. Marmorstein and P. B. Sigler, Dept. of Biochemistry and Molecular Biology, 920 East 58th Street, University of Chicago, Chicago, IL 60637.

By contrasting the recently solved and refined crystal structure of the unliganded, and inactive *trp* aporepressor with the structure of the liganded, active repressor (1), we can visualize the structural transition induced by the co-repressor ligand, L-tryptophan, that creates a high affinity recognition surface. The unique dimer interface formed by the three interlocking helices of each subunit (residues 16-64) forms a single globular domain at the center of the molecule that resists tertiary or quaternary structural changes on binding the co-repressor. Flanking this solid central core, two flexible "reading heads" that contain the helix-turn-helix motif form a snug fit with the cognate surface in the operator's major groove. The shape and orientation of each head depends upon a tryptophan molecule wedged between the helix-turn-helix motif and the core. When the tryptophan is removed, the reading heads collapse towards the center of the molecule, changing their shape and orientation; but the central core remains unchanged. The electrostatic field, which is probably the principal "adhesive" between the protein and DNA in both specific and nonspecific interactions, remains approximately the same.

Operator recognition is mediated primarily, but not exclusively, by non-polar interactions. Presumably the free energy of binding gained in sequence-specific interactions through the release of ordered water and counter ions is augmented by enhanced electrostatic attraction caused by a closer fit and locally diminished dielectric constant. Progress will be reported on the structure determination of crystalline repressor/operator complexes.

1. Schevitz, R. W., et al. (1985). Nature **317**, 782-786.

N 004 STRUCTURAL STUDIES OF TWO DNA-BINDING PROTEINS: CAP AND KLENOW FRAGMENT, T. A. Steitz, P. Freemont, J. Warwicker, B. Engelman, S. Schultz, G. Shields, J. Friedman, V. Derbyshire and C. Joyce, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511

Current structural studies of the catabolite gene activator protein (CAP) address the issues of how it recognizes a specific DNA sequence, how the binding of the small molecules cyclic AMP to CAP activates its specific DNA binding and how the binding of this protein to DNA activates RNA polymerase. Recent calculations of the electrostatic charge potential on CAP and model building suggest that the DNA bound to CAP may be more sharply bent than was incorporated into an earlier model for the CAP·DNA complex (1). The sharply kinked DNA bound to CAP produces a larger DNA binding site of 28-30 b.p., consistent with solution data. While CAP·cAMP will not crystallize as a complex with duplex DNA fragments shorter than 22 b.p., co-crystals have been obtained of the CAP·cAMP·DNA complex using either 28 or 29 base-pair duplex DNA. The X-ray diffraction pattern of these 0.5 mm long crystals is different than that obtained from crystals of CAP·cAMP.

The 3.3 Å resolution crystal structure of the Klenow fragment complexed with nucleoside monophosphate (2) is being refined at 2.6 Å resolution and has suggested a number of hypotheses that are being tested by mutagenesis. To establish the function of the large 400 amino acid C-terminal domain the DNA specifying it has been cloned and the protein product purified; it has polymerase but no exonuclease activity (3). Mutant Klenow fragment proteins having altered residues in the expected active site of the 3'-5' exonuclease activity have been made. In one such mutant, Asp 424 has been changed to Ala resulting in altered 3'-5' exonuclease activity. High resolution crystal structure analysis of this mutant protein shows that while it still binds nucleoside monophosphate identically with wild type protein, a metal ion appears to be missing from the active site. The separation of the polymerase and exonuclease active sites by 20-30 Å raises the question as to whether the polymerase active site plays any active role in recognizing misincorporated nucleotides. Large crystals of Klenow fragment complexed with DNA have been obtained that diffract well and are nearly isomorphous with the solved crystal form.

(1) Weber, I.T. and Steitz, T.A. PNAS 81: 3973-3977 (1984).
(2) Ollis, D., Brick, P., Hamlin, R., Xuong, N.G. and Steitz, T.A. Nature 313: 762-766 (1985).
(3) Freemont, P.S., Ollis, D.L., Steitz, T.A. and Joyce, C.M. Proteins 1: 66-73 (1986).

Enzyme Mechanisms

N 005 THE EVOLUTION OF ENZYME FUNCTION. Jeffrey D. Hermes and Jeremy R. Knowles,* Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138.

There are two criteria that an enzyme must fulfill before we may say that it has reached the end of its evolutionary development as a catalyst. First, the transition state of highest free energy must be that for the diffusion 'on' of the less stable substrate (so that the catalyzed reaction is diffusion-limited) and secondly, the most stable ground state at the ambient substrate levels in vivo, must be that of the free enzyme plus the more stable substrate (so that the reaction in vivo runs below saturation). An enzyme system that obeys these constraints cannot become a more efficient catalyst, provided that the reacting species diffuse freely and provided that the ambient substrate levels do not alter. Any changes that are made in such 'perfect' enzymes by the methods of site-directed mutagenesis must either be silent, or deleterious, in catalytic terms. One of many enzymes that appears to satisfy the criteria of catalytic perfection is triosephosphate isomerase, and several changes of active site amino acids have indeed led to mutant enzymes of sharply reduced activity. While the nature of such kinetic damage can be informative in terms of enzyme mechanism, the creation of "imperfect" isomerases also allows us to study the forward evolution of enzyme function. Accordingly, we have taken the gene for a mutant isomerase that is approximately 500-fold less active than the wild-type, have subjected it to heavy random mutagenesis, and have selected for transformants that synthesize less sluggish isomerases. Starting with a mutant enzyme in which the active-site Glu_{165} has been changed to Asp, we have found a second site suppressor mutant that produces an enzyme that is 50-fold more active. The suppressor site has been identified as a change from Ser_{96} to Pro. That is, while the enzyme with Asp_{165} is 500 times less active than wild-type (which has Glu_{165}), the enzyme with Asp_{165} and Pro_{96} is only 10 times less active than wild-type (which has Glu_{165} and Ser_{96}). Our current experiments involve both the kinetic characterization of this pseudo-revertant isomerase, as well as efforts to find, from it, a third-site suppressor of yet higher activity.

N 006 MOLECULAR BASES FOR BACTERIAL RESISTANCE TO ORGANOMERCURIALS, Christopher T. Walsh, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.
Microorganisms acquire resistance to organomercurials and inorganic mercuric salts by enzymic conversion of mercurials to elemental mercury (Hg^{O}). Mercury resistant bacteria have genes organized in an inducible mer operon involved in uptake and processing of toxic mercurials. The two enzymes incoded by mer genes are (a) an organomercury lyase and (b) mercuric ion reductase. We have cloned both genes into overproduction vectors and expressed the lyase and reductase as 3% and 10% of the soluble cell protein respectively and purified each enzyme to homogeneity. The lyase is a 22K monomer with no detectable cofactors or metals and cleaves aryl and aklyl mercuric compounds to Hg^{II} and the parent hydrocarbon. Mechanistic studies in support of an SE_2 type of protonolytic cleavage will be presented for this unique organometallic cleaving enzyme.

The second enzyme mercuric ion reductase is also unusual in its ability to reduce Hg^{II} to Hg^{O} as NADPH is oxidized to NADP. This enzyme is a dimeric flavoprotein with structural homology to glutathione reductase, including a redox active site disulfide, conferring 4 electron redox capacity on the enzyme. Experiments involving site-directed mutagenesis altering either the cys_{135} or cys_{140} partner in the disulfide and analyzing the functional consequences will be presented.

Protein Folding

N 007 BPTI FOLDING MUTANTS, Stephen Anderson*, Cara Berman Marks*, David Powers*, Björn Nilsson+, Hossein Naderi+, Phyills A. Kosen+, and I.D. Kuntz+, *Department of Biocatalysis, Genentech, Inc., S. San Francisco, CA 94080, and +Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143.
We have developed a heterologous E. coli expression system (1) that produces native, correctly-folded bovine pancreatic trypsin inhibitor (BPTI). This system has been used to produce mutants of BPTI that have altered folding properties. Mutants lacking the 14-38 disulfide bond fold readily into the native conformation at 37 degrees; however, at lower temperatures the rate refolding in vitro of these mutants is greatly reduced. Mutants lacking the 30-51 disulfide bond also fold readily into the native conformation, but contrast to the 14-38 disulfide minus mutants, these refold in vitro at rate that is equal to or greater than the rate at which the wild type protein refolds. Attempts are currently underway to characterize the intermediates produced by these mutants during refolding. Other mutants will also be discussed.
(1) C.B. Marks et al. (1986). J. Biol. Chem. 261, 7115.

N 008 SIDECHAIN INTERACTIONS CONTROLLING α-HELIX STABILITY, Robert L. Baldwin, Dept. of Biochemistry, Stanford University Medical Center, Stanford CA 94305.

Intrahelical sidechain interactions are important in stabilizing the α-helix formed by C-peptide (residues 1-13 of ribonuclease A) in aqueous solution. Without such stabilizing interactions, α-helix formation should not be observable in aqueous solution for any 13-residue peptide, according to the Zimm-Bragg equation and using host-guest data for the Zimm-Bragg parameters. Chemically synthesized peptides have been used to analyze the interactions. By varying the charge on the N-terminal residue, it has been found that charged group-helix dipole interactions play a major role in controlling helix stability. Consequently, the effect of a charged group on helix stability depends on its position in the helix and direct evidence for such position effects, which are not included in the Zimm-Bragg model of α-helix formation, has been found. The structure of the helix formed by residues 3-13 in intact RNase A appears to be closely similar to the C-peptide helix. (1) As reported earlier, the endpoints of the C-peptide helix are similar to those in RNase A. (2) ^{1}H-NMR studies of sidechain chemical shifts show changes near Glu 2 as a result of the substitution Arg 10 → Ala, which suggests that the charged sidechains of Glu 2 and Arg 10 interact in the peptide helix as in RNase A. (3) The association constant for binding a C-peptide analogue to S-protein is directly correlated with the extent of peptide helix formation, as expected if the peptide helix is the same as in RNase S. Analogues of the longer peptide (1-15) are used for binding studies. These results support the framework model of protein folding in which a framework of secondary structure is formed before the tertiary structure. They suggest that experiments on peptide helix formation can be used to predict the locations of α-helices in proteins. These experiments are the work of Peter Kim, Susan Marqusee, Colin Mitchinson, John Osterhout and Kevin Shoemaker; the peptides were synthesized and characterized by Eunice York and John Stewart, Department of Biochemistry and Biophysics, University of Colorado Medical School, Denver, CO.

N 009 MUTAGENESIS AND PROTEIN FOLDING, A. M. Beasty, M. S. Gittelman, C. S. Herndon, M. R. Hurle, J. T. Manz, J. J. Onuffer, K. M. Perry, T. Stackhouse, N. A. Touchette, N. Tweedy and C. R. Matthews, Department of Chemistry, Penn State University, University Park, PA 16802.

Mutagenesis is proving to be a powerful tool for investigating the mechanism of protein folding and for probing the nature of the forces that stabilize proteins. Replacement of individual amino acids in the alpha subunit of tryptophan synthase and dihydrofolate reductase has shown that certain residues play key roles in limiting folding; others affect the stability but are not involved in the rate-limiting step. In the case of dihydrofolate reductase, inspection of the x-ray structure suggests a correlation between involvement in secondary structure and the effects on folding and stability. Pairwise amino acid replacements permit one to assess the potential for interaction between the two residues. Nonadditive effects of the replacements on the stability provide direct evidence for such interactions; kinetic studies pinpoint when the interactions are established. Examples of each of these phenomena will be presented.

N 010 CORRELATIONS BETWEEN STRUCTURE AND THERMODYNAMIC STABILITY OF PHAGE T4 LYSOZYME. Tom Alber, Jeff Bell, Sean Cook, Julie A. Nye, Daopin Sun, Keith Wilson, Joan Wozniak and Brian W. Matthews. Institute of Molecular Biology, University of Oregon, Eugene, OR 97403

The lysozyme of bacteriophage T4 lysozyme has been used to investigate the structural basis of the thermodynamic stability of proteins. A collection of temperature sensitive (ts) mutants of this protein has been isolated and characterized to locate residues that contribute to stability. Reversible thermal denaturation experiments show that the ts mutant proteins are at least one to four kcal/mole less stable than the wild-type lysozyme at 42°C. The mutations are clustered in the C-terminal domain of the protein. All types of noncovalent interactions are altered, suggesting that all types can contribute to protein stability. The high resolution X-ray crystal structures of six of the ts mutant proteins provide examples of both localized and dispersed structural changes in response to mutation.

Site directed mutagenesis has been used to make multiple substitutions at selected sites to determine the relative magnitudes of specific interactions. For example, the thermal stabilities and X-ray crystal structures of lysozymes with 13 different substitutions for Thr 157 were determined. The lysozymes that make hydrogen bonds to position 157 are all more stable than the mutants that do not. This correlation between native structure and thermal stability suggests that (1) the hydrogen bonds to Thr 157 make a larger contribution to stability than the interactions of the side chain γ-methyl group and (2) interactions in the folded protein dominate the changes in the free energy of the unfolded states caused by mutations at this site. A similar analysis of the structure and thermal stability of 10 substitutions of Pro 86 shows that (1) some hydrogen bonds in the folded state do not correlate with protein stability and (2) statistical measures of helix forming potential do not predict the effect of mutations at this helical site. One general conclusion from these structural studies of selected and site directed mutants is that the ability to undergo conformational changes makes proteins surprisingly tolerant of amino acid substitutions.

Dynamics and Energetics of Protein Structure

N 011 STRUCTURAL LOCALIZATION OF FUNCTIONAL ENERGETICS USING MUTANTS, Gary K. Ackers, Dept. of Biology, The Johns Hopkins Univ., Baltimore, MD 21218.

A useful strategy for localizing the functional energetics within a protein structure is that of "Mapping by Structure-Function Perturbation" (1). In this method a series of functional perturbations (i.e., deviations of functional behavior from that of the native protein) are mapped against the structural locations of the modified sites, obtained over a range of site locations. The modifications are treated as arbitrary perturbations of structure at specific locations, in contrast to the conventional approach of trying to interpret their local stereochemistry. The map yields information on the structural locations of functional events and on the pathways of coupling within a protein molecule.

This strategy provides a possible solution to one of the most difficult problems of connecting structural changes with functionally-significant events: that of knowing whether a given structural difference observed (say by NMR or x-ray crystallography) in a local part of a macromolecule is really of functional significance. If an observed structural difference has only small energetic consequences in relation to that required for function, or if the local energetic effect is compensated within the molecule (e.g., by entropic effects), the observed structural changes could be ancillary. When the function of interest is an energetic property (i.e., the Gibbs energy of ligand binding), this problem is bypassed by directly mapping functional perturbations against structural locations of the modified sites.

By using this approach with an extensive series of 52 mutant and chemically-modified hemoglobins we discovered that (a) the only structural region where single-site modifications give large perturbations in cooperative free energy is the $\alpha^1\beta^2$ intersubunit contact region. (b) At those sites the magnitudes of perturbations in free energy of subunit interaction are drastically altered by ligation of the four heme sites. It follows that these altered amino acid residues can be used as "thermodynamic reporter groups" to monitor (and map) the pathways of intramolecular "communication" within the tetrameric structure that respond to heme-site ligation. This approach provides much new information on the nature of the cooperative switching mechanism, and is being applied to a number of systems.

1. Ackers, G. K. and Smith, F. R. (1985), Ann. Rev. Biochem. 54, 597-629.

N 012 PROTEIN FOLDING: THREE APPROACHES, D. Eisenberg, R.J. Almassy, C. Janson, M. Gribskov, M. Yamashita, M. Wesson and D.C. Rees, University of California, Los Angeles 90024.

The relationship of the amino acid sequences of proteins to their three-dimensional structure has been studied by three methods. The first is protein crystallography, which has yielded the structure of bacterial glutamine synthetase, a 12-subunit enzyme (M_r = 620,000). Unusual aspects of the protein fold include 12 cylindrical active sites at the subunit interfaces, and a C-terminal "helical thong" that is buried within a neighboring subunit.

The second is the PROFILE method. The profile of a protein fold is a matrix that describes the family of amino acid sequences that fold in a given way. The method can be illustrated with the globin and immunoglobulin families. Any new amino acid sequence can be compared to a profile to determine if it belongs to the family fold of the profile.

The third method consists of energy calculations of folding and ligand association. Conventional energy terms are supplemented by atomic solvation parameters (ASPs) which describe the hydrophobic effect. These ASPs show that metal ions bind to protein sites that are hydrophilic and are surrounded by hydrophobic groups.

N 013 MOLECULAR DYNAMICS SIMULATIONS OF INTRA- AND INTERMOLECULAR INTERACTIONS OF PROTEINS, Terry P. Lybrand and J.A. McCammon, Department of Chemistry, University of Houston, Houston, TX 77004.

Molecular dynamics computer simulation techniques, such as the thermodynamic cycle--perturbation method (1,2), have been used to investigate structural and energetic aspects of substituent modification in proteins and protein-ligand complexes. Relative free energies of binding have been computed for various sugars (e.g. galactose, fucose, arabinose) to the arbinose binding protein and for several antiviral compounds to the coat proteins of human rhinovirus 14. These computer simulation methods not only predict relative free energies of binding for a series of ligands to a protein receptor site quite reliably, but can also provide insight regarding 'atomic resoulution' details of the intermolecular interactions. We are now using the computer modeling methods to assist in the design of new antiviral compounds with improved binding properties. These simulation techniques are also being used to examine the conformational and thermodynamic consequences of amino acid substitution of modification in proteins. Such studies should provide useful information and guidance to aid in the design of proteins with altered function and/or stability proterties.

1) T.P. Lybrand, J.A. McCammon, and G. Wipff, Proc. Natl. Acad. Sci. USA, 83, 833-35 (1986)
2) T.P. Lybrand, I. Ghosh, and J.A. McCammon, J. Amer. Chem. Soc., 107, 7793-94 (1985).

Protein Modification

N 014 CONSTRUCTION AND USE OF NEW E. COLI NONSENSE SUPPRESSOR GENES, Jeffrey H. Miller, Jean-Michel Masson and Lynn G. Kleina, University of California, Los Angeles CA 90024; Jennifer Normanly and John Abelson, California Institute of Technology, Pasadena CA 91125.

Amber suppressor genes have been constructed for use in amino acid substitution studies as well as protein engineering. The genes for a set of E. coli tRNAs were encoded by four to six oligonucleotides which were annealed and ligated into a vector. Each anti-codon was altered to read the amber codon UAG. The suppressor genes are expressed constitutively from a synthetic promoter, derived from the promoter sequence of the E. coli lipoprotein gene. To verify that the suppressors insert the predicted amino acids, the genes were employed to suppress an amber mutation in a protein coding sequence. N-terminal sequence analysis of the resultant proteins reveals which amino acids are being inserted. To date, the suppressors derived from tRNAs inserting the following amino acids have been synthesized and examined: histidine, glutamic Acid, phenylalanine, cysteine, alanine, valine, lysine, glycine, isoleucine, proline, arginine, aspartic acid, asparagine, threonine, and methionine. Strong suppressors that insert the correct amino acid, as defined by protein chemistry, include those derived from histidine, alanine, phenylalanine and cysteine tRNAs. Strong suppressors have also been generated from glutamic acid, isoleucine, valine, arginine and proline inserting tRNAs, but the amino acid inserted has not yet been determined by protein chemistry, although it appears to be correct from genetic criteria. Suppressors derived from methionine and one of the isoleucine inserting tRNAs result in the insertion of glutamine. Also, the suppressor derived from a glycine-inserting tRNA inserts 50% glycine and 50% glutamine.

In summary, by using gene synthesis and additional alterations to increase the efficiency of suppressors, we have created a collection of suppressors that can insert, in turn, up to 15 different amino acids at an amber site. We are employing this collection to examine amino acid replacements in a number of proteins, including the lac repressor.

N 015 EFFECTS OF AMINO ACID SUBSTITUTIONS ON THE DENATURED STATE OF STAPHYLOCOCCAL NUCLEASE, David Shortle and Alan K. Meeker, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

A collection of more than 50 mutant forms of staphylococcal nuclease containing one, two, or three different amino acid substitutions have been analyzed using solvent denaturation to quantitate the stability of the native conformation to reversible denaturation (1). By monitoring the intrinsic fluorescence of the single tryptophan residue at position 140, an apparent equilibrium constant K was determined over a range of urea or guanidinium chloride concentrations C. The data are interpretted using the two-state approximation to obtain the midpoint concentration Cm and the derivative $m = d(\log K)/dC$, a quantity which reflects the differential interaction of the denaturant with the native and the denatured states. A number of amino acid changes were found which resulted in sizeable reductions in m, whereas a few others resulted in significant increases. Similar quantitative changes in the value of m were found for a number of other denaturants (e.g., potassium thiocyanate, lithium bromide, n-propanol, etc.). One plausible explanation for these altered patterns of denaturant action is that amino acid substitutions can modify the solvent-accessible surface area of the denatured state, possibly via chain-chain interactions involving hydrophobic groups.

In an effort to characterize these putative changes in the "statistical structure" of the denatured state, model systems of the denatured state which consist of large fragments of nuclease lacking either a few carboxy-terminal or amino-terminal amino acids are being developed. These fragments exhibit CD spectra that suggest most of the secondary structure found in the native state has been lost. Preliminary gel filtration data demonstrate significant changes in the Stokes radius of the 1→136 fragment upon changing one or two amino acid residues.

(1). D. Shortle and A.K. Meeker. PROTEINS: Structure,Function,Genetics 1:81-89 (1986).

N 016 STRUCTURE-FUNCTION STUDIES ON YEAST HEME PROTEINS USING IN VITRO MUTAGENESIS, Michael Smith, Robert L. Cutler, David B. Goodin, A. Grant Mauk and Gary J. Pielak. Department of Biochemistry, Faculty of Medicine, University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia, Canada V6T 1W5. Oligonucleotide-directed mutagenesis is an ideal technique for protein structure-function studies in cases where questions about the functional role(s) of specific amino-acid residues are being asked. Recent improvements in the methodology (T.A. Kunkel, 1985) has made oligonucleotide-directed mutagenesis into a very efficient and convenient and routine procedure. Two studies on yeast heme proteins will be discussed to illustrate this point. In the case of iso-1-cytochrome c, the functions of conserved amino-acid residues and defining the properties of the mutant proteins. These studies (in collaboration with N. Liang and B.M Hoffman) have demonstrated a specific role of Phe-87 in electron transfer and further studies on the crystal structures of the mutant proteins (in collaboration with G. Louie and G.D. Brayer) are correlating changes in amino-acid residues with protein structure. In the case of cytochrome c peroxidase, studies have been directed at examining the possible role(s) of two amino-acid residues, Trp-51 and Met-172, in the formation and structure of the hydrogen peroxide-activated form of the enzyme.

Peptide Design and Structural Predictions

N 017 THE DESIGN, SYNTHESIS, AND CHARACTERIZATION OF A FOUR-HELICAL BUNDLE PROTEIN, W. F. DeGrado, S . P. Ho, P.C. Weber and Z. R. Wasserman, E. I. du Pont de Nemours & Company, Central Research & Development Department, Wilmington, DE 19898; and D. Eisenberg, Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA 90024.

Proteins consisting of the 4-α helical bundle motif are fairly common in nature (Weber & Salemme; Nature 287: 82-84, 1980). They consist of four helices which pack in an antiparallel manner and at an angle of about 20° to one another. An idealized representation of such a protein can be described in terms of nine modular units:

N Terminus-Helix-Loop-Helix-Loop-Helix-Loop-Helix-C Terminus

Although the naturally occurring proteins have great diversity in function and possess little sequence homology, their tertiary structures as determined by crystallography are strikingly similar. Common forces must therefore be acting on these diverse polypeptide sequences to produce similar packing arrangements. We postulate these forces to be largely hydrophobic and entropic in nature. Through careful consideration of such first principles, we should be able to design a protein which has the aforementioned tertiary structure. As mentioned previously, such proteins can be broken up into 9 modular units. In our incremental approach (Fig. II), we first designed and optimized the sequence of an amphiphilic α-helix using glutamic acid, lysine and leucine residues only. Such peptides tetramerize in solution, evidence for which comes from gel permeation chromatography experiments and concentration dependent circular dichroism studies. The best of these peptides has a ΔG° (tetramerization) of around -25 kcal/mol. Building on the optimized alpha helices, we next designed a short sequence of amino acids to link two helices together in a hair-pin loop. The second of our two designs met with success so that now dimerization of Helix-Loop-Helix peptides gives the 4 helical bundle tertiary structure. Efforts are now underway to synthesize and characterize a single polypeptide (about 75 amino acids) consisting of Helix-Loop-Helix-Loop-Helix-Loop-Helix.

N 018 DESIGN OF PEPTIDES FROM HORMONES TO ENZYMES, E.T. Kaiser, Lab of Bioorganic Chemistry and Biochemistry, The Rockefeller University, New York, NY 10021,

The systematic design of protein tertiary structure poses a formidable challenge in protein engineering. Enroute to our eventual goal of designing tertiary structure several years ago we began to construct models for a variety of surface active peptides and proteins where to a first approximation tertiary structure can be neglected. The molecules for which we have built successful models based on secondary structural characteristics include apolipoproteins A-I and B, melittin, a host of peptide hormones such as calcitonin, calcitonin gene-related peptide, B-endorphin and growth hormone-releasing factor, the signal region of E. coli alkalinephosphatase, and the chemotactic peptide platelet factor 4. Part of our current effort is focussed on the total synthesis of two enzymes, a methotrexate resistant dihydrofolate reductase and ribonuclease T_1, as well as of structural analogs of these enzymes. Among the problems we are attacking is the question whether or not we can redesign whole secondary structural regions of the enzymes, while maintaining proper folding and catalytic activity.

Membrane Proteins

N 019 THE PHOTOSYNTHETIC REACTION CENTER FROM RPS. VIRIDIS; H. Michel, J. Deisenhofer, K.A. Weyer, O. Epp, and F. Lottspeich
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The photosynthetic reaction center (RC) from the purple bacterium Rhodopseudomonas viridis contains four protein subunits (L, M, H, and a cytochrome), and various pigments. Well-ordered three-dimensional crystals could be obtained (1). An X-ray structure analysis allowed the calculation of an electron density map at 3 A resolution, and a model for the chromophore arrangement was derived (2). The photosynthetic pigments are found in the central part of the complex. The arrangement of the pyrrole ring systems shows twofold symmetry. The polypeptide chains could be traced (3). Concomittantly the amino acid sequences of the protein subunits were determined by DNA and protein sequencing (4,5). They were incorporated into the atomic model of the RC. The L and M subunits form the central core of the RC and interact with the photosynthetic pigments. They show very similar protein folding containing five membrane spanning helices each. The H subunit is anchored to the membrane by an amino-terminal helix, the remainder forms a globular domain on the cytoplasmic side. The cytochrome is anchored to the membrane by covalently linked fatty acids which are not visible in the electron density, and it is attached to the L and M subunits on the periplasmic side by polar interactions. General aspects of membrane protein structure, like the distribution of charged residues, will be discussed. Differences in pigment-protein interactions (6) may explain, why only one of two branches of pigments is used for light-driven electron transfer.

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(2) Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H., (1984) J. Mol.Biol. 180, 385-398
(3) same authors as (2) (1985) Nature 318, 618-624
(4) Michel, H., Weyer, K.A., Gruenberg, H., and Lottspeich, F. (1986) EMBO J. 4, 1667 - 1672
(5) Michel, H., Weyer,K.A., Gruenberg, H., Dunger, I., Oesterhelt, D., and Lottspeich, F., (1986) EMBO J.5, 1149 - 1158
(6) Michel, H., Epp, O., and Deisenhofer, J., (1986) EMBO J.5 in press

Computer Graphics Lecture

N 020 UNDERSTANDING PROTEIN ARCHITECTURE THROUGH SIMULATED UNFOLDING, Richard J. Feldmann, Division of Computer Research and Technology, National Institutes of Health, Bethesda, Maryland 20892.

The construction of a model of protein from its amino acid sequence is one of the important unsolved problems facing molecular biology. The success of DNA sequencing techniques means that there is an exponential growth in the number of DNA sequences and hence protein sequences. Thinking about the folding of a protein from its primary structure has focused on the use of molecular mechanics programs to eventually go from a random starting configuration to a folded structure comparable to the crystal structure of the protein. Recently Bernard Brooks and I developed a new way of representing the peptide backbone of a protein as a smooth wire in three dimensional space. The representation is calculated using CHARMM running on the Apollo network and the Star Technologies ST-100. When this representation was applied to all the proteins in the Brookhaven National Laboratory's Protein Data Bank a really startling insight occurred. It is possible in this representation to begin to think about logically and graphically unfolding any or all the proteins from the crystal structure backwards to the unfolded state. The core nucleating events in the folding of the protein become readily apparent. This will in turn enable us to write a set of rules to direct the folding thus short circuiting the unrealistically large amounts of computation needed by the direct methods. A set of stereo slides using this smooth wire representation has been prepared and distributed. This paper will focus on interpreting these images and extracting primitive rules about protein folding.

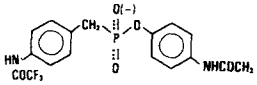
Viruses and Antibody Structures

N 021 THE THREE-DIMENSIONAL STRUCTURE OF POLIOVIRUS: ITS BIOLOGICAL IMPLICATIONS, James M. Hogle and David J. Filman, Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037.

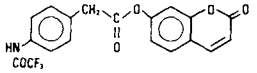
We have solved the structure of the Mahoney strain of type 1 poliovirus at 2.9 Å resolution. The alpha carbon model has been interpreted in terms of its implications for the role of post-translational cleavage in the assembly of the virion, and the location of the antigenic sites. We have recently completed a complete atomic model for the structure which is currently being refined. We anticipate that this model will be a rich source of information for the design and interpretation of experiments with polio and related viruses. Several examples of experiments currently in progress will be discussed. 1) Upon examination of our high resolution maps and models we noted a feature in the electron density maps which could not be assigned to capsid protein. This density could be reasonably fit with a C16 to C18 hydrocarbon. The site is of particular interest since a nearly identical site has been found by Rossmann and his colleagues to be the binding site in rhinovirus 14 for a class of drugs which stabilize the virus against heat denaturation and inhibit infection by preventing uncoating. In collaboration with Dr. Marie Chow we have been investigating the possibility that cellular lipid occupies this site. While the in vitro labelling experiments have failed to find the hydrocarbon in the mystery site, they have serendipitously shown that VP4 and the precursor VPO of polio and several other Picornaviruses are myristoylated apparently at their amino termini. 2) We have begun to investigate the structure an altered form of the virus which is induced by attachment to the cell. The transition is blocked by the antiviral drugs described above and is apparently required for cell entry and virus uncoating. Using antibodies against synthetic peptides and proteolytic probes Carl Fricks in our laboratory has shown that this form of the virus has exposed the amino terminus of VP1 which is normally inside the virus (it has long been known that this form of the virus has also released VP4). The exposure of the amino terminus of VP1 results in an increased lipophilicity of the virus and permits attachment to liposomes. 3) In collaboration with Dr. Philip Minor we are investigating the point mutation which confers temperature sensitivity on the Sabin strain of type 3 poliovirus, and the location of second site mutations in several neurotropic revertants isolated from vaccine associated cases of type 3 poliomyelitis which result in a return to a non temperature sensitive phenotype. We have found that the ts mutation and all of the second site reversions are located in the interface between fivefold related protomers. We are currently collecting high resolution diffraction data from superb crystals of Sabin 3. This structure will be used to initiate a series of experimental and molecular modeling studies design to investigate temperature sensitivity in type 3 poliovirus.

N 022 ANTIBODIES AS ENZYMIC CATALYSTS, Alfonso Tramontano, Kim D. Janda and Richard A. Lerner, Department of Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

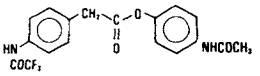
Monoclonal antibodies have been elicited to haptens in which an organophosphorus moiety imparts the stereoelectronic properties of an esterolytic transition state for analogous carboxylic esters. Some of these antibodies were found to react specifically and stoichiometrically with activated esters that share certain structural features with the hapten. This is explained as a combining site directed acyl transfer, facilitated through the stabilization of a tetrahedral intermediate or transition state by antibody-ligand binding interactions. Studies on the effect of chemical modification of amino acids in the protein implicate the involvement of a histidine and a tyrosine residue of the combining site in the acyl transfer mechanism. One of these residues is presumed to be acylated in the reaction.[1] These same antibodies are also shown to behave as enzymic catalysts with the appropriate ester substrates. These substrates are distinguished by the structural congruence of both hydrolysis products with haptenic fragments, as well as the correspondence of the acyl center with the phosphono group of the hapten. Mechanisms are proposed to account for the divergent chemical behavior of these esters with the antibodies. These are based on the difference in leaving group ability between the hydrolysis substrates and esters which covalently combine with the antibody. The antigenic phosphonates are potent inhibitors of this reaction in accord with their assigned role as transition state analogs. These experiments demonstrate that antibody-antigen binding may be directed to chemical processes, according to the prevailing theory which relates binding energy to enzyme function. The generation of artificial enzymes through transition state stabilization by antibodies has long been expected as a corollary to Pauling principle catalysis. These observations provide evidence towards the fulfillment of that prediction.



Haptenic Ligand



Stoichiometric Reagent



Esterolysis Substrate

1. A. Tramontano, K.D. Janda, R.A. Lerner, Proc. Natl. Acad. Sci. (USA) 83:6736-6740, 1986.

N 023 SPECIFICITY OF ANTIBODY-ANTIGEN INTERACTIONS. A.Amit, G.Bentley, G.Boulot, V.Guillon M.Harper, R.Marriuzza, R.Poljak and C.Rojas. Immunologie Structurale, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris, FRANCE.

The ability to produce monoclonal antibodies against a given antigen provides an excellent means to study systematically those factors responsible for the high specificity of antibody-antigen interactions. Recent structural and immunochemical work in our laboratory has been directed towards this end using hen egg lysozyme (HEL) as an antigen. Lysozyme was chosen not only because of its well characterised 3-D structure and antigenic properties but also because natural variants may be readily obtained from other avian species to test the fine specificity of the antibody-antigen interactions.

The X-ray crystal structure of the complex formed by HEL and the Fab D1.3 has revealed a large complementry interface between the two proteins which cannot readily accommodate heterologous antigens. Thus, a single amino acid change at position 121 of the lysozyme interface renders binding between D1.3 and lysozymes from some other species undetectable. Subtle differences in the affinities of anti-HEL Fabs for different lysozymes may be observed, however, as in the case of the Fab D11.15 which binds pheasant egg lysozyme more strongly (heteroclitic antibody).

The conformation of HEL shows no significant change upon binding to D1.3, and immunobinding studies with other anti-HEL Fabs strongly suggest that this observation may be generally true. Indeed, the preservation of the native conformation of antigenic proteins may be an important factor in regulating the immune response by anti-idiotype antibodies which bear the 'internal image' of the antigen. With this in mind we have begun a structural study of a complex formed between D1.3 and the Fab fragment from its antibody.

Enzyme and Protein Recruitment

N 024 TAILORING ENZYMATIC PROPERTIES THROUGH MULTIPLE MUTATIONS, David A. Estell, Thomas P. Graycar, Brian C. Cunningham and James A. Wells, Genencor, Inc. and Genentech, Inc., South San Francisco, CA 94080.

One of the key questions in comparing two evolutionally related proteins is how differences in protein sequence relate to differences in the properties of the proteins. The subtilisins from B. licheniformis (subtilisin Carlsberg) and B. amyloliquifaciens (subtilisin BPN') have different substrate specificities and can differ by as much as 10 fold in turnover number, depending on the substrate. Although the sequences of the two enzymes differ in 86 out of 275 positions, we have found that the properties of subtilisin BPN' can be made to resemble those of the Carlsberg enzyme by making mutations at only three of the 86 positions. Replacement of Glu156, Gly169 and Tyr217 of subtilisin BPN' with the corresponding residues of subtilisin Carlsberg (Ser156, Ala169 and Leu217) results in an enzyme with kinetic properties and performance that are essentially undistinguishable from those of subtilisin Carlsberg.

N 025 PROTEIN ENGINEERING OF HUMAN α_1-ANTITRYPSIN AND SUPEROXIDE DISMUTASE: ALTERATIONS IN STABILITY AND ACTIVITY, Robert Hallewell, Philip Barr, Ian Bathurst, Lawrence Cousens, Guy Mullenbach, Steven Rosenberg, Robin Carrell[1], Peter George[2], Elizabeth Getzoff[3], John Tainer[3], James Travis[4]. Chiron, Emeryville, CA. 94608, [1]Cambridge University, U.K., [2]Christchurch Hospital, New Zealand, [3]Scripps Institute, La Jolla, CA. 92307, [4]University of Georgia, Athens, GA. 30602.

Human α_1-antitrypsin (α_1-AT) is a serine protease inhibitor whose main physiological function is to protect the lower respiratory tract from proteolysis by neutrophil elastase. When levels of functional α_1-AT in blood are reduced due to genetic deficiency or oxidative inactivation (mediated by neutrophils or cigarette smoke) an increased suceptibility to premature lung aging and emphysema is observed. The specificity and regulation of α_1-AT activity resides primarily in a short exposed segment of amino acids (residues 348-359) which functions as a substrate analogue to trap and inactivate target enzymes. By measuring rate constants for several α_1-AT mutants purified from yeast we have shown that much of the specificity of the inhibitor resides at the Met^{358} position. For example an excellent thrombin inhibitor can be made by substituting Arg at this position. Oxidation of the Met^{358} by neutrophil H_2O_2 is the probable mechanism for local inactivation of α_1-AT allowing inflammatory foci to form. Under some conditions oxidative inactivation can lead to disease states such as emphysema and adult respiratory distress syndrome. Derivatives of α_1-AT with Ala^{358} or Val^{358} residues will be described which inactivate neutrophil elastase as efficiently as Met^{358} and which are resistant to oxidative inactivation.

Human Cu,Zn SOD (HSOD) is a dimer of two identical subunits of 153 amino acids whose main function is to protect cells from the toxic effects of the superoxide radical ($O_2^{\cdot-}$). Superoxide dismustases have therapeutic applications because they are antinflammatory and prevent post-ischemic damage. The HSOD monomer consists of a Greek key β-barrel to which the Cu is liganded by 4 histidines and from which two functionally important loops emerge. One loop binds the Zn and the other is involved in electrostatic channelling of $O_2^{\cdot-}$ towards the $O_2^{\cdot-}$ pocket adjacent to the Cu ion. During SOD catalysis the Cu is reversibly oxidised and reduced by successive encounters with $O_2^{\cdot-}$. Electrostatic channelling is thought to account for the high rate constants for both reactions ($2x10^9\ M^{-1}\ s^{-1}$) which are probably faster than the diffusion controlled limit. We have investigated the function of the species conserved residues (Thr^{137}, Arg^{143}) forming the $O_2^{\cdot-}$ pocket by substitution at both positions with all 19 amino acids. Our results indicate that both residues are important for SOD activity. Replacement of the positively charged Arg^{143} with smaller uncharged residues (Ala^{143}, Ile^{143}) reduces the specific activity about 10-fold whereas replacement with negatively charged residues (Gly^{143}, Asp^{143}) reduced activity more than 100-fold. Replacement with the positively charged Lys^{143} reduces the specific activity only 2-fold. These results indicate that Arg^{143} has an important function in local electrostatic attraction of the $O_2^{\cdot-}$ radical.

N 026 CREATION OF CHIMERIC PROTEINS TO ALTER CATALYTIC SPECIFICITY: β-LACTAMASE D, D-CARBOXYPEPTIDASE, John Richards, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

β-Lactamases have been proposed to be evolutionary relatives of D,D-carboxypeptidases-transpeptidases (such as PBP5 from E. coli or R61 from Streptomyces) that cross-link the bacterial cell wall. This kinship is apparent both in amino acid sequence homologies and in three-dimensional structural similarities between the two classes of enzymes. Our objective has been to change a β-lactamase (RTEM-1), which has no detectable D,D-carboxypeptidase activity, to an enzyme into which such activity has been recruited. To this end we have explored a number of mutants involving changes of one or two amino acids in the region of the active site serine to create sequences typically found in D,D-carboxypeptidases; these simple mutants still provide phenotypic resistance to penicillin antibiotics, many are significantly less stable thermally and toward proteolysis than the parent β-lactamase and none showed any carboxypeptidase activity.

With the idea in mind that introduction of much larger stretches of peptide chain with a sequence characteristic of a D,D-carboxypeptidase may be necessary to cause the desired change in catalytic specificity, we created a chimeric protein containing a 30 amino acid insert from the PBP5 enzyme (M.W. 44,500) in place of a 29 amino acid region of RTEM β-lactamase (M.W. 28,500); the region spans the active site serine*.

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                                  50          *       78
β-Lactamase:                      -DLNSGKiLes-frpeeRFpmmStfKvllcG-
PBP5 Sequence:                    -DLNSGKvLaeeqnadvRrdpaSltKmmtsG-
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The changes involve 18 point mutations plus an insertion and creates a mutant that differs in 7% of its amino acids from the parent β-lactamase. The mutant protein does not confer phenotypic resistance to penicillin antibiotics (the activity of the purified mutant toward ampicillin is less than 10^{-5} that of the wild type enzyme). The chimeric mutant is a relatively stable protein, and most interestingly given our objective, that has indeed acquired considerable D,D-carboxypeptidase activity having about 3% the activity of PBP5 toward diacetyl L-lys-D-ala-D-ala.

This provides an example of how on an essentially β-lactamase skeleton one can recruit the catalytic specificity of a much larger, but evolutionarily and structurally related enzyme. It also provides a protein into which further random mutations can be introduced to enhance the D,D-carboxypeptidase activity.

N 027 STRATEGIES FOR NOVEL AND HIGHER ENZYME ACTIVITIES, Paul Schimmel, Lynne Regan Matthew Toth, and Ruth Starzyk, Department of Biology, Massachusetts Institute Institute of Technology, Cambridge MA 02139.

Three systems are under development to explore the potential for selection and design of enzymes with higher or novel activities. These are applied to enzymes--certain aminoacyl tRNA synthetases--for which there is, as of yet, little three dimensional structural information. The emphasis, therefore, is on development of general approaches which can be applied to enzymes regardless of whether detailed structural information is available. We have made gene constructions where the activity of an enzyme is reduced to a level that is insufficient to assure cell survival. Random mutagenesis, directed to the part of the coding region known to encode active site amino acids, yields mutant enzymes which enable cell growth. One mutant has been characterized in detail; it has a higher intrinsic catalytic activity because of enhancement of specific kinetic parameters. A second selection system has been designed to select for changes in amino acid specificity. Phenotypes consistent with specificity alteration have been obtained and mutant enzymes are now being characterized further. A third system explores the potential for building chimeric enzymes which have novel activities. This is attempted by making polypeptide constructions in which the reactive intermediate from one enzyme is brought into proximity of a substrate that is normally bound to a different enzyme. Reaction of the reactive intermediate with the substrate then yields a novel product. Through a variety of polypeptide constructions, we have ascertained where stable connections can be made between domains of two different enzymes. A few stable chimeras have been made and are under investigation for production of a novel product.

N 028 ALTERATION OF SUBSTRATE SPECIFICITY OF SUBTILISIN BY PROTEIN ENGINEERING

James A. Wells,* David B. Powers,* Brian C. Cunningham,* Richard R. Bott,* Thomas P. Graycar,+ and David A. Estell,+. *Department of Biocatalysis, Genentech, Inc. and +Research Department, Genencor, Inc., South San Francisco, CA.

Substrate specificity is determined by a complex and poorly understood mixture of chemical binding forces that include steric and hydrophobic effects, hydrogen bonding-electrostatic interactions. Replacement of two residues in the substrate binding cleft of subtilisin, Gly166 and Glu156, separately and in combination has allowed us to begin to quantitate the contribution of these forces to substrate transition-state binding. Bulky substitutions in the binding cleft can diminish binding of large substrates while retaining affinity for small substrates (1). Hydrophobic substitutions can increase affinity for hydrophobic substrates. Charged substitutions increase affinity for complementary charged substrates while lowering affinity for similarly charged substrates (2). Thus, it is possible to "recruit" new enzyme specificities with increased affinities into substilisin. Quantitative evaluation of chemical forces by protein engineering should provide a data base for rational design of ligand and substrate binding sites.

(1) D. A. Estell, T. P. Graycar, J. V. Miller, D. B. Powers, J. P. Burnier, P. G. Ng and J. A. Wells (1986) Science 233 659-663.
(2) J. A. Wells, D. B. Powers, R. R. Bott, T. P. Graycar and D. A. Estell (1986) Submitted.

N 029 TRANSPLANTING BINDING SITES IN ANTIBODIES,

Alexander R. Duncan, Jefferson Foote, Peter T. Jones, Martine E. Verhoeyen and Greg Winter, Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England.

We have tried to "humanise" mouse monoclonal antibodies by transplanting the variable domain of a mouse monoclonal antibody onto human constant domains (1). Furthermore, a variable domain itself has been humanised by assembling complementarity determining regions from a mouse antibody with the framework regions of a human (2). We have also tried by site-directed mutagenesis of the mouse γ2b CH_2 domain, to identify those residues which bind complement (C1q) and cell receptors.

1. Neuberger, M.S. et al. (1985), Nature 314, 268-270.

2. Jones, P.T. et al. (1986), Nature 321, 522-525.

DNA-Protein Complexes

N 100 CRYSTALLOGRAPHIC REFINEMENT OF THE LARGE PROTEOLYTIC (KLENOW) FRAGMENT OF E. COLI DNA POLYMERASE I AT 2.75 A RESOLUTION, Lorena Beese and Thomas A. Steitz, Dept. Molec. Biophys. & Biochem., Yale Univ., New Haven, CT 06511

Klenow fragment, the C-terminal 605 amino acid polypeptide (MW 68,000) from E. Coli DNA polymerase I, retains both DNA polymerase and 3'-5' exonuclease activities. Crystals of the cloned Klenow fragment (space group P4(3), a=b= 102.9 A, c=85.8 A) were used to determine the structure of the protein complexed with dTMP at 3.3 A resolution (Ollis et al. [1986] Nature 313, 762-766); however, data were collected to 2.6 A resolution. The protein model is being refined using the Konnert-Hendrickson restrained least squares procedure and phase extension. After stereochemical idealization, the R-factor calculated from the starting model was 0.38 to 3.3 A resolution. The R-factor dropped to 0.29 after 12 cycles of refinement using the stereochemical weights suggested by Hendrickson (Meth. Enzymol. 115, 252-270 [1986]). The resolution has been extended to 2.75 A by phasing sucessive 0.2 A shells of data. At present, the model yields an R-factor of 0.25 at 2.75 A resolution. The reduction in the R-factor was in part due to the addition of solvent molecules and metal ions. The R-factor has currently reached a minimum and rebuilding using "omit map" procedures is in progress. Results from the refinement, in particular details of the dTMP binding site, will be discussed. Research was supported by American Cancer Society grant NP-421 and USPHS grant GM-22778.

N 101 MUTATIONAL ANALYSIS OF THE ARC REPRESSOR AND ITS OPERATOR, James U.Bowie, Andrew K. Vershon, Robin Kelley, Robert T. Sauer, MIT, Cambridge, MA 02139

Mutations altering 70 percent of the amino acids in the Arc repressor have been isolated and many of these proteins have been characterized in terms of their folding and DNA binding characteristics. We have purified mutant proteins which have greater, equal, or lower stablity than wild type. Mutations in amino terminal residues do not affect protein stability but greatly diminish DNA binding. We believe these amino acids constitute major determinants of operator recognition, yet are unlikely to be part of the familiar helix-turn-helix motif used by other repressor proteins.

We have also synthesized transition and transversion mutations at every position in the Arc operator. These mutants have allowed us to determine which bases in the operator are involved in recognition by Arc and to assess their individual contributions to the binding energy.

N 102 DNA BINDING BY E. COLI *TRP* REPRESSOR, Jannette Carey, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305

A new assay has been developed that allows quantitative measurement of DNA binding by E. coli *trp* repressor. The assay is based on the polyacrylamide gel retardation method of Garner and Revzin (Nucl. Acids Res. 1981 9, 3047) in which the gel mobility of a DNA fragment is reduced by a bound protein. The method was optimized for *trp* repressor by systematic study of gel electrophoresis conditions.

The *trp* repressor regulates three E. coli operons, and each operator site had been identified previously. The apparent dissociation constant for repressor binding to the trpEDCBA biosynthetic operon is around 1 nM when measured by the gel assay, consistent with previous estimates. Two kinds of complexes are seen in the gel at these low repressor concentrations. DNase footprinting confirms that these complexes are located at the operator site. At repressor concentrations above about 100 nM, additional, non-operator sites are also protected from DNase, and in the gel assay these complexes are greatly retarded. In the absence of L-tryptophan, operator-specific complexes are not observed but nonspecific complexes are still formed. Both specific and nonspecific binding is remarkably salt-resistant. Tritiated *trp* repressor has been prepared by in vivo labeling and is being used to detemine the stoichiometries of the various complexes observed in the gel assay.

N 103 ANIONS AFFECT THE IN VITRO FUNCTION OF ENDONUCLEASE ECO RI. S. Cayley and M.T. Record Jr., University of Wisconsin, Madison 53706

Environmental variables dictate the stability and function of protein-DNA interactions *in vitro*. For instance, increasing the *in vitro* K^+ concentration drastically affects both the rate and extent of the formation of Eco RI-DNA complexes (1). Here we show the critical importance of anionic solution components in determining the functional activity of Eco RI.

K^+ and glutamate are the predominant intracellular ionic species in *E. Coli* and the *in vivo* concentrations of these ions can accumulate to greater than 0.9 and 0.25 M, respectively, as part of the osmotic adaptability of the organism. Comparison of the extent of DNA cutting by Eco RI as a function of KCl or KGlu concentration *in vitro* indicates the rate of cleavage is not only highly sensitive to the K^+ concentration but also to the types and concentrations of anions present. Interestingly, substitution of Glu^- for Cl^- increases the rate of cleavage of DNA dramatically in solutions of high K^+ content. Since Cl^- is not a primary anion in *E. coli*, the results in KGlu are more likely to reflect the physiological function of the enzyme. Our studies indicate that anions drastically affect the stability and function of protein-DNA interactions. Kinetic studies therefore provide insight into the determinants of complex formation not obtainable from structural studies alone.

(1) Jen-Jacobsen *et. al.*, JBC 258: 14638-14646, 1983.

N 104 STRUCTURAL DYNAMICS OF RecA-ssDNA COMPLEXES IN THE PRESENCE OF LexA REPRESSOR. C-F. Chang, S.N. Slilaty, J.W. Little and W. Chiu. Department of Biochemistry, University of Arizona, Tucson, AZ 85721.

RecA protein is known to form a complex with ssDNA in the presence of Mg++ as the only additive to the buffer. Structural studies of frozen-hydrated complexes using ϕX174 ssDNA revealed that the contour length and the helical pitch distance are 0.98 μm and 7.5 nm, respectively. RecA protein is also known to interact with the cellular repressor, LexA, and effect its cleavage *in vivo* and *in vitro*. The *in vitro* cleavage reaction requires ssDNA, Mg++ and a nucleoside triphosphate. Analysis of the structural dynamics of RecA-ssDNA complexes in the presence of LexA provides a basis for understanding the mechanism of protein-protein interaction and RecA-dependent repressor cleavage. We found that, when wild type LexA was added to preformed RecA-ssDNA complexes in the absence of a nucleotide triphosphate, the contour length and helical pitch distance increased to 1.24+0.07 μm (n=27) and 8.64+0.45 nm (227 turns), respectively. A similar increase in both parameters was observed for a noncleavable mutant of LexA, KA156. No change in either parameter was found, however, when LexA cleavage products were added. These observations suggest that intact LexA, but not LexA cleavage fragments, bind and alter the structure of the complex. The finding that cleavage fragments do not bind to the complex is consistent with the results of another experiment in which the rate of RecA-dependent cleavage of wild type LexA, in the presence of ATP[S], was unaffected by added LexA cleavage products. The effects of a noncleavable LexA mutant on the structural dynamics of RecA-ssDNA complexes, formed in the presence of ATP[S], will be discussed.

N 105 STRUCTURE OF THE ACTIVE SITE OF E. COLI RNA POLYMERASE, Peter P. Chuknyiski, Joseph M. Rifkind, Rajasekharan P. Pillai, Edward Tarien and Gunther L. Eichhorn, National Institutes of Health, National Institute on Aging, Gerontology Research Center, Laboratory of Cellular and Molecular Biology, Baltimore, Maryland 21224.

The basic molecular processes responsible for RNA synthesis occur at the active site of RNA polymerase. Since the active site of *E. coli* RNA polymerase consists of an initiation site (IN) and an elongation site (EL), it is possible to map out the structure of the complete active site by studying the interrelationship between IN and EL. Both IN and EL have metal ions bound to them - Zn(II) and Mg(II), respectively, and both of these ions can be replaced by Mn(II). The distances between Mn(II) on each site and substrate atoms on the same site have been previously determined in the laboratories of Mildvan and Wu and our own laboratory. Complete mapping of the active site requires one more distance - that between the metals at the initiation and elongation sites. Such a metal-metal distance can be determined by EPR measurements, since the EPR spectrum of a paramagnetic metal is affected by another paramagnetic metal in its proximity. We were able to obtain the distance between two Mn(II) ions, substituted for Zn(II) and Mg(II) in the IN and EL, respectively. In the absence of substrate the distance is (5.2 ± 0.4)A and in the presence of ATP substrates in both IN and EL the distance is about 4.8A. From these distances and metal substrate distances we have constructed models of the active site of the enzyme which lead to a mechanism of the translocation of substrate from EL to IN during RNA synthesis.

N 106 CHARACTERIZATION OF THREE EcoRI ENDONUCLASE MUTANTS WITH ALTERED DNA-PROTEIN CONTACTS DESIGNED TO CHANGE SPECIFICITY. J.P.Day, N.O.Reich, P.Hager, J. Rosenberg*, J.McClarin*, J.Grable*, H.W.Boyer, P.J.Greene. UCSF, San Francisco, CA 94143. * Univ. of Pittsburgh, Pittsburgh, PA 15260.

Mutants of the EcoRI endonuclease were created by site directed mutagenesis in an attempt to alter specificity. The mutations were chosen, based on the crystal structure, to change the hydrogen bonding characteristics of the amino acid side chains responsible for recognition of the canonical DNA sequence. The mutants generated were Glu144-> Asp(ED144), Arg145->Lys (RK145), and Arg200->Lys(RK200). These were purified to homogeneity. We measured kcat and Km on pBR322 and pBR322 lacking the RI site. We also determined the salt and pH optima. All of the mutants cleave the canonical sequence; however, they all accumulate the nicked intermediate. Linear DNA production by ED144 is increased at low salt to a nearly wildtype level. kcat values are well below wildtype kcat in RK145 and RK200. The specificity constants (kcat/Km) are different from wildtype. Canonical to noncanonical kcat/Km ratios indicate altered specificity. ED144 has increased specificity for the canonical site. The rate limiting step in the wildtype is the off rate from nonspecific DNA after cleavage. If this step is also limiting in the mutants, then hydrogen bonds involved in recognition of the canonical site are normally made to some extent in nonspecific DNA. Completely changing specificity is difficult or perhaps impossible to accomplish by making a single amino acid change. One might expect enzymes to have a structure resistant to specificity changes by a single amino acid substitution, especially if a specificity change would be a lethal event.

N 107 ROLE OF GLU181 IN DNA-SEQUENCE RECOGNITION BY THE CATABOLITE GENE ACTIVATOR PROTEIN (CAP): R. Ebright,[1,2] A. Kolb,[2] H. Buc,[2] T. Kunkel,[3] J. Krakow,[4] W. Davies,[5] and J. Beckwith.[1] [1]Harvard Medical School, Boston MA, USA, [2]Institut Pasteur, Paris, France, [3]NIEHS, Research Triangle Park NC, USA, [4]Hunter College, New York NY, USA, and [5]Allelix, Toronto, Canada.

The consensus DNA site for CAP is illustrated below; the site is 22 base pairs in length and exhibits 2-fold sequence symmetry:

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A A - T G T G A - - - - - - T C A C A - T T
T T - A C A C T - - - - - - A G T G T - A A
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Previously, we have presented evidence that Glu181 of CAP makes a direct H-bonded contact with 2-fold related positions 7 and 16 of the DNA site (Nature 311:232, 1984; PNAS 81:7274, 1984). Here, we have constructed 19 amino acid substitutions at residue 181 of CAP using site-directed mutagenesis. The interactions of wild-type CAP and of the 19 substituted CAP variants with DNA sites containing A:T, C:G, G:C, and T:A at positions 7 and 16 are being assessed. At this writing, complete data are available for the interactions of wild-type CAP, CAP(Glu181 $\rightarrow$ Val), and CAP(Glu181 $\rightarrow$ Leu). The data indicate that the strength of the H-bond by Glu181 is approximately -0.7 kcal/mol.
Parallel experiments are in progress with respect to Gln18 of Lac repressor--i.e., the residue of Lac repressor equivalent in its position within the homologous α-helix-turn-α-helix DNA binding motif to Glu181 of CAP.

N 108 PROTEIN-DNA RECOGNITION IN THE ARABINOSE OPERON OF E. COLI, Christopher Francklyn and Nancy Lee, University of California at Santa Barbara, Santa Barbara, California 93106

The specificity elements involved in the interaction of the arabinose operon specific regulatory protein (araC) with the sequences required for transcriptional activation have been studied using site directed mutagenesis and classical genetic techniques. Particular positions in the initiator site where araC binds (araI) were chosen for substitution based on their predicted location in the major grooves of the araC-araI complex, as suggested by DNAase footprinting and chemical modification experiments. These mutants were then screened to obtain a suitable candidate for a secondary site reversion experiment, i.e. the isolation of an araC protein with altered nucleotide specificity. Two such araC proteins were isolated by direct selection and then tested against various araI base substitutions to obtain a profile of their base specificity. The results of these experiments suggest that at least two different portions of the araC carboxy terminus may be important in determining the sequence specificity of the protein.

N 109 ANALYSIS OF NONCANONICAL DNA CLEAVAGE BY ECORI ENDONUCLEASE MUTANTS. P.W. Hager, H.W. Boyer, J.P. Day, N.O. Reich, and P. Greene: University of California, San Francisco, CA 94143. J. Rosenberg, J. Grable, R. Love: University of Pittsburgh, Pittsburgh, PA 15260.

It has been proposed that DNA sequence specificity for the EcoRI endonuclease results from the formation of twelve hydrogen bonds between bases exposed at the major groove of the EcoRI site and six amino acid side chains (3 from each subunit) in the enzyme. (Rosenberg and Greene, DNA 1, 117-124 (1982), Rosenberg et al., Science, in press) Conservative replacements in the 3 amino acids identified in the X-ray crystallographic structure as responsible for specificity were made (Glu 144 to Asp, Arg 145 to Lys, and Arg 200 to Lys). These changes failed to alter the site of primary cleavage. However, all of these proteins nick noncanonical DNA sequences in pBR322 lacking the EcoRI site. The location of these single strand cleavage sites is being determined for the wild type and mutant enzymes. The identification of the sequences recognized by the different enzymes and the hierarchy of the rates at which these sequences are hydrolyzed will contribute to our understanding of how the endonuclease discriminates between DNA sequences.

N 110 FUNCTIONAL DOMAINS OF DNA POLYMERASES DEFINED BY PROTEIN SEQUENCE HOMOLOGIES AND MUTANT PHENOTYPES, J.D. Hall, University of Arizona, Tucson, AZ, 85721.

The goal of this work is to identify functionally important domains in the DNA polymerase from herpes simplex virus type 1. I have used a combined approach of computer and genetic analysis to accomplish this goal. Computer analysis has revealed highly conserved regions which appear in the polymerase protein sequences from herpes simplex, from other animal viruses (other herpes viruses, vaccinia virus and adenovirus) and from two bacteriophages (ϕ29 and T4). The fact that these similarities occur between very distantly related species suggests that they encompass active sites. Their significance to polymerase activity will be discussed. A second method to identify active site regions involves isolation of polymerase mutants with altered abilities to recognize nucleoside triphosphates. Such mutant enzymes are fully functional but exhibit altered substrate specificities and, hence, may carry mutations which affect active site function. I will discribe certain mutants from herpes simplex virus which incorporate nucleoside triphosphate analogues at abnormal levels or which exhibit altered frequencies of base-substitution errors during replication. Some of these mutations map at (or near) the highly conserved regions described above, giving further support to the functional importance these regions.

N 111 DOMAIN ANALYSIS OF THE cII TRANSCRIPTIONAL ACTIVATOR PROTEIN FROM PHAGE LAMBDA, Yen Sen Ho*, Michael E. Mahoney+, Daniel L. Wulff+ and Martin Rosenberg*, *Dept. of Molecular Genetics, SmithKline and French Laboratories, 709 Swedeland Rd, Swedeland, PA 19479, and +Dept. of Biological Sciences, State University of New York at Albany, Albany, NY 12222. The bacteriophage λ transcriptional activator protein cII is a DNA-binding protein. It coordinately regulates transcription from three phage promoters, P_{RE}, P_I and P_{aQ}. A representative set of 25 single amino acid mutations spanning the entire cII protein were cloned into the E. coli expression vector system pKC30 and used for structure-function relationship studies. The results of the biochemical and genetic studies indicated that the cII protein could be subdivided into several distinct domains. The region extends from amino acid position 26 to 45 is important for the sequence-specific DNA binding. Biochemical studies demonstrated that though these mutant proteins retained their ability to form tetramer, yet they failed to activate transcription and lost their ability for sequence-specific DNA binding. Two regions of cII protein, extended from amino acid position from 8 to 25 and 45 to 71 respectively, are important for maintaining the tetrameric structure of cII protein. They are defective both in tetramer formation and positive regulation. We have strong evidence to show that the defect in regulation function is resulted from the inability of these variants to form the active tetrameric structure of the protein. The NH_2-terminal is highly flexible and carboxyl-terminal of cII protein only partially influences cII function presumably by structural effects.

N 112 SITE DIRECTED MUTAGENESIS OF THE KLENOW FRAGMENT OF E. COLI DNA POLYMERASE I, Catherine M. Joyce, Victoria Derbyshire, Andrea Polesky and Nigel D.F. Grindley, Yale University, New Haven, CT 06510.

The Klenow fragment of DNA polymerase I is the only DNA polymerizing enzyme for which high resolution structural data are available. The structure determined by T. Steitz and co-workers shows that the molecule has two domains (1). A variety of biochemical and model-building experiments suggest that the two domains correspond to the two enzymatic activities of Klenow fragment: polymerase and 3′-5′ (editing) exonuclease (2,3). We are using site-directed mutagenesis to locate active site regions and to identify functionally important amino acid side chains. In investigating the 3′-5′ exonuclease active site, we have changed residues that have been shown crystallographically to interact with dNMP, a competitive inhibitor of the reaction. In this way we have obtained a mutant enzyme that lacks exonuclease activity, and one in which the kinetics of the reaction are altered. We have also made several changes in the polymerase domain of the molecule, focussing on a region that we consider likely (based on model building) to contain the catalytic site. All of our mutant proteins are being characterized both *in vitro* and *in vivo*, when introduced as single-copy genes into *E. coli*.

(1) Ollis, D.L. *et al.* (1985) Nature 313: 762-766.
(2) Joyce, C.M. *et al.* (1986) UCLA Symposium: Protein Strucutre, Folding and Design, (D. Oxender, editor), in press.
(3) Freemont, P.S. *et al.* (1986) Proteins 1: 66-73.

N 113 STRUCTURAL AND FUNCTIONAL IMPORTANCE OF THE INTRINSIC ZINC IN GENE 32 PROTEIN, THE SINGLE-STRANDED DNA BINDING PROTEIN FROM T4 PHAGE, Kathleen M. Keating, David P. Giedroc, Kenneth R. Williams, William H. Konigsberg and Joseph E. Coleman, Yale University, New Haven, CT 06510.

Gene 32 protein (gp32) isolated from bacteriophage T4 infected *E. coli* contains one mole of tightly bound Zn(II) per mol of protein. A linear incorporation of three molar equivalents of 1 p-hydroxymercuriphenylsulfonate (PMPS) results in a linear release of 1.1 mol of Zn(II) from the protein. Reversal of formation of the gp32-PMS complex with thiol in the presence of EDTA results in a zinc-free apo-gp32. Cd(II) and Co(II) can be exchanged with the intrinsic Zn(II) ion. The spectroscopic properties of the Co(II) and Cd(II) substituted gp32 as well as the PMPS data suggest that the Zn(II) is coordinated through at least three cysteines in gp32. The decrease in thermostability that accompanies removal of the Zn(II) as well as the inability of the apo-gp32 protein to form a protease-resistant core suggest that the Zn(II) makes a significant contribution to the conformational stability of native gp32. In addition, fluorescence quenching, poly[d(A-T)] melting and nuclease digestion studies on the apo-gp32:ssDNA complex all indicate that the Zn(II) is essential for maintaining the high affinity of gp32 for ssDNA. Three of the four cysteines in gp32 are located in a tyrosine-rich sequence that spans residues 72-116 and that previous proton NMR studies have implicated in DNA binding. Taken together, these data suggest that by coordinating with the sulfhydryl groups on the cysteine side chains in the tyrosine-rich region of gp32, Zn(II) stabilizes conformations that are directly involved in cooperative DNA binding.

N 114 AMINO ACID SUBSTITUTIONS IN THE N-TERMINUS OF *LAC* REPRESSOR; EFFECTS ON DNA BINDING Lynn G, Kleina, Jean-Michel Masson, and Jeffrey H. Miller. Department of Biology and Molecular Biology Institute, U.C.L.A., Los Angeles, CA. 90024.

Understanding the relationship between protein structure and function is a complex and interesting problem. Many DNA-binding proteins including the *lac* repressor, contain a helix-turn-helix motif with similar amino acid composition at analogous sites. These proteins are still able to distinguish between and bind to their specific, short operator DNA sequences (14-22 b.p.) that may be present only as a single copy in the genome. Our approach to understanding the specificity of the *lac* repressor uses *E.coli* strains carrying amber suppressors which can insert different amino acids in response to amber (UAG) codons. Fourteen amino acids can be inserted in response to an amber site. We are systematically saturating the first 60 positions of the *lac* repressor known to encode operator binding to determine precisely which residues are important for operator contact. Preliminary data suggests that certain residues are invariable, other positions allow conservative substitutions, whereas others are non-specific.

N 115 SITE DIRECTED MUTAGENIC STUDIES OF THE MNT REPRESSOR FROM PHAGE P22.
Kendall L. Knight and Robert T. Sauer, Department of Biology, M.I.T., Cambridge, MA. 02139

The Mnt repressor is an 82 residue protein that binds as a tetramer to a unique, symmetric 17 bp operator sequence. Previous work demonstrated that the Mnt protein contacts both the front and backside of the operator helix(1). The cI repressor of phage lambda is the only other sequence specific DNA binding protein known to make both front and backside operator contacts, using the N-terminus as a flexible arm to wrap around the helix(2,3). Other work on the Mnt protein suggests that N-terminal residues are responsible for contacts on the front side of the helix(4). Based on these results, and the sequence homology between the N-terminal arm of cI(Ser-Thr-Lys-Lys-Lys-...) and the C-terminus of Mnt(...Tyr-Lys-Lys-Thr-Thr) we constructed a series of deletion mutants lacking 3 or more of the C-terminal residues. Results of assays performed using purified mutant proteins show that, although the operator affinity decreases by up to 10^4, backside contact is retained in each case. Hydroxy radical footprints of each of the mutant Mnt/operator complexes show that a similar pattern of protection is afforded by wild type Mnt and all of the C-terminal deletion mutants. A complete charaterization of the DNA binding properties, oligomeric state, and stability of these mutants will be presented.

Recently, using both oligonucleotide-directed mutagenesis and cassette mutagenesis we have constructed a series of N-terminal Mnt mutants. Data will be presented regarding the properties of these mutants as well.

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3- Eliason,J.L., Weiss,M.A., and Ptashne,M.(1985) Proc. Natl. Acad. Sci. 82, 2339-2343.
4- Youderian,P., Vershon,A.K., Bouvier,S., Sauer,R.T., and Susskind,M.M.(1983) Cell 35, 777-783.

N 116 A DNA STRUCTURE EFFECT MODULATES THE DNA AFFINITY OF 434 REPRESSOR AND CRO, G.B. Koudelka, M. Ptashne, Harvard Univ. Cambridge MA. 02138.

To investigate the role that the central 6 basepairs (bp)of the 14 bp, partially rotationally symmetric binding site of phage 434 Repressor and Cro have in modulating DNA binding affinity, the effect of symmetric and asymmetric base substitutions at these operator positions on the DNA affinities of Cro and Repressor were determined. Both Repressor and Cro discriminate only slightly between symmetrical substitutions of A:T or T:A at positions 6 or 7; asymmetric base substitutions that create poly A sequences at the center of the operator dramatically increase the DNA affinity of both proteins. Furthermore, symmetrical substitution of G:C or C:G at positions 6 or 7 equally decrease binding affinity. These effects are not a result of protein contacts to the major groove because neither protein protects the major groove functional groups of the bases at position 6 and 7 from chemical modification, and the proteins bind equally well to operators containing inosine or adenine at positions 7 and 8. At least for Repressor, the discrimination between A/T and G/C bp at positions 6-9 is not the result of a protein contact to the minor groove, since mutagenesis of the only group capable of contacting the minor groove yields a protein that still discriminates between G and A at these positions. The above results are consistent only with the idea that substitutions at operator positions 6-9 exert their effect on the DNA affinity by adjusting the flexibility and/or conformation of the DNA at the center of the operator. Such DNA structural effects could modulate the DNA affinity of the proteins by adjusting the geometry of DNA contacts that are made by the recognition helicies of the proteins.

N 117 IDENTIFICATION OF A REGION IN THE T3 AND T7 RNA POLYMERASES THAT IS REQUIRED FOR SPECIFIC PROMOTER RECOGNITION, W. T. McAllister, K. Joho, N. McGraw, C. Morris, J. Klement, M. Moorefield, SUNY-Health Science Center at Brooklyn, Brooklyn, New York, 11203.

The RNA polymerases that are encoded by bacteriophage T7 and its relatives are highly specific for their respective promoters. To determine the mechanism by which these polymerases discriminate their promoters, we have approached the problem from two directions. First, we have compared the consensus sequence of the T3 and T7 promoter signals, and have systhesized mutant promoters which change individually bases at positions where the two promoters differ. The properties of the mutant promoters in vivo and in vitro indicate that a change at -11 (approximately one turn of the helix up-stream from the start site) is sufficient to change the specificity of the promoters. Secondly, we have examined the predicted amino acid sequences of the T3 and T7 RNA polymerases, and by taking advantage of the cloned genes for these two polymerases, we have constructed hybrid polymerase genes in which part of the polymerase structure is determined by the T3 gene and part of the polymerase structure is determined by the T7 gene. In this way, we localized the region of the RNA polymerase that is responsible for specific promoter recognition to a region that lies between 76 and 91% of gene length. This region of the RNA polymerase gene bears significant homology to other sequence specific DNA binding proteins, but is unrelated to the bi-helical domain used by repressor-type molecules of E. coli.

N 118 CASSETTE MUTAGENESIS OF ECORI ENDONUCLEASE. Michael C. Needels, Norbert O. Reich, Herbert W. Boyer, and Patricia Greene: Univeristy of California, San Francisco, CA 94143. John Rosenberg, John Grable, and Robert Love: University of Pittsburgh, Pittsburgh, PA 15260.

From inspection of the X-ray crystal structure of the DNA-EcoRI endonuclease recognition complex, regions of the protein which are responsible for binding specificity and catalysis were inferred. Based on this structural information, we have subjected the EcoRI endonulease gene to site directed mutagenesis. In the first round of mutagenesis we used mismatched oligonucleotides to introduce a small number of conservative substitutions into the putative recognition regions. In order to extend this analysis, we have now engineered several unique restriction sites into the EcoRI endonuclease gene bracketing potentially important regions. For example, we have introduced sites which bracket the N-terminal residues of each of the recognition helices. Also, the introduction of flanking BstEII and SpeI sites around residue 130 has allowed us to test a model for the mechanism of phosphodiester hydrolysis. Based on the crystal structure of the protein-DNA complex, it is possible that Lys 130 participates in general acid catalysis of this reaction. Employing cassette mutagenesis, we have generated mutants of the EcoRI endonuclease gene which encode a variety of different amino acids at position 130. The mutant gene products are being characterized and the effect on catalysis of different substitutions at position 130 is being assessed.

N 119 MULTI-STEP BINDING OF recA PROTEIN TO DUPLEX DNA, B. Franklin Pugh and Michael M. Cox, Department of Biochemistry, School of Agriculture and Life Sciences, University of Wisconsin-Madison, Madison, WI 53706.

The recA protein of Escherichia coli promotes DNA strand exchange. RecA protein rapidly binds ssDNA cooperatively to form a contiguous nucleoprotein filament. In the presence of ATP this complex is capable of interacting with duplex DNA to search for homology and pair complementary strands. The inability to detect significant binding to duplex DNA under optimal strand exchange conditions has generally been assumed to represent an unfavorable binding equilibrium, which results in a net dissociation of recA protein from the paired DNA. We and others have shown, however, that recA protein remains associated with the nascent heteroduplex DNA. This does not represent a paradox. Our results show that there is no thermodynamic requirement for dissociation of recA protein from the heteroduplex DNA product. Instead, there is a slow step in the association pathway which is overcome by the process of strand exchange. This kinetic barrier to association involves a pH- and ATP-dependent initiation (nucleation) of DNA unwinding. The final product of this process is a cooperatively bound nucleoprotein filament of recA protein and extensively unwound duplex DNA, which is capable of hydrolyzing ATP. Once binding is initiated propagation into a nucleoprotein filament is fast and pH-insensitive.

N 120 QUANTITATIVE ANALYSIS OF SPECIFICITY IN THE RECOGNITION OF OPERATOR DNA SEQUENCES BY CRO AND λ REPRESSORS. A. Sarai and Y. Takeda. NCI, NIH, Bethesda, MD 20892, and NCI-FCRF, Frederick, MD 21701.

To understand the mechanism of recognition of specific operator sequences by repressor proteins we have studied quantitatively the interactions of Cro and λ repressors with their operators. We chemically synthesized 21-mer of OR1, made all three substitutions at each base pair in half of the pseudo-symmetrical operator sequence, and measured the binding constants of the repressors to these DNAs by the filter binding assay. Our findings are as follow. (1) Base pairs at the 2nd, 4th and 6th positions are crucial for the bindings of both repressors. Most of the base substitutions at these positions are fatal and destabilize these repressor complexes with up to 3 Kcal/mol of free energy change. The substitutions at other positions exhibit milder effects. (2) Our results have confirmed most of the interactions proposed in the molecular models of repressor-operator bindings (Ohlendorf et al., 1982; Lewis et al., 1983). However, there are some significant differences. (3) The free energy change due to hydrogen bonds is estimated to be between 0.8 and 2.6 Kcal/mol, and the free energy change due to hydrophobic interactions between T's methyl group and hydrocarbon regions of amino acid to be between 0.5 and 1.8 Kcal/mol. These interactions are the specificity determinants. (4) Free energy changes are additive within a limit (we define the binding within this limit the "specific binding"), but not additive (cooperative) outside of this limit (we define the binding outside of this limit the "nonspecific binding"). (5) Differential operator bindings between Cro and λ repressors can be explained by a few key amino acid differences between these two repressors.

N 121 CRYSTALLIZATION AND PRELIMINARY CHARACTERIZATION OF CAP-cAMP-DNA COMPLEXES, Steve C. Schultz, George C. Shields & Thomas A. Steitz, Yale University, Mol. Biophys. and Biochem., New Haven, CT 06511. Catabolite gene activator protein (CAP) from E. coli. binds to specific operator regions of several genes in the presence of cAMP and regulates transcription of these genes [Pastan, I. & Adhya, S. (1976) Bacteriol. Rev. 40, 527-551]. CAP is a 45,000 dalton dimer composed of two identical subunits, and the X-Ray crystal structure of the CAP-cAMP complex has been solved [McKay, D.B. & Steitz, T.A. (1981) Nature 290, 744-749]. Models have been proposed for the interaction of CAP-cAMP with its operator DNA based on structural and electrostatic complementarity [Weber, I.T. & Steitz, T.A. (1984) Proc. Natl. Acad. Sci. USA 81, 3973-3977]. Recent evidence indicates that CAP bends its operator DNA [Wu, H.M. & Crothers, D.M. (1984) Nature 308, 509-513], possibly greater than 90°.

We have obtained crystals with CAP-cAMP in the presence of oligonucleotides that are 28 and 29 base pairs in length. HPLC analysis of these crystals demonstrates that they contain the oligonucleotides. Preliminary characterizations indicate that the CAP-cAMP complex with the 29 base pair oligonucleotide crystalizes as a centered lattice with apparent cell dimensions of approximately 103A X 180A X 128A. Currently, we are attempting to grow larger, more ordered crystals by varying the lengths of oligonucleotides and conditions used for crystallization.

N 122 RECA PROTEIN PROMOTES COMPLETE PARANEMIC PAIRING BETWEEN HOMOLOGOUS DNA SUBSTRATES, Brian C. Schutte and Michael M. Cox, Department of Biochemistry, School of Agriculture and Life Sciences, University of Wisconsin-Madison, Madison, WI 53706.

As a first step in DNA strand exchange, recA protein of E.coli forms a filamentous complex on single-stranded DNA which contains stoichiometric (1 recA monomer per 4 nucleotides) amounts of recA protein. RecA protein monomers within this complex hydrolyze ATP with a turnover number of 25 min^{-1}. Upon introduction of homologous duplex DNA to initiate strand exchange, this rate of ATP hydrolysis drops by 33%. The decrease in rate is complete within two minutes and the rate of ATP hydrolysis then remains constant during and subsequent to the strand exchange reaction. This drop is completely dependent upon homology in the duplex DNA. Furthermore, the magnitude of the drop is linearly dependent upon the length of the available homologous region in the duplex DNA. This implies that all of the available homology in the incoming duplex DNA is detected very early in the DNA strand exchange reaction, with the duplex DNA paired paranemically with the homologous ssDNA in the complex throughout its length. These and other results imply that the duplex DNA is paired with the ssDNA, but remains outside the nucleoprotein filament. If an homologous free end is available, the minus strand of this duplex DNA is brought from outside the complex into the interior of this complex as it becomes part of the heteroduplex DNA product. Finally, the results also support the notion that all of the ATP hydrolysis which occurs during DNA strand exchange plays a direct role in the reaction.

N 123 **GENETIC ANALYSIS OF THE DNA BINDING SITE OF THE LexA PROTEIN OF E. coli K-12,** **A.T. Thliveris and D.W. Mount. University of Arizona, Tucson, AZ, 85721.**

The LexA protein of E. coli is a repressor of at least 20 operons in the SOS regulon. The amino acid sequence of LexA protein shows sequence homology to regions in other repressors such as phage lambda cI and Cro proteins which correspond to the helix-turn-helix structural motif for DNA binding of these repressors. We have isolated several mutant repressors which are defective in DNA binding. Genetically, the mutations generating these repressors are dominant to lexA$^+$, indicating that the mutant proteins can poison normal protein by forming defective, mixed dimers. Several sequence changes in the presumptive helix-turn-helix motif will be discussed.

N 124 **THE DNA-BINDING DOMAINS OF THE BACTERIOPHAGES Mu AND D108 TRANSPOSASE PROTEINS AND NEGATIVE REGULATORY PROTEIN NER.** Peter P. Tolias, Chantal Autexier and Michael S. DuBow, Dept. of Microbiology & Immunology, McGill University, Montreal, QC, Canada, H3A 2B4.

Bacteriophages Mu and D108 are related temperate, transposable phages. It is the ner proteins of these two phages that regulates transposase expression and the transposase (A) protein that is absolutely required for the transposition of the phage genomes. To identify the functional domains of these proteins, we cloned and expressed various segments of the respective A proteins (transposase) and ner proteins. For transposase, we found that fusion proteins containing the amino-termini of the Mu and D108 A proteins can specifically bind to the Mu right-end. We divided the amino terminal 13 kDa of the D108 A protein in half and found that each half is independently capable of binding to the Mu attR site. We then purified one of these fusion proteins (which contains only 13 kDa of the amino terminus of the Mu A protein) and found that this fusion protein binds to the Mu right-end at the apparent consensus sequence 5'-PuCGAAA-3'. This sequence represents a subset of the consensus A protein binding sites of Mu and D108 DNAs.

The D108 and Mu ner genes, which code for λ cro-like proteins, are ~ 50% homologous at the protein level, yet their DNA-binding is markedly different. Mu ner, like λ cro, binds to a small 12 bp sequence of dyad symmetry, while D108 ner binds to an 11 bp sequence present as a perfect inverted repeat in its operater. The cloning of the DNA-binding domains of these two ner proteins, and further subcloning of the transposase protein, will allow us to determine how two related (ner) proteins recognize two different DNA sequences, and to continue the functional dissection of a DNA-catalytic (transposase) enzyme.

N 125 EFFECT OF A cAMP-INDEPENDENT MUTATION ON CRYSTAL STRUCTURE OF CATABOLITE GENE ACTIVATOR PROTEIN, Irene T. Weber[1], Gary L. Gilliland[1,2], James Harman[3] and Alan Peterkofsky[3], Center for Chemical Physics, National Bureau of Standards, Gaithersburg MD 20899[1]; Genex Corporation, 16020 Industrial Drive, Gaithersburg MD 20877[2]; Laboratory of Biochemical Genetics, The National Heart, Lung and Blood Institute, Bethesda MD 20892[3]

E. coli NCR91 synthesizes a mutant form of catabolite gene activator protein (CAP) in which alanine 144 is replaced by threonine. This mutant, which also lacks adenylate cyclase activity, has a CAP* phenotype; in the absence of cAMP it is able to express genes that normally require cAMP. CAP91 has been purified and crystallized with cAMP under the same conditions as used to crystallize the wild type CAP-cAMP complex. X-ray diffraction data were measured to 2.4Å resolution and the CAP91 structure was determined using initial model phases from the wild type structure. A difference Fourier map calculated between CAP91 and wild type showed the two alanine to threonine sequence changes in the dimer and also a change in orientation of cysteine 178 in one of the subunits. The CAP91 coordinates were refined by restrained least-squares to an R factor of 0.186. Differences in the atomic positions of the wild type and mutant protein structures were analyzed by a vector averaging technique. There were small changes that included concerted motions in the small domains, in the hinge between the two domains and in an adjacent loop between β-strands 4 and 5. The mutation at residue 144 apparently causes changes in the position of some protein atoms that are distal to the mutation site.

N 126 MUTATIONAL ANALYSIS OF THE ECORI ENDONUCLEASE, Stephen Yanofsky[1], Herb Boyer[1], John Grable[2], Judith McClarin[2], John Rosenberg[2] and Patricia Greene[1], [1]University of California at San Francisco, San Francisco, Ca. 94143, [2]University of Pittsburgh, Pittsburgh, PA 15260.

Using in vitro mutagenesis with hydroxylamine a large number of null mutants of the EcoRI endonuclease have been generated. Fifty per cent of the 121 mutants examined by western blot tested positive against anti-RI. The entire endonuclease gene from twenty-seven western positive mutants was sequenced by using dideoxy sequencing directly from double stranded plasmid DNA. Twenty of these were single base change substitutions, with 10 of the mutants falling within the region from ala 139 to glu 144. Examination of the mutants with respect to the structure of the EcoRI endonuclease-DNA co-crystal reveals that all of the null mutants map at either the protein-DNA interface or at the subunit-subunit interface. Molecular weight determination of purified protein on a BioSil TSK250 HPLC column demonstrates that wild type EcoRI endonuclease runs as a dimer of 60Kd. Three mutants with alterations at the protein-DNA interface (AT139, GS140 and RQ203) run as dimers while 3 mutants mapping at the subunit-subunit interface (EK144, EK152 and GR210) run as monomers.

Enzyme Mechanisms, Folding

N 200 n-PROPANOL INDUCED 'NATIVE-LIKE FOLD' OF α-GLOBIN: A MOLECULAR TRAP IN THE SEMISYNTHESIS OF α-GLOBIN, A.S. Acharya, Y.J. Cho and K.S. Iyer, Rockefeller University, New York, NY

Staphylococcus aureus V8 protease catalyzed condensation of the unprotected complementary fragments of α-globin, namely α_{1-30} and α_{31-141}, occurs in the presence of 25% n-propanol at pH 6.0 and 4°C. Glycerol, the commonly used organic cosolvent was not very efficient. Apparently, the use of n-propanol has brought out a more specific influence on the conformation of α-globin or its fragments than merely decreasing the acidity of the α-carboxy group of the carboxyl component. Indeed in the presence of 25% n-propanol, the α-helical content of α-globin increased, it was nearly 80% of that of native α-chain. Propanol also induces α-helical structure in RNase-S-peptide. The digestibility of RNase-S-peptide by V8 protease at Glu(9) is not influenced by n-propanol. On the other hand, n-propanol protected the peptide bonds of Glu-23, Glu-27, and Asp-47 of α-globin from protease digestion; a selective cleavage at Glu(30) occurs in the presence of n-propanol. Similar high selectivity of Glu-30 to V8 protease digestion is also seen with α-chain. Thus, n-propanol besides increasing the α-helical conformation of α-globin, appears to induce the packing of the α-helical segments of the globin, presumably with a topology comparable to that of heme containing chain. It is suggested that the 'native-like fold' of α-globin in n-propanol, provides a molecular trap mechanism for shifting the equilibrium of the hydrolytic reaction in favor of proteosynthesis. (Supported by NIH Grant HL-27183, and a Grant-in-Aid from NYHA. ASA is an Established Fellow of NYHA)

N 201 CORRELATIONS BETWEEN DIFFERENTIAL SCANNING MICROCALORIMETRY (DSC) AND CLASSICAL ENZYME TEMPERATURE-ACTIVITY PROFILES, John O. Baker, Melvin P. Tucker, Kuniyasu Tatsumoto, Kenneth K. Oh, Karel Grohmann, and Michael E. Himmel, Biotechnology Branch, Solar Fuels Research Division, Solar Energy Research Institute, Golden, CO 80401.

Beta-D-glucosidases (EC 3.2.1.21) as a group represent rather ubiquitious O-glycosyl transferases, since these enzymes are found in most species in both the plant and animal kingdoms. It is likely, therefore, that beta-glucosidases can be found in nature with wide ranges of temperature stability and optima, thereby providing excellent subjects for comparative studies of protein thermal unfolding. Three beta-glucosidases have been purified by high-performance size exclusion chromatography on a Toya Soda 3000 SWG column followed by Fast Protein Liquid Chromatography on a Pharmacia Mono-Q anion-exchange column. These enzymes include the beta-glucosidase forms I and II from Aspergillus niger, the beta glucosidase secreted by Trichoderma reesei, and the cell-wall-associated beta-glucosidase from the cellulolytic thermophile, Acidothermus cellulolyticus. The temperature activity profiles and DSC thermograms of the purified enzymes have been determined under optimal conditions of pH, ionic strength and required cationic concentration. Based on these results, we present a preliminary evaluation of DSC as a predictive tool for selecting beta-glucosidases with high temperature optima.

N 202 DOMAIN STRUCTURE IN TWO TRYPTOPHAN-REGULATED ENZYMES OF AROMATIC BIOSYNTHESIS, Ronald Bauerle, Soon-Cheol Hong, Jill Ray and John Hess, University of Virginia, Charlottesville VA 22901.

In enteric bacteria, L-tryptophan (Trp) regulates carbon flow in aromatic biosynthesis by feedback inhibition of two branch-point enzymes: (1) the Trp-sensitive 3-deoxy-D-arabinoheptulsonate-7-phosphate synthase [DAHPS(Trp)], one of the three isozymes catalyzing the first step of the common aromatic pathway and (2) the anthranilate synthase-anthranilate phosphoribosyl transferase (AS-PRT) complex, the first enzyme of the Trp pathway. DAHPS(Trp) is a homodimer encoded by aroH and AS-PRT is a heterotetramer encoded by trpE and trpD. Feedback inhibition is mediated in each by Trp binding sites on the AroH and TrpE polypeptides. We are comparing domain structure and organization in AroH (347 residues) and TrpE (520 residues) as a model system for the study of the molecular evolution of feedback-regulated enzymes.

Mutagenic and proteolytic studies of TrpE have shown that the carboxy-terminal half of the polypeptide comprises a catalytic domain containing the active site for AS activity. Photoaffinity labelling experiments indicate that the feedback site is separate from the active site and is located within the amino-terminal half of TrpE. In contrast, mutagenic probing of AroH indicates the presence of overlapping catalytic and regulatory sites. We tentatively conclude that the structural basis of feedback regulation by Trp is different in the AS and DAHPS enzymes and probably evolved in each by distinct mechanisms, i.e. gene fusion in the former and divergence from an unregulated ancestral molecule in the latter.

N 203 STRUCTURAL AND FUNCTIONAL STUDIES ON RIBONUCLEOTIDE REDUCTASE, J.B. Clements, I. Nikas, J. McLauchlan, *W. Taylor and H.S. Marsden, MRC Virology Unit, Church Street, Glasgow G11 5JR, Scotland: *Department of Crystallography, Birkbeck College, London, UK.

Ribonucleotide reductase (RR) is an essential enzyme for DNA synthesis; it catalyses reductive conversion of ribonucleotides to deoxyribonucleotides. Herpesviruses encode an RR enzyme comprised of large (RR1) and small (RR2) subunits. Previously (Dutia et al., Nature 321, 439, 1986), we showed that a synthetic peptide corresponding to the C-terminal 9 amino acids of RR2 specifically inhibited the activity of herpes simplex virus RR, and the proposed mechanism involves interference with RR1/RR2 complex formation. For RR1 and RR2, our amino acid sequence comparisons between herpesviruses, mouse, surf clam and E.coli have revealed blocks of conserved amino acids of presumed functional significance. One block with conserved glycines, present in RR1, has a predicted secondary structure of β turn α and resembles a dinucleotide substrate binding fold. The herpes simplex virus RR1, unlike any of the other RR1 subunits studied, has an additional N-terminal domain of about 320 amino acids. This additional domain has a predicted β sheet structure and its role in RR activity is being studied.

N 204 PROBING MEMBRANE PROTEIN DYNAMICS USING FLUORESCENCE ENERGY TRANSFER. T. Gregory Dewey, Department of Chemistry, University of Denver, Denver, Co 80208

A new fluorescence energy transfer technique has been developed to measure structural changes in confomational intermediates of membrane bound proteins. A novel application of modulation excitation spectroscopy has been used to determine the position of the retinal chromophore in bacterial rhodopsins. Absorption of light by the retinal in these proteins results in a photocycle in which the retinal goes through a number of intermediate states. Phase modulation of fluorescence energy transfer is used to determine the distance from a lipid fluorescent donor to the retinal for a variety of photocycle states. Concentrations of the photocycle intermediates were varied by changing the frequency of modulation of the actinic light that drives the photocycle. Measurements of retinal absorbance and fluorescence quenching due to energy transfer were made with phase-sensitive detection. Fluorescence probes from different locations within the lipid bilayer were used to measure distances to the retinal. For bacteriorhodopsin, there is a significant difference in location of the retinal in the ground state and in each of two photocycle states. This demonstrates the conformational flexibility of bacteriorhodopsin as it proceeds through its photocycle.

N 205 LINEAR AND CYCLIC β - TURNS. Gerald D. Fasman and Miklos Hollosi, Brandeis University, Waltham, MA 02254.

To investigate the chiroptical properties of flexible linear and rigid cyclic models of β-Turns, linear tetrapeptides (1) and bridged cyclic system (2) is reported. (1) Z-Gly^1-X^2-Y^3-Gly^4-O Stearyl (Methyl); (2) cyclo[Gly^1-X^2-Y^3-Gly^4-$NH(CH_2)_n$-CO], X = Pro,Gly,Ser,Ser-(OBu^t), Y = Pro,Gly,Ser,Ser(OBu^t), Asp(OBu^t), Glu(OBu^t) and Leu, n = 2 or 4. The circular dichroism (CD) of many of the linear models suggests the existence of more than one species in solution. Some models exhibit typical class B, C or D spectra [Woody (1974) in Peptides, Polypeptides and Proteins (Eds. Blout, Bovey, Goodman, Lotan) John Wiley, P. 338-350]. Z-Gly-Gly-Pro-Gly-OSt has a class C CD spectrum in acetonitrile, and a type III β-turn conformation in nonpolar solvents. Linear models, Z-Gly-Ser-Y-Gly-OSt [Y = Asp(OBu^t), Glu-(OBu^t) or Ser(OBu^t)] show a class B spectra with a very strong positive band <200 nm. Z-Gly-Ser-Ser(OBu^t)-Gly-OSt, in dil. $CHCl_3$ soln., assumes a distorted type II β-turn, held rigid by an extended system of intramolecular H-bonds. Cyclo[Gly-Ser(OBu^t)-Ser(OBu^t)-Gly-NH-$(CH_2)_4$-CO] and cyclo[Gly-Pro-Gly-Gly-NH-$(CH_2)_4$-CO] have a class B spectra in aqueous solution. FT-IR and NMR studies indicate that both models have a type II β-turn. The third bridged system, cyclo[Gly-Pro-Ser(OBu^t)-Gly-NH-$(CH_2)_4$-CO] has a class C spectrum in H_2O and adopts a type I β-turn fixed by 1←4 and O^{γ}...NH intramolecular H-bonds. This is the first class C spectrum reported for a conformationally mobile system in H_2O. A set of subspectra for β-turns, with reduced band intensities is suggested for use in the CD analysis of conformation of polypeptides in solution.

N 206 KINETIC ANALYSIS OF DIHYDROFOLATE REDUCTASE MUTANTS, Carol A. Fierke and Stephen J. Benkovic, The Pennsylvania State University, University Park, PA 16802.

A kinetic scheme is presented for the E. coli dihydrofolate reductase which predicts steady-state kinetic parameters and full time course kinetics under a variety of substrate concentrations and pH. This scheme was derived from measuring association and dissociation rate constants and pre-steady state transients using stopped-flow fluorescence and absorbance spectroscopy. The binding kinetics suggest that during steady state turnover product dissociation follows a specific, preferred pathway in which tetrahydrofolate (H_4F) dissociation occurs after NADPH replaces $NADP^+$ in the ternary complex. This step, H_4F dissociation from the E·NADPH·H_4F ternary complex, has been proposed as the rate-limiting step for steady state turnover at low pH because $k_{off} = V_{max}$. The rate constant for hydride transfer from NADPH to dihydrofolate (H_2F), measured by pre-steady state transients, has a deuterium isotope effect of 3 and is: rapid, $k_{hyd} = 950\ s^{-1}$; essentially irreversible, $K_{eq} = 1700$; and pH dependent, $pK_a = 6.5$, reflecting ionization of a single group in the active site. This kinetic scheme has been used to analyze the effects of several single amino acid substitutions in the active site of dihydrofolate reductase on individual rate constants and on steady state turnover.

N 207 PROTEIN FOLDING AND INTRACELLULAR TRANSPORT, Mary-Jane Gething*, Karen McCammon*, Karl Normington, Chuck Chao and Joe Sambrook, Department of Biochemistry and *Howard Hughes Medical Institute, University of Texas Health Science Center, Dallas, TX 75235.

The hemagglutinin of influenza virus is synthesized as monomeric subunits that are co-translationally translocated across the membrane of the rough endoplasmic reticulum (ER). We have shown using chemical crosslinking, velocity sedimentation, protease sensitivity and antibodies specific for native or denatured epitopes that folding and assembly of HA monomers into trimeric structures takes approximately 7-10 minutes and is completed before the protein leaves the ER (1). Evidence will be presented to show that folding is the rate limiting step in transport of HA from the ER. Mutants of hemagglutinin that fail to be transported from the ER are blocked at different stages of the folding pathway. Unfolded molecules of hemagglutinin are associated with BiP, a cellular protein of 77kD that has been shown previously to bind to IgG heavy chain in the ER of certain myelomas (2). The reason why assembly of native structures is required for transport of proteins through the exocytotic pathway will be discussed.

1. Gething, M.J., McCammon, K. and Sambrook, J. (1986) Cell 46, 939-950.
2. Bole, D.G., Hendershot, L.M. and Kearney, J.F. (1986) J. Cell Biol. 102, 1558-1566.

N 208 BPTI HAS A COMPACT STRUCTURE WHEN THE DISULFIDE BONDS ARE REDUCED. E. Haas and D. Amir, Dept. Life Sciences, Bar Ilan University, Ramat-Gan, ISRAEL.

A series of four BPTI derivatives, labeled with a donor of excitation energy at residue 1 and an acceptor at one of the of lysine residues (15,26,41,46) were prepared. Steady state excitation spectroscopy and fluorescence decay measurements were used to determine interresidue distance distributions. Our data indicate that reduced denatured BPTI (in 6M GuHCl) is not a random coil. Instead, a bimodal distribution of interprobe distances is obtained for the derivative labeled at residues 1 and 26. This is interpreted as showing the existance of two subpopulations, one with an extended conformation and one with a very compact conformation. The average distribution of distances ($\langle r \rangle^{\frac{1}{2}}$) between probes attached at residues 1 and 15 in reduced BPTI in shorter than that found in the native state. Thus, the N-terminal segment of the chain must stretch when the protein folds. The distances between residues 1 and 41 and 1 and 46 are wide, with large contributions from very short distances. These results show that even in 6M GuHCl, strong local interactions direct specific but flexible packing of reduced BPTI. When diluted into 0.5M GuHCl solution under reducing conditions, reduced BPTI collapses into a more compact form with high transfer eficiencies. Thus, our measurements suggest that a first step in folding may be a fast collapse into a non-native compact ("molten") globule which is then reorganized into the native oblate conformation, stabilized by the formation of the disulfide bonds and other specific interactions.

N 209 DENATURATION OF RECOMBINANT HUMAN GM-CSF BY GUANIDINE HYDROCHLORIDE. A NOVEL METHOD TO DETERMINE THE NATIVE-DENATURED TRANSITION BY THE EXPOSURE OF BURIED DISULFIDE TO SOLVENT, Lewis S. Hanna, Rosemarie Rattoballi and David R. Thatcher, Biogen Research Corp., Cambridge, MA. 02142.

The denaturation of recombinant human granulocyte-macrophage colony stimulating factor GM-CSF by guanidine.HCl was studied by a novel method which detects the degree of exposure of buried disulfide bond(s) to solvent.
T.W. Thannhauser, Y. Konishi and H.A. Scheraga, Anal. Biochem. 138, 181 (1984) have developed a quantitative method to determine the number of disulfide bonds in proteins. In this method the disulfide bonds are broken by excess sodium sulfite; one cysteine being sulfonated and the other remaining reduced as a free sulfhydryl group. Free -SH group is quantitated by its reaction with disodium 2-nitro-5 thiosulfobenzoate (NTSB) in the presence of excess Na_2SO_3 to produce 2-nitro 5-thiobenzoate (NTB).
We have shown that sulfonation of unfolded Human GM-CSF in 3M guanidine.HCl leads to the modification of its four cysteine residues. The NTB produced indicate that these cysteines are involved in two disulfide bridges. Sulfonation of Human GM-CSF in absence of denaturant was not detected, leading to the conclusion that these two disulfide bridges are buried. Plotting the initial rate of sulfonation vs. the concentration of guanidine.HCl produced a transition curves very similar to those produced by UV difference spectrophotometry. The denaturation of Human GM-CSF by guanidine.HCl indicated one transition at 2.0 * 0.1M.
In conclusion the rate of the production of NTB is dependent on the degree of reagent accessability to the disulfide bond, at fixed reaction conditions of concentrations, pH and temperature.

N 210 ELECTROSTATIC INTERACTIONS IN PROTEINS. B. Honig[1], K. Sharp[1], M.K. Gilson[1], R. Fine[2], R. Hagstrom[3]. [1]Dept. of Biochem. & Molecular Biophysics, Columbia Univ., N.Y., N.Y. 10032. [2]Dept. of Biological Sciences, Columbia Univ., N.Y., N.Y. 10027 & Dept. of Structural Biology, Brookhaven Nat'l. Lab., Upton, N.Y. 11973. [3]High Energy Div., Argonne National Laboratory, Argonne, IL. 60439.

We have recently developed a general approach to the treatment of electrostatic interactions in proteins. The protein is treated as a low dielectric medium containing real and partial charges whose coordinates are known from x-ray data. The solvent is treated as a high dielectric medium which contains a simple electrolyte. Electric fields inside and outside the protein are obtained by solving the Poisson-Boltzmann equation. The theoretical basis for this model, including its range of validity and the appropriate choice for the dielectric constant of a protein will be discussed. A finite difference algorithm which solves the Poisson-Boltzmann equation for molecules of arbitrary shape will be introduced. The method has been applied to problems of solvent screening of interatomic electrostatic interactions and the role of helix dipoles in protein stability and function. The electrostatic potential around the enzyme Cu,Zn superoxide dimutase will also be described. The role of this potential in determining the rate of substrate diffusion is considered in light of stochastic dynamics simulations that include an accurate description of the shape of the protein.

N 211 AN IONIC STRENGTH DEPENDENT CONFORMATIONAL CHANGE IN THE Fc FRAGMENT OF HUMAN IGG, Joyce E. Jentoft and Richard Rayford, Case Western Reserve University, Cleveland, OH 44106

The Fc portion of the IgG molecule mediates a wide range of physiological responses related to the humoral immune system. However, there have been few structure-function studies of the Fc fragment. The solution properties of Fc fragments derived from a human monoclonal IgG1 (Tu) and from a normal pooled human source (Cohn Fraction II) were studied by ^{13}C NMR, using reductively ^{13}C-methylated fragments (N. Jentoft & D.G. Dearborn (1979) J. Biol. Chem. 254 4359-4365). The multiple methyllysyl resonances from each Fc fragment and the wide range of the pKa values indicate that the lysine residues in Fc fragments experience a variety of microenvironments. Each resonance is sensitive to changes in solution conditions and to alterations in the local conformation of the protein. At very low ionic strengths (10 mM) the ^{13}C NMR spectra of Fc-Tu were significantly different from those of Fc-Cohn at all pH values while at ionic strengths 0.1 M, the chemical shift limits and pKa values were similar. Ionic strength dependent changes in the conformation of Fc-Cohn and Fc-Tu were confirmed by circular dichroism in the 200 to 250 nm range and by fluorescence spectroscopy. These results imply that 1) Fc fragments undergo a large change in conformation between 10 and 100 mM, and 2) that small differences in sequences can give rise to different conformation energy minima for these fragments.

N 212 CHARACTERIZATION OF INTERMEDIATES IN REFOLDING BOVINE TRYPSINOGEN. Albert Light, Thomas J. Odorzynski, and Jeffrey N. Higaki, Department of Chemistry, Purdue University, West Lafayette, IN 47907.

The in vitro folding of bovine trypsinogen was accomplished with the mixed disulfide of the fully reduced protein and glutathione, with cysteine as the disulfide interchange catalyst. Samples were quenched with time and analyzed quantitatively with isoelectric focusing and with size-exclusion HPLC. The initial open structure, with a large hydrodynamic volume and a pI of 5.2, progressed through a number of principal intermediates, which decreased in hydrodynamic volume and increased in pI, until the compact stable structure of native trypsinogen was formed. A kinetic analysis of the appearance and disappearance of the intermediates identified precursor-product relationships. The conformational changes were fewer as the structure became globular and compact. The rate determining step was a change from an apparent molecular weight of 39,000 to that of the native trypsinogen. Although many intermediates were observed in going to 39,000, the last step apparently was a single transition to the native molecule; other intermediates were not detected. The identification of the principal intermediate species immediately suggested a plausible refolding pathway.

N 213 STRUCTURE-FUNCTION STUDIES OF PHOSPHOGLYCERATE KINASE, Maria T. Mas, Zenaida E. Resplandor and Arthur D. Riggs, Beckman Research Institute, Duarte CA 91010.

Phosphoglycerate kinase (PGK) is a monomeric protein composed of two domains of approximately equal size, connected by a hinge. It has been proposed that binding of substrates initiates a hinge-bending motion of the two domains, which results in the closure of an active site cleft. A hinge region consists of the α-helix (residues 185-199), which constitutes a covalent link between both lobes, and the carboxy-terminal end of the polypeptide chain (residues 387-415). Site directed mutagenesis has been applied to study the structural basis of the domain movement. The importance of the Glu-190 — His-388 interaction for the conformational transition between the open and closed forms of PGK has been investigated by oligonucleotide-directed mutagenesis of Glu-190 (Glu → Gln and Glu → Asp) in the hinge region of yeast PGK. Both mutations resulted in a decreased catalytic efficiency of the enzyme. The Michaelis constants for ATP and 3-phosphoglycerate were essentially unchanged. These results suggest that Glu-190 is important for the domain movement, while it is not directly involved in the substrate binding. Chemical modification studies of these mutant enzymes indicate that the introduced mutations alter the conformation of the carboxy-terminal end of the molecule. Both mutants exhibited increased susceptibility to proteolytic degradation.

N 214 SELENOCYSTEINE INCORPORATION INTO PROTEINS: BOVINE, MOUSE AND HUMAN cDNAs ENCODING GLUTATHIONE PEROXIDASE. Guy T. Mullenbach, Azita Tabrizi, Bruce Irvine, Graeme I. Bell and Robert A. Hallewell. Chiron Research Laboratories, Chiron Corp., Emeryville, California 94608, USA.

Selenocysteine ($-CH_2SeH$), an analog of serine ($-CH_2OH$) and cysteine ($-CH_2SH$), is found in a number of bacterial proteins and at least one mammalian protein. Because selenocysteine possesses a redox potential and chemical reactivity different from these analogs, its artificial placement in other proteins offers a way of altering protein function and of better understanding the role its analog plays within the unaltered protein. The elucidation of this residue's mode of incorporation thus represents an important step in artificially achieving conservative replacements of active site cysteine, serine and other residues of various redox proteins and enzymes by selenocysteine via protein engineering techniques.

To this end we have focused upon glutathione peroxidases (bovine, mouse and human), a mammalian enzyme which possesses an active site selenocysteine residue wherein the Se atom is apparently cotranslationally, rather than posttranslationally incorporated, and which catalyzes the reduction of hydrogen peroxide and various alkyl, aryl and lipid peroxides as follows:

$$H_2O_2 + 2GSH \rightarrow H_2O + GSSG \quad \text{and} \quad ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$$

We present evidence derived from cDNA sequences of glutathione peroxidase from these three species which indicates that: (1) an internal, in-frame, opal nonsense codon (TGA) encodes the selenocysteine residue in these species by a nonsense suppression process and that (2) translation is cytosolic and therefore does not utilize the extended codon-reading capacity exhibited by mitochondria. In order to understand the high reading specificity of this TGA we have (3) analyzed the role nucleotide context of this TGA plays in translation by examining homology of flanking regions of these species. (4) Context features which are found to be conserved between species are compared with unrelated genes wherein TGA is utilized as a true termination signal. And (5) recombinant expression studies of glutathione peroxidase are presented which relate to methods of artificial introduction of selenocysteine into other proteins.

N 215 ROLE OF CALMODULIN IN PULMONARY TUBERCULOSIS.
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Calmodulin, a calcium binding protein was estimated from the membranes of the monocytes of patients suffering from Pulmonary tuberculosis. Patients who were diagnosed with AFB +ve in sputum and chest Xray were subjects for the study. Monocytes were homogenised and treated at 90°c for five minutes and centrifuged at 100000g for 30 minutes. Calmodulin was estimated by Radio Immuno Assay and expressed in total protein concentration. The values were as under:

Number of patients	Calmodulin in ng/5x10^5 cells/ml	Calmodulin in ngm/mg of protein
10	10.27	149.859

The same experiments are now being conducted in normal healthy controls. The effects of calmodulin to alter the membrane transport for calcium ions and its immune effects are being studied.

N 216 ANALYSIS OF THERMOSENSITIVE MUTANTS OF THE ENZYME ADENYLATE KINASE FROM ESCHERICHIA COLI. Emil F. Pai, Georg H.W. Haase, Martin Brune and Fred Wittinghofer, Max-Planck-Institute for Medical Research, Dept.of Biophysics, Jahnstrasse 29, D-6900 Heidelberg, FRG.

The adenylate kinase genes from several thermosensitive mutants of E. coli have been cloned and sequenced thereby establishing the point mutations responsible for thermolability of the gene product. Overproducing strains were constructed carrying ts-genes in the genome as well as in the plasmids. They allow rapid purification of large amounts of thermolabile protein making crystallization attempts and NMR studies feasible.

Contrary to a recent report, the proteins purified from those strains show specific activities similar to those of wild type enzymes. The overproducing strains also grow at high temperature, although much slower than does wild type. High synthesis rates probably counteract the destruction of the protein by elevated temperature. At present we are determining the kinetics of the mutant enzymes.

We have made double mutants and are presently analyzing natural revertants. In vitro mutagenesis is used to construct further mutants in order to test predictions based on these anlyses.

N 217 FACTORS AFFECTING REFOLDING WITHIN THE COLLAPSED STATE OF GLOBULAR PROTEINS, Roger H. Pain and Stewart Craig, Department of Biochemistry, University of Newcastle upon Tyne NE1 7RU, U.K.

Evidence exists for several globular proteins folding through a collapsed, globular state which has largely native secondary structure but more mobility and access of aromatic residues to solvent than the active native state to which it folds. Such a state has been studied for β-lactamase from S.aureus with the following results. 1) The conformational rearrangements can be blocked by single amino acid replacements, suggesting a critical interaction between folding units or domains. 2) Solvent viscosity has no effect on the kinetics showing that the rearrangement does not involve major changes in molecular shape. 3) Agents that stabilise the native state increase the rate of conformational rearrangement leading to the conclusion again that the equilibrium between open and collapsed states is pulled to the latter but that in terms of hydrophobic interaction the collapsed state cannot be collapsed further. 4) The rate of rearrangement is strongly pH dependent, probably dependent on a key carboxyl group. 5) A scheme will be proposed which includes a further pH dependent equilibrium between two open states which can condense to two identifiably different collapsed states.

N 218 **DISULFIDE BONDS AND THE *IN VITRO* STABILITY OF T4 LYSOZYME VARIANTS**

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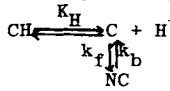
A disulfide bond has been engineered into cloned T4 lysozyme by site directed mutagenesis[1,2]. This crosslinked protein exhibits increased stability against irreversible thermal inactivation[2] as well as reversible thermal unfolding relative to the wild-type protein[3]. Replacement of free cysteines by thiol-specific agents or by mutagenesis further enhances the stability toward thermal inactivation of both crosslinked and uncrosslinked lysozymes[4,5]. Evidence suggests that free cysteines are involved in oxidation processes leading to inactive higher molecular weight aggregates[4,5]. The rate of inactivation of non-crosslinked T4 lysozymes by both conformational and oxidative pathways varies with temperature in a manner consistent with a dependence of inactivation on prior unfolding[3,5]. The disulfide appears to inhibit conformational routes of inactivation at 60-90°C, including at temperatures where the crosslinked form is unfolded. Disulfide crosslinked variants which also contain a temperature-sensitive mutation (R96H or A146T) are more stable than wild-type against irreversible thermal inactivation, although they are less stable to reversible unfolding. These results suggest that a disulfide bond can stabilize a protein toward irreversible inactivation by a mechanism independent of its effect on the thermodynamic stability of the folded chain. Construction of other disulfides, in progress, will probe the generality of this conclusion.

[1]Perry, L. J., Heyneker, H. L., and Wetzel, R., *Gene*, 1985, *38*, 259.
[2]Perry, L. J., and Wetzel, R., *Science*, 1984, *226*, 555.
[3]Wetzel, R., Perry, L. J., Becktel, W. J., and Baase, W. A., *manuscript in preparation.*
[4]Perry, L. J., and Wetzel, R., *Biochemistry*, 1986, *25*, 733.
[5]Perry, L. J., and Wetzel, R., *manuscript in preparation.*

N 219 β-LACTAMASE-CATALYZED ACYL TRANSFER TO AMINO ACIDS, R. F. Pratt and Chandrika P. Govardhan, Wesleyan University, Middletown, CT 06457.
Class A and class C β-lactamases have been shown to catalyze acyl transfer from specific depsipeptide substrates to amino acid acceptors. The steady-state kinetics of the aminolysis reaction catalyzed by the class C β-lactamase of Enterobacter cloacae P99 indicate an ordered reaction mechanism with the amino acid binding first. This result provides the first evidence for an extended substrate binding site on β-lactamases, which links them more closely, in a functional sense, to the bacterial cell wall D-alanyl-D-alanine transpeptidases.

N 220 EFFECTS OF POINT MUTATIONS ON THE ALKALINE ISOMERIZATION OF ISO-1-CYTOCHROME c FROM Sacchromyces Cerevisiae: Latha Ramdas & Barry T.Nall Department of Biochemistry & Molecular Biology, University of Texas Medical School at Houston, Houston, Texas-77225.

Replacements of the highly conserved residue, proline71, with valine, isoleucine or threonine results in a shift in the apparent pK of the alkaline transition of iso-1-cytochrome c. Equilibrium studies in which the decrease in the absorbance at 695nm is monitored with increasing pH gives the apparent pK's of 8.88, 7.18, 6.96 and 6.86 for Pro71 iso-1-ms, Val71 iso-1-ms, Ile71 iso-1-ms and Thr71 iso-1-ms respectively. Kinetic studies have been carried out by stopped-flow mixing. The data for the wild type (Pro71 iso-1-ms) are in accord with a mechanism proposed by Davis et.al (J.Biol.Chem.249, 2624)



An expanded mechanism is required to explain the data obtained for the mutant proteins.

N 221 EFFECT OF DELETING FUNCTIONAL DOMAINS OR CYSTEINE RESIDUES ON THE FOLDING AND TRANSPORT OF INFLUENZA HEMAGGLUTININ, Mark S. Segal, Jackie Bye*, Jianhua Zhang, Joe Sambrook, and Mary-Jane Gething*, Department of Biochemistry and *Howard Hughes Medical Institute, University of Texas Health Science Center, Dallas, TX 75235

In recent studies we have shown that attainment of the correct trimeric structure is required for export of newly-synthesized influenza hemagglutinin (HA) from the endoplasmic reticulum (1). The close linkage between folding, oligomerization and transport raises the question of how mammalian cells discriminate between folded and unfolded proteins and how unfolded proteins are retained in the ER. A possible candidate to carry out these functions is the cellular protein BiP, which is resident in the ER (2) and interacts with HA during the early stages of its biosynthesis. For wild-type HA this interaction is transient; however mutant HAs, as well as other proteins (2) that are defective in export to the Golgi, remain bound to BiP during the period that they remain in the ER. To distinguish whether BiP recognizes general features of an unfolded protein, such as exposed hydrophobic surfaces, or whether it recognizes specific signals, such as unpaired, reduced cysteine residues, two sets of HA mutants have been made that lack either whole functional domains or specific cysteine residues. These mutants have been expressed in CV-1 cells using SV40-HA vectors. The analysis of the folding, assembly, and intracellular transfer of the variant HA molecules will be presented.

1. Gething, M.J., McCammon, K. and Sambrook, J. (1986) Cell 46, 939-950.
2. Bole, D.G., Hendershot, L.M. and Kearney, J.F. (1986) J. Cell Biol. 102, 1558-1566.

N 222 SIMULATION OF ELECTROSTATICALLY ENHANCED DIFFUSION OF SUPEROXIDE TO SUPEROXIDE DISMUTASE. Kim Sharp[1], Richard Fine[1,2], Klaus Schulten[1] and Barry Honig[1]. [1]Dept. of Biochemistry and Molecular Biophysics, Columbia University, New York, NY, 10032, [2]Dept of Biological Sciences, Columbia University, New York, NY, 10027, and Dept. of Structural Biology, Brookhaven Natl. Lab., Upton, NY 11973. Diffusion of superoxide to the active site of superoxide dismutase in the presence of the electrostatic field of the protein is simulated by means of brownian dynamics. The field is calculated from the protein shape and charge distribution derived from the crystal coordinates by a finite difference solution to the Poisson-Boltzmann equation (Klapper et al., Proteins 1, 47 (1986)). The complex field and protein shape are accurately represented in the simulation by mapping them on a fine grid, and indexing into this grid using the instantaneous position of the superoxide trajectory. With no field, the efficiency (E) of finding the active site copper compared to the whole protein is less than 4%. At an ionic strength of 0, the field increases E by an order of magnitude. Neutralization of the catalytically important ARG 141 decreases the association rate by 30%. The rate decreases with increasing ionic strength for both the native and modified ARG 141 enzyme. Neutralization of all lysines reduces the rate 100 fold for zero ionic strength, but reverses the dependence on ionic strength. These changes in association rate with ionic strength and amino acid modification parallel the measured changes in enzyme catalytic rate, indicating that the latter depends on the field enhanced diffusion of the substrate to the active site.

N 223 INTERMEDIATES IN PENICILLIN BIOSYNTHESIS, Nicholas J. Turner, Jack E. Baldwin and Andrew E. Derome, Dyson Perrins Laboratory, University of Oxford, U.K.

A key reaction in the biosynthesis of penicillins is the oxidative cyclisation of the tripeptide δ-L-(α-aminoadipoyl)-L-cysteinyl-D-valine (LLD-ACV) to isopenicillin N, catalysed by the enzyme isopenicillin N synthase (IPNS). Kinetic studies are in accord with the reaction proceeding via a monocyclic β-lactam intermediate in which the carbon-nitrogen, but not the carbon-sulphur bond has been formed; to date no spectroscopic detection of this putative intermediate has been achieved. We have therefore used high-field n.m.r. as an approach to this problem.

(a): ^{13}C n.m.r. has been used to monitor C-N bond formation via observation of ^{13}C-^{15}N heteronuclear spin-coupling. Incubation of the appropriately labelled substrate revealed a low intensity doublet (J_{CN}=5Hz) in addition to the expected doublet (J_{CN}=5Hz) assigned to the product [^{13}C,^{15}N]-isopenicilllin N. Its structure remains to be determined.

(b): By using a combination of spin-echo difference and a selective excitation sequence, the conversion of ^{13}C-ACV to isopenicillin N has been followed by detection of only those resonances marked by a ^{13}C heteronuclear coupling.

N 224 STRUCTURE, STABILITY, AND FOLDING OF STAPHYLOCOCCAL NUCLEASE, Eldon L. Ulrich, Andrei T. Alexandrescu, Charles B. Grissom, David A. Mills, and John L. Markley, University of Wisconsin, Madison, WI 53706.

^{1}H and ^{13}C NMR spectroscopy have been used to investigate the pH and thermal stability of nuclease, originally from S. aureus (Foggi-strain), and several nuclease variants cloned and overproduced in E. coli. ^{1}H NMR chemical shifts and the pH dependence of peaks assigned to conserved histidine residues of wild-type (wt), and the single amino acid mutants D21Y (Asp-21 replaced by tyrosine), V23F, H46Y, Y85F, H124L, and H124R are all quite similar. The H124L mutant is slightly more stable than the wt protein in high pH denaturation studies. By contrast, significant spectral differences are observed between the wt protein and the single mutant F76V and the double mutant F76V,H124L, including the absence and/or large shifts in the positions of high-field methyl peaks and the presence of multiple peaks for individual histidine C_{ϵ} protons with intensities much larger than those observed for the wt protein. The thermal denaturation temperatures for the wt and F76V proteins are 54 °C and ~42°C, respectively. The F76V mutation appears to reduce the stability of the wt structure and leads to increased concentrations of protein in alternative conformations. Multiple conformations are still observed when the F76V mutation is combined with the psuedo-revertant mutation H124L. All of the proteins studied (except for the active site mutant D21Y) are catalytically active with Kcat/Km values similar to that of the wt protein. (Supported by NSF Grant DMB-841022 and USPHS NIH Grants GM35976 and RR02301.)

N 225 THE STRUCTURE OF YEAST INORGANIC PYROPHOSPHATASE Donald Voet, Chemistry Department, University of Pennsylvania, Philadelphia, PA 19104

Yeast inorganic pyrophosphatase (PPase), a dimeric protein of 64,000 kdal, crystallizes in the space group $P2_1$ with an entire dimer in the asymmetric unit. We have obtained an electron density map of PPase at 2.5 Å resolution. The X-ray data of the native enzyme and its $Hg(SCN)_4^{2-}$ derivative were collected by rotation photography at -60°C and the resulting intensities were processed by Fourier-Bessel scaling. The structure was solved by the SIR-SAS method as supplemented by the iterative applications of Wang's solvent leveling algorithm and 2-fold symmetry averaging.

N 226 NMR STUDIES OF UBIQUITIN AND ENGINEERED UBIQUITIN MUTANTS P.L. Weber, S. Brown, L. Mueller, J.A. Marsh, T.R. Butt, & D.J. Ecker Smith Kline and French Laboratories, Swedeland, Pennsylvania 19479

We have initiated NMR studies of ubiquitin with the goal of determining the different structures that this protein forms in solvents with high and low dielectric constants. A second pursuit is investigating the requirement for the extremely conserved amino acid sequence of ubiquitin, which is presumably required for the distinct folding patterns seen using CD (1). This is being investigated through analysis of the folding of ubiquitin and several engineered mutants; our progress in both pursuits will be reported.

(1) K.D. Wilkinson & A.D. Mayer, personal communication.

N 227 REFOLDING STUDIES ON RECOMBINANT HUMAN TRANSFORMING GROWTH FACTOR ALPHA, Marjorie E. Winkler, Genentech, Inc., South San Francisco, CA 94080

Transforming growth factor-α (TGFα) is a 50 amino acid polypeptide, which contains 3 disulfide bonds. The polypeptide shares a 35% homology with epidermal growth factor. The protein is expressed in *E. coli* as a fusion in which 17 amino acids of the *TrpLE* protein are joined to the amino terminus of TGFα through a methionine moiety. To prepare full length TGFα, the TGFα fusion protein was refolded and then the fusion protein removed by reaction with CNBr. The folding pathway of full length, reduced TGFα has been investigated using reverse phase HPLC to separate intermediates in the refolding pathway. The disulfide structures of several of the cysteine blocked refolding intermediates have been studied and seem to be similar to each other, containing a non-native disulfide bond between Cys16 and Cys21. The products obtained after refolding full length TGFα have been compared to those produced when the TGFα fusion protein is reduced and then refolded. The presence of the fusion peptide (which does not contain any cysteine residues) interferes with the refolding, resulting in low yields of active fusion TGFα.

N 228 STRUCTURE OF PHOSPHATE-FREE RIBONUCLEASE REFINED AT 1.25 Å RESOLUTION, Alexander Wlodawer and Gary L. Gilliland, Center for Chemical Physics, National Bureau of Standards, Gaithersburg MD 20899; Lennart Sjolin and L. Anders Svensson, Department of Inorganic Chemistry, Chalmers Institute of Technology, Gothenburg, Sweden

Phosphate-free bovine pancreatic ribonuclease crystals are isomorphous with the previously determined phosphate or sulfate substituted species ($P2_1$, a=30.18 Å, b=38.4 Å, c=53.32 Å, ρ=105.85 deg). Data were collected on two crystals using a Nicolet area detector, with 25,732 unique reflections measured. Structure was refined with Hendrickson - Konnert's program PROLSQ, to the final R factor of 0.15. Thirteen side chains had to be modeled with two alternate positions due to local disorder. All of them, except Gln 11, are located on the molecular surface. The discrete conformers have no unusual torsion angles and their interactions with the solvent and with other atoms of the protein are similar to those residues modeled with a single conformation. Structure of bound solvent was extensively modified compared to the previous model, and 187 waters and two tertiary butanol molecules were included. We observed interesting water clusters containing two alternate sets of sites, with the nearest water molecules spaced approximately 1.4 Å. It appears that the absence of an ion in the active site of ribonuclease does not change the structure of the protein in any appreciable way, with the possible exception of allowing multiple conformations of Gln 11.

N 229 ELECTROSTATIC INTERACTIONS IN PROTEINS: TOWARDS MORE ACCURATE MICROSCOPIC MODELS, S.J. Wodak, I. Couplet, D. Van Belle, M. Prevost, Universite Libre de Bruxelles, Brussels, Belgium

We analyze an alternative to the commonly used approximation for evaluating electrostatic interactions in proteins. It consists in including effects due to induced protein dipoles in addition to permanent dipoles, and takes into account departures from pairwise interactions. Results from computations performed using the refined electrostatic force-field on a number of proteins show that polarizability contributes significantly to the pattern of local electrostatic fields within the proteins matrix. Highlights of these results, in particular, on local variations in the induction effects and on the influence of the protein matrix (through induction) on the macro-dipoles of alpha helices will be discussed.

Structural Dynamics and Predictions; Peptide Design; Protein Modification

N 300 COVALENT HYDROGEN BOND MIMICS: AN APPROACH TO SHAPING PEPTIDES, Thomas Arrhenius, Lin C. Chiang, Richard A. Lerner and Arnold C. Satterthwait, Research Institute of Scripps Clinic, La Jolla, California 92037.

Protein-protein interactions form the bases of many medically important processes. The tight binding and high selectivity of proteins for one another are presumed to be the consequence of a precise matching of three-dimensional shapes. Peptides of identical shape and flexibility to the binding domains of proteins are expected to show the same binding characteristics.

We have developed a strategy for introducing secondary structure into peptides which can be adapted to solid-phase peptide synthesis. In this approach, covalent mimics of the hydrogen bond are tested for their potential to fold peptides into helices, beta-sheets and tight turns. Our results indicate that a hydrazone-hydrocarbon link ($N-NH=CH-CH_2-CH_2$, $N-NH=CH-CH=CH$), and a N-aminomethylamidine link ($N-CH_2-NH-C=N$) can serve as covalent mimics for the hydrogen bond (N-H...O=C-NH).

N 301 Enhancing Protein Stability through Point Mutations: Theoretical Studies on Sequence Modifications for Incorporation of Disulfide Bridges. V.N. Balaji*, Shashidhar N. Rao+, Stephen W. Dietrich* and Peter A. Kollman+. *Computer Assisted Drug Discovery Department, Discovery Research, Allergan, Inc., 2525 Dupont Drive, Irvine CA 92715. +Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143.

A combination of Balasubramanian ϕ-ψ plots and β-carbon distance plots derived from the known X-Ray structures of globular proteins are extended to suggest mutation sites for enhancing protein stability. These possible mutation sites include incorporation of cysteine residues (leading to disulfide bridge formation) and proline residues (in order to retain the native folding pattern of the polypeptide backbone). Novel geometric parameters have been derived to suggest probable cysteine mutation sites in a protein for formation of additional disulfide bridges. As an example, we have applied this approach to suggest probable sites of mutation in subtilisin to enhance its stability.

N 302 **PROGRESS TOWARD A COMPUTATIONAL MOLECULAR BIOLOGY**

Paul A. Bash, U. C. Singh, Robert Langridge, Peter Kollman, Dept. of Pharmaceutical Chemistry, University of California, San Francisco 94143

Thermodynamic perturbation theory implemented with molecular dynamics is described together with its application to problems in drug and protein design. The perturbation method allows one to transform or "mutate" a system between any two states that can be suitably parameterized. This method is applied to the protein thermolysin complexed with phosphonamidate and phosphonate ester inhibitors. The atomic structure of these protein-inhibitor complexes has been determined (Matthews et. al.) and they are nearly identical, yet their binding constants differ by three orders of magnitude (Bartlett and Marlowe). We have calculated the difference between the free energy of binding of these inhibitors with a result of 4.2 ± 0.54 kcal/mol which compares remarkably well with the experimental value of 4.1 ± 0.1. In preparation for the use of this method in protein design, we simulated the free energies of solvation for all classes of amino acids. Calculated values are all within about 10% of relevant experimental data and provides the necessary experience to apply these methods to protein design. An example of its use on a site-specific mutant in dihydrofolate reductase and a prediction of the effects of such a change in trypsin will be presented. The resultant close correspondence between calculated and experimental values are very encouraging and lead us to believe that this technique will provide a significant advance toward a rational approach to both drug and protein design.

N 303 EPIDERMAL GROWTH FACTOR BINDING PROTEIN: A MODEL SERINE PROTEASE FOR ENZYME ENGINEERING, Michael Blaber, Paul J. Isackson and Ralph A. Bradshaw, Dept. Biol. Chem, Univ. California, Irvine, CA 92717.
Both epidermal growth factor (EGF) and nerve growth factor (NGF) are isolated from the adult mouse submandibular gland as high molecular weight complexes containing specific glandular kallikreins (serine proteases) that may play a role in the processing of the precursors of the growth factors. These enzymes, termed EGF binding protein (EGF-BP) and NGF γ-subunit (γ-NGF), are entirely exclusive in these interactions. Noting that previous reports of EGF-BP were inconsistent with our partial protein sequence data, we have reexamined authentic preparations of EGF-BP and isolated a corresponding full length cDNA. This sequence is significantly different from any previously reported sequence for EGF-BP. Both the nucleotide and amino acid sequences of this EGF-BP are quite similar to those of γ-NGF, showing 92% and a 85% identities, respectively. Not unexpectedly, model building suggests very similar 3-D structures. As members of a large family of glandular kallikreins with a high degree of homology yet distinct substrate specificities, they are excellent models for the application of site-directed mutagenesis to determine the molecular basis of their growth factor selectivity. Supported by USPHS NS19964 and DK32465.

N 304 HELICAL FORMATION OF AN ISOLATED FRAGMENT FROM BOVINE GROWTH HORMONE, D.N. Brems, S.M. Plaisted, and E.W. Kauffman, Control Division, The Upjohn Company, Kalamazoo, Michigan 49001

Folding studies of bovine growth hormone (bGH) have demonstrated that the secondary structure (helix) is stable in the absence of tertiary structure (Brems et al. 1985, Biochemistry 24, 7662). As a result, we have searched for small peptides derived from bGH that when isolated have measurable helix structure.

One peptide, 109-133, was found to be near 100% helical in aqueous solutions as determined by circular dichroism. The helical content of this fragment is dependent on pH and peptide concentration. This helical structure is most likely amphipathic. We suggest that the unexpected helical stability of this peptide is due to specific electrostatic interactions and intermolecular packing of the hydrophobic surfaces of the amphipathic helix.

N 305 PROTEIN ENGINEERING OF SUBTILISIN: PROTEASES OF ENHANCED STABILITIES Bryan, P., Pantoliano, M., Rollence, M., Wood, J., Howard, A., Ladner, B. and Poulos, T., Genex Corporation, Gaithersburg, MD 20902 We have created subtilisin variants of enhanced stability using two complementary strategies. The first was to design changes based on a highly refined 1.3 angstrom structure of wild type subtilisin. These site-directed mutations have focused on building in disulfide bonds and modifying calcium binding sites known to be critical for stabilization. We have characterized more than a dozen disulfide variants by measuring resistance to thermal inactivation and by determining the temperature of unfolding using differential scanning calorimetry (DSC). In two cases the disulfide bond-containing variants were significantly more stable than wild type. We also employed a second strategy of introducing mutations in vitro into random sites within the cloned subtilisin gene and screening for proteases of increased stability. A number of these were sequenced and further characterized kinetically and by DSC. The crystal structures of five stable variants have been determined to 1.8 angstroms with R-factors of 14%. The x-ray data suggest why these variants are more stable. Using the information obtained from site-directed and random mutagenesis approaches we have constructed multiple site-directed variants which exhibit the combined stabilities of the single-site variants. To date our most stable subtilisin variants are more than fifty-fold more stable than wild type.

N 306 ANALYSIS OF PROTEIN TARGETING TO REGULATED SECRETORY GRANULES IN AtT-20 CELLS: THE ROLE OF THE N-TERMINAL DOMAIN OF TRYPSINOGEN. T.L. Burgess, L. Matsuuchi, C.S. Craik & R.B. Kelly, Dept of Biochemistry & Biophysics, University of California, San Francisco, CA 94143

AtT-20 cells (an endocrine cell line derived from mouse pituitary) maintain two pathways for secretion (Gumbiner & Kelly. 1982. Cell 28, 318). One of these paths involves classical dense core secretory granules and is regulated by secretagogues. We have shown previously that the heterologously expressed exocrine protein, trypsinogen, is targeted into these regulated secretory granules in AtT-20 cells (Burgess et al. 1985. JCB 101, 639). To identify the information contained within this protein which targets it to the regulated pathway, oligonucleotide-directed mutagenesis has been used to create mutations in trypsinogen. Among the mutants we have made is an exact deletion of the N-terminal "pro" sequence, as well as a signal peptide swap with a constitutive protein. Both of these mutant proteins are efficiently synthesized and secreted by AtT-20 cells. We are currently characterizing the secretion properties of these and other mutant proteins made in AtT-20 cells.

N 307 PRODUCTION OF SUBTILISINS HAVING INCREASED ALKALINE STABILITY BY RANDOM MUTAGENSIS METHODS, Brian C. Cunningham*, Robert M. Caldwell[+] and James A. Wells*. *Department of Biocatalysis, Genentech, Inc. and [+] Research Department, Genencor, Inc., So. San Francisco, CA., 94080.

Subtilisin, a bacterial serine protease, undergoes irreversible autolytic inactivation at high pH. Modifications of a α-thiol misincorporation mutagenesis method (1), on the cloned B. amyloliquefaciens subtilisin gene (2) in M13, allowed highly efficient (>30%) random mutagenesis of the entire 1kb coding sequence. A replica plating method was developed to screen for mutant subtilisins that had increased resistance to inactivation at alkaline pH. Several mutants of increased stability were isolated, and when combined produced double and triple mutants of even greater alkaline stability. Random mutagenesis by oligonucleotides (RMBO) was applied over these sites in 30 codon segments to further focus the mutagenesis events. The combination of these methods has produced a multiply mutated subtilisin having greater stability to autolytic inactivation at alkaline pH.

1. Shortle, D. and Lin, B. (1985) Genetics 110, 539.
2. Wells, J.A., Ferrari, E., Henner, D.J., Estell, D.A. and Chen, E.Y. (1983) Nucleic Acids Res 11, 7911.

N 308 FREE ENERGY OF DIMERIZATION OF GRAMICIDIN HYBRID CHANNELS. J.T. Durkin[1], R.E. Koeppe II[2], and O.S. Andersen[1] ([1]Dept. Physiol. Biophys., Cornell Univ. Med. Coll., New York, NY 10021; [2]Dept. Chem. Biochem., Univ. Arkansas, Fayetteville, AR 72701).

The linear gramicidins form symmetric dimeric channels in bilayer membranes. The formation of hybrid channels by two chemically dissimilar gramicidin analogues demonstrates that the two parent channels have the same structure (Durkin et al., 1986, Biophys. J. 49: 118-121). Because the sequence strictly alternates between L and D amino acids, analogues differing in length by one residue form hybrid channels with a gap of one hydrogen bond. Single-channel data can measure the energetic cost of this gap. The number of hybrid channels relative to the number of parent channels gives the ratio of formation rate constants for the hybrid channel relative to the parent channels, and thus the difference in free energy of activation between the hybrid and the parents. The average lifetime of each channel is the inverse of the dissociation rate constant; the ratio of kinetic constants then gives the difference in equilibrium free energy between the hybrid and the parents. The concentration of monomers is unknown and does not matter, as all channel types are observed in the same reaction mixture (membrane). For three hybrid channels formed by analogues differing in length by one residue, the difference in free energy of activation is near zero, suggesting that no refolding of the peptide is required to form the hybrid channel. The equilibrium free energy difference is $\simeq$ +2 kcal/mole, a reasonable value for a hydrogen bond in an apolar environment. These data illustrate one way to measure differences between a modified protein (produced, e.g., by site-directed mutagenesis) and a reference structure.

Supported by NIH grants GM 21342 and GM 34968.

N 309 PROTEIN STABILITY AND SURFACE HYDROPHOBICITY, Anthony L. Fink, Brian Goldman, Debbie Joy and Michael Waldren, Department of Chemistry, University of California, Santa Cruz, CA 95064.
The relationship between thermal stability and the hydrophobic, neutral polar and charged fractional surface areas in the unfolded and native states has been investigated. In order to minimize other effects we compared the stability of members of the trypsin family, which have varying fractional surface hydrophobicity but similar tertiary folds. Points on the molecular surface were assigned scores based on the attributes of the nearest atom. Atom hydrophobicities were determined on the basis of the electronegativity of the adjacent atoms. The effect of hydrophobic (ethanol), "neutral" (ethylene glycol) and polar (sorbitol) cosolvents on the position of the thermal unfolding transition was determined. A number of possible relationships between hydrophobicity and charge, in the unfolded and native states, were investigated. There is a strong positive correlation between stability and the ratio of the relative hydrophobic area buried on folding and the fraction of the native surface which is charged. The net stability is far more sensitive to the hydrophobicity of the solvent than to the fraction of hydrophobic surface in either native or unfolded states. Implications of these findings to protein engineering will be discussed.

N 310 EXPRESSION OF PROTHROMBIN AND THE B CHAIN OF THROMBIN IN ESCHERICHIA COLI AND YEAST. Dana M. Fowlkes, Fayanne E. Thorngate and Harold Smith. University of North Carolina at Chapel Hill, NC 27514
Active thrombin consists of two polypeptides, the A and the B chains, linked by a disulfide bond. The B chain contains the Ser, Asp, and His residues common to serine proteases. Starting from a cDNA clone of bovine pre-proprothrombin and using site-directed deletion mutagenesis, a construct was made in an M13 phage vector which expresses only the B chain in E. coli. Thrombin specific amidolytic activity was detected in lysates from cells infected with phage containing the B chain construct. Fibrinogen, a physiological substrate, acts as a competitive inhibitor in these assays. The biosynthetic B chain from E. coli also interacts with heparin and antithrombin III, but not low molecular weight inhibitors such as DAPA or NAPAP. These data indicate that the B chain contains many, but probably not all, of the determinants for thrombin's various biological activities. There was no detectable activity in this lysate unless the proteins were denatured with 6 Molar guanidinium HCl and allowed to refold in high salt buffer. To increase the yields of synthesized polypeptides, we have expressed prothrombin and the B-chain of thrombin in yeast. Unlike the polypeptides made in E. coli, prothrombin from yeast is biologically similar to bovine prothrombin in all respects tested except for its ability to be activated with Factor Xa.

N 311 STRUCTURAL DYNAMICS AND REACTIVITY IN HEMOGLOBIN AND MYOGLOBIN, Joel M. Friedman, AT&T Bell Laboratories, Murray Hill, NJ 07974.
The equilibrium structures and properties of hemoglobin and myoglobin have been extensively characterized. As a consequence these proteins are ideal model systems for exploring on a microscopic level the relationship between structural dynamics and functional properties. Modulation of ligand binding properties by the protein structure has been shown to occur at the heme. Using resonance Raman scattering it has been shown that the iron-proximal histidine linkage is both responsive to protein structural changes and correlates with changes in the potential energy barrier controlling rebinding. Focussing on this structure sensitive and functionally important degree of freedom we have used time resolved Raman scattering to probe transient species of Hb and Mb from 25 picosecond to 10's μ seconds subsequent to photodissociation of the ligand bound starting material. These studies suggest a hierarchy of dynamical process each of which influences the functional properties. Currently Raman hole burning experiments at both ambient and cryogenic temperatures are being pursued to determine the relationship between both static and dynamic conformational heterogeneity and functional properties.

N 312 **STEADY-STATE AND TIME-RESOLVED FLUORESCENCE SPECTROSCOPY OF THE SINGLE TRYPTOPHAN IN BOVINE GROWTH HORMONE.** Henry A. Havel, Paul A. Elzinga and E. Wayne Kauffman, Control R&D, The Upjohn Company, Kalamazoo, MI 49001

The study of protein structure and dynamics has received considerable attention lately from both a theoretical and experimental viewpoint. One way to approach these studies experimentally is to compare the structure and dynamics for a protein under different solution state conditions, such as the comparison of native and unfolded structures; a more useful comparison would involve native and folding intermediate structures. Such an approach can be followed with bovine growth hormone (bGH, somatotropin) as it has been determined previously that the equilibrium folding process for bGH involves at least one monomeric and one self-associated intermediate. A characterization of structure and dynamics has been obtained by the analysis of steady-state and time-resolved fluorescence data for the single tryptophan in bGH in several structural forms (native, monomeric intermediate, self-associated intermediate, unfolded, etc.). Results obtained thus far include: 1) steady-state fluorescence emission differences among the forms indicate intramolecular quenching of fluorescence in the native state which is much reduced in the other forms, 2) fluorescence lifetime measurements in the native form reveal three components (0.5, 3 and 7 ns) while in the unfolded form only two components are found (2 and 4 ns), and 3) addition of extrinsic quenching agents shows that the tryptophan in all bGH forms is more accessible to a nonpolar quencher (trichloroethanol) than to a polar nonionic (acrylamide) or ionic (iodide) quencher. A structural interpretation of the folding process for this protein will be presented as deduced from these and other observations of the spectroscopic behavior of the tryptophan residue.

N 313 IMPROVED HYDROPHILICITY PLOTTING METHOD FOR MEMBRANE PROTEINS. Thomas P. Hopp, Immunex Corporation, Seattle, Washington. 98101.

Hydrophilicity (or hydrophobicity; hydropathy) plotting methods are used for determining surface oriented or buried portions of protein sequences. The profiles generated by these procedures have an optimal match to the actual structural features of globular proteins when the averaging group length (window) is set at six amino acids. However, it has become common practice to use wider windows, ranging from 11 to 21 residues to facilitate the identification of membrane spanning segments of proteins. This practice is useful for the membrane spanning segments, but destroys all of the information that could be obtained concerning the globular portions of the membrane protein being studied. We have developed a procedure that makes it possible to identify transmembrane segments while retaining the six residue window regions of a given sequence. The key to this method is the realization that glycine, serine and threonine residues all exhibit a dual nature, and can readily be accommodated at the surface or in buried portions of protein structures. When this is taken into account during hydrophilicity analysis, plots are generated that are useful both for locating membrane associated regions, and for identifying other key features such as antigenic sites and other surface features of proteins.

N 314 GEOMETRICAL PRINCIPLES GOVERNING STRAND PARITY IN EIGHT STRAND PARALLEL BETA BARREL PROTEINS, I. Lasters, Ph. Alard, S. Wodak and M. Claessens, Plant Genetic Systems Universite Libre de Bruxelles

Analysis of geometrical properties and packing arrangements of the beta sheet portion in seven distinct parallel beta barrel domains has been carried out. The principal features of these structures arise from the right handed twist of the beta sheets and from the manner in which they fold upon themselves. The preferred packing mode between sheets is orthogonal. But unlike orthogonal sheet packing with anti-parallel strands (Chothia & Janin, 1982), those with parallel strands clearly display geometrical properties of regular parabolic hyperboloids (Salemme, 1983). The closed nature of the hyperboloid surface, and topologiacl requirements of sheet packing are shown to impose constraints on sound parity in the barrel.

N 315 HIGH LEVEL EXPRESSION OF BIOLOGICALLY ACTIVE HUMAN ATRIAL NATRIURETIC PEPTIDE FROM MULTIPLE JOINED GENES IN E. COLI

Michael Lennick, Joel Haynes and Shi-Hsiang Shen, Connaught Research Institute, Toronto, Canada. A method is described which allows human Atrial Natriuretic Peptide (hANP) to be synthesized in stable form and with high yield in E. coli. In the final expression system, eight copies of the synthetic hANP gene were linked in tandem, separated by codons specifying a short removable linker. This sequence was in turn joined to the 3' end of a fragment containing the lac promoter and a leader sequence coding for the first seven N-terminal amino acids of beta-galactosidase. The expressed multidomain protein accumulated intracellularly into stable inclusion bodies and was easily purified by urea extraction of the insoluble cell fraction. The purified protein was cleaved into monomers and trimmed to the authentic C-terminus by successive enzyme digestions and then folded by gentle oxidation with potassium ferricyanide. Assays of the folded material in the non-diuretic rat assay have shown its biological activity to be comparable to that of chemically-synthesized hormone.

N 316 COMPUTER MODELING OF YEAST iso-1-CYTOCHROME c - YEAST CYTOCHROME c PEROXIDASE COMPLEXES. Vanessa R. Lum, Gary D. Brayer, A. Grant Mauk, and Michael Smith, Life Sciences Research Laboratories, Eastman Kodak Company, Rochester, NY 14650, and the Department of Biochemistry, University of British Columbia, Vancouver, B. C. V6T 1W5 Canada.

To facilitate interpretation of functional studies on wild-type (WT) and mutant forms of bakers' yeast iso-1-cytochrome c (CYT C) (e.g., Pielak et al., Nature, 313, 152 (1985); Hampsey et al., J. Biol. Chem., 261, 3259 (1986)), a detailed model for the three-dimensional structure of the complex of WT-CYT C and bakers' yeast cytochrome c peroxidase (CCP) was constructed. The model was built using the published coordinates for CCP (Finzel et al., J. Biol. Chem., 259, 13027 (1984)); x-ray data recently obtained for CYT C; and the protein-protein residue pair interactions specified by Finzel and Poulos (Peptide Prot. Rev., 4, 115 (1984)). The resulting coordinate set was then subjected to molecular mechanics calculations (Brooks et al., J. Comp. Chem., 4, 187 (1983)) as implemented in the program BIOGRAF (Biodesign, Inc., Pasadena, CA). The structures and surface properties of mutants at selected surface residues of CYT C (e.g., positions 87, 43, and 25) have also been calculated. The possible effects of the mutation-induced alteration in protein surface on the interaction of CYT C with CCP will be discussed. (Supported in part by NIH Grant GM-33804.)

N 317 THE ROLE OF INTRAHELICAL ION-PAIRS IN α- HELIX FORMATION BY SYNTHETIC PEPTIDES.

Susan Marqusee and Robert L. Baldwin, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305.

Synthetic peptides have been designed to investigate systematically the role of intrahelical hydrogen-bonded ion-pairs in α-helix formation. The design was based on a simple alanine backbone plus the interaction under investigation. Amino acids of opposite charge (glu and lys) were placed three or four residues apart to introduce potential hydrogen-bonded ion-pairs. The resulting 16- and 17- residue long peptides are predicted to be too short to show measurable helicity by the Zimm-Bragg equation using parameters obtained from host-guest studies on long polymers.

The peptides can form relatively stable isolated helices in water (85% helix under optimum conditions). Helix formation is sensitive to the spacing of the oppositely charged residues, indicating that the geometry and energetics of intrahelical ion-pair formation are specific. The pH titration and salt sensitivity of helix content for a peptide with glu-lys pairs four residues apart is consistent with stabilization of the helix by hydrogen-bonded ion-pairs. The titration of a peptide with glu-lys pairs three residues apart is not. Consistent with these results, Maxfield and Scheraga (Macromolecules 8, 491-493 (1975)) have noted that glu residues in protein helices show a statistical preference for positively charged amino acids four residues away. These model peptides should allow the construction of predictive schemes based on realistic model compounds.

N 318 STRUCTURAL AND DYNAMIC STUDIES OF BACTERIOPHAGE T4 LYSOZYME USING PROTON NMR AIDED BY ^{13}C AND ^{15}N ISOTOPIC LABELING. Lawrence P. McIntosh, David C. Muchmore, and Frederick W. Dahlquist, Inst. of Molecular Biology, Univ. of Oregon, Eugene, OR 97403; Richard H. Griffey, Univ. of New Mexico School of Medicine, Albequerque, NM 87131; and Alfred G. Redfield, Dept. of Biochemistry, Brandeis Univ., Waltham, MA 02254.

We have developed a strategy to investigate the structure and dynamics of selected, isotopically labeled regions of larger proteins (up to 40kD) using proton NMR. The resonances from protons directly bonded to ^{13}C or ^{15}N nuclei are detected in two-dimensional ^{1}H vs. ^{13}C or ^{15}N "forbidden echo" (multiple quantum) spectra. Furthermore, the nuclear Overhauser effects from or to isotopically tagged protons are selectively observed in 1D isotope-directed or -detected NOE (IDNOE) spectra or in 2D isotope-directed NOESY spectra. In the forbidden echo spectrum of uniformly ^{15}N-labeled T4 lysozyme (18.7kD), we have resolved ca. 160 resonances from ^{15}N-bonded protons in the backbone and sidechains of this protein. This spectrum can be simplified by partial $^{1}H/^{2}H$ exchange. These resonances are identified by amino acid class through selective incorporation of ^{15}N-labeled amino acids, and are assigned to specific residues by multiple ^{13}C and ^{15}N labeling, IDNOE measurements, and mutational substitutions. We are presently studying the structure and dynamics of wild type and temperature sensitive T4 lysozymes by using forbidden echo spectroscopy to measure the the amide and indole hydrogen exchange kinetics of these proteins. This strategy is very general and will allow NMR studies of larger proteins of known sequence in a selective, site-specific manner.

N 319 OVERPRODUCTION AND ENZYMATIC CHARACTERIZATION OF NATIVE AND MUTANT RNaseT1 ENZYMES IN *E. coli*., Yvonne McKeown, Chris Thoen, Patrick Stanssens and Gaston Matthyssens, Plant Genetic Systems, Gent, Belgium

RNaseT1 of *Aspergillus oryzae* is a guanyloribonuclease which hydrolyses RNA endonucleotically to 3′ phosphomono and oligonucleotides. By virtue of the availability of a body of information concerning its chemico-biological properties and 3-dimensional structure, RNaseT1 lends itself readily to studies on protein structure-function relationships. We have previously described the cloning of a synthetic RNaseT1 gene (R. Quaas et. al., in press) and we now report its successful overproduction in *E. coli*. Expression has been achieved by placing a lacZ-RNaseT1 fusion gene under the control of the phage lambda P promoter. Following temperature of the promoter, the B-galactosidease-RNaseT1 fusion protein is present at 5-10% total cell protein. This hybrid protein is enzymatically inactive and is precipitated intracellularly. Cyanogen bromide cleavege of the purified fusion protein and renaturation of the products yields material which on the basis of three different assays we conclude to be native RNaseT1 enzyme. Using the alternate selection method for site-directed mutagenesis developed in this laboratory (P. Stanssens et. al., in preparation), we have introduced a number of mutations into the gene, addressing questions on the catalytic mechanism and substrate specificity of the protein. Biochemical and enzymatic characterization of some of these mutants will be presented.

N 320 THE CONSTRUCTION OF NEW PROTEINS - DESIGN, SYNTHESIS AND CONFORMATIONAL PROPERTIES OF POLYPEPTIDES WITH 'βαβ'-TOPOLOGY. M. Mutter, Institute of Organic Chemistry, University of Basel, CH-4056 Basel, Switzerland.

The construction of artificial proteins is one of the most fascinating prospects in the field of peptide chemistry. According to a general strategy based on characteristic topological features of proteins, the design of polypeptides adopting a well defined three-dimensional conformation is achieved by the assembly of amphiphilic peptide blocks with stable secondary structures (1). The amphiphilic nature of the helical and β-structure blocks acts as a major driving force for intramolecular folding. The present state of art in chemical peptide synthesis and structure modification is implicated opening new perspectives for the de novo design of artificial proteins. The design, synthesis and conformational properties of a number of folding units with βαβ-topology are presented. The propensity of this concept for the design of conformational epitopes is discussed (2).

(1) M. Mutter, Angew. Chem. Int. Ed. Engl. 24, 639 (1985).
(2) M. Mutter, K.-H. Altmann, K. Müller, S. Vuilleumier, Th. Vorherr, Helv. Chim. Acta 69, 985 (1986).

N 321 The Investigation of α-helix Formation in Peptides by Two-Dimensional NMR Spectroscopy.
John J. Osterhout, Jr. and Robert L. Baldwin. Stanford University Medical School, Stanford, California, 94305

Helix formation in isolated peptides has been studied in earlier work by synthesizing analogues of the C peptide (residues 1-13 from RNase A , produced by cyanogen bromide cleavage) and measuring the helix content by circular dicroism. These studies are aimed at understanding the intrahelical sidechain interactions that stabilize the helix. One result of this work is the production of peptides with increased helix content (up to approximately 80% helix) at low temperatures. These peptides are ideal for the study of helix formation by two-dimensional NMR. There are several goals: to observe alpha helix formation by a means other than CD, to detect specific interactions, and to localize alpha helix formation along the peptide. The basic procedure is to measure the nuclear Overhauser effect (NOE) between nuclei which would be expected to be in proximity only when the α-helix is present. The interactions of interest are designated $d_{na}(i, i+3)$,$d_{ab}(i, i+3)$ $d_{nb}(i, i+3)$and $d_{nn}(i,i+1)$ (The notation is according to Wüthrich et. al. (1984) J. Mol. Biol. 180:715-740). These interactions represent NOE's across one turn of the helix (between the ith residue and the amino acid three residues along the chain) except the $d_{nn}(i,i+1)$ interaction which is between consecutive amide protons. Phase-sensitive 2D nuclear Overhauser (NOESY) experiments are employed to detect these interactions. The initial results of phase-sensitive NOESY experiments run in D_2O indicate that it is possible to detect some $d_{ab}(i,i+3)$ NOE's at low temperature (where the helical conformation is highly populated) and that these are not present in the control experiments performed at high temperature. Other NOEs are detected which indicate that certain side chains have restricted motion under conditions of alpha helix formation. Experiments are in progress in H_2O to detect the NOEs arising from the amide protons.

N 322 COMPUTER SIMULATION OF DIFFUSE X-RAY SCATTERING FROM PROTEIN CRYSTALS.
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Motions of proteins in crystals give rise to two effects in X-ray diffraction patterns. There is a general fall-off in the intensity of the Bragg diffraction with scattering angle, and 'diffuse' scattering appears in the background. In principle, this X-ray scattering between the strong Bragg peaks contains information about the deviations of the protein structure from its average.

Studies of the diffuse scatter of tropomyosin crystals have yielded information about the direction, extent, and coupling of motions of these molecules in the lattice (Boylan and Phillips, Biophys. J., 49, p. 76-78). It may be possible to examine the dynamic modes of globular proteins, as well. Towards this goal, computer programs have been developed for simulating the diffuse X-ray scattering from myoglobin crystals using various models for the dynamic behavior of the protein. Different models result in different diffuse scattering patterns. If suitable corrections for contributions of incoherent (Compton) scattering and the scattering from the bulk water can be devised, these patterns can be compared with actual diffuse scattering measurements to yield information about the dynamic behavior of the protein in the lattice.

Supported by NIH grant AM32764. GNP is an Established Investigator of the American Heart Association.

N 323 THE TRANSFECTED HUMAN GH GENE DISPLAYS ALTERNATIVE SPLICING; JHARNA RAY, BRUCE A KUO, STEPHEN A. LIEBHABER AND NANCY E. COOKE, The Howard Hughes Medical Institute and the Departments of Human Genetics and Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

Transgenic cell lines containing the human growth hormone (hGH) and hGH-variant (hGH-V) genes have been established in order to study the expression of these two highly homologous genes. These genes were inserted into a bovine papillomavirus shuttle vector under the transcriptional control of the mouse metallothionein gene promoter and the resultant recombinants were transfected into mouse C-127 cells. The cells containing the hGH gene secrete two sizes of hGH, a major species of 22 kDa and a minor species of 20 kDa in the same relative proportions as synthesized by the human pituitary. S1 nuclease analysis of the mRNA from these cells confirms that the 20 kDa hGH is encoded by an alternative splicing product of the hGH gene transcript in which the normal exon 3 splice-acceptor site is bypassed for a secondary site 15 codons within the exon. In contrast to these results, analysis of cells expressing the hGH-V gene, which is identical to the hGH gene at both the normal and the alternative exon 3 splice-acceptor sites does not result in the synthesis of a 20 kDa product. Furthermore, substitution of exon 3 and its flanking intron sequences within the hGH gene for the corresponding segments of the hGH-V gene eliminates the synthesis of the 20 kDa product. These results firmly establish that both the major 22 kDa and the minor 20 kDa forms of hGH synthesized by the human pituitary are the products of a single hGH gene and that the alternative splicing of the hGH gene transcript is regulated by sequences outside the splice site consensus sequence.

N 324 SITE-DIRECTED MUTAGENESIS OF T4 ENDONUCLEASE V - ALTERATIONS WHICH DECREASE DIMER SPECIFIC BINDING AND GLYCOSYLASE ACTIVITIES BUT NOT THE ENDONUCLEASE ACTIVITY, Adrian Recinos, III and R. Stephen Lloyd, Vanderbilt University, Nashville, TN 37232.

The DNA sequence of the denV gene of bacteriophage T4 which encodes the DNA repair enzyme, endonuclease V, was previously reconstructed behind the hybrid λ promoter $O_L P_R$ in a plasmid vector. The $O_L P_R$-denV sequence was subcloned in M13mp18 and used as template for the construction of site specific mutations in the denV structural gene in order to investigate structure/function relationships between the primary structure of the protein and its various DNA binding and catalytic activities. The Lys-130 position of the wild-type endonuclease V has been postulated to be involved in the apurinic endonuclease activity of the enzyme. The denV codon for K130 was changed to H130 or G130, and each denV mutant was subcloned into a pEMBL expression vector. The following parameters were examined for these cells or for extracts from cell lysates: (1) colony forming ability after UV irradiation, (2) kinetics of phosphodiester bond scission at pyrimidine dimer sites, (3) kinetics of N-glycosylase activity to produce AP sites at pyrimidine dimers, and (4) binding affinities for pyrimidine dimer sites in DNA. The three endonuclease V gene products consistently gave parallel levels of relative function - K130>H130>G130>>control denV. Cell lysates containing K130, H130 and G130 have equal endonuclease activities for DNA containing apurinic sites. Cells harboring K130, H130 and G130 denV genes accumulate equivalent levels of denV-specific RNA. These results suggest that the ability of the mutant enzymes to bind DNA at dimer sites has been compromised. However, this dimer-specific binding appears not required for the AP-endonuclease activity. Supported by NIH ES00267.

N 325 PREDICTION OF PROTEIN TERTIARY STRUCTURE FROM Cα ATOMIC COORINATES

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Modelling of protein structures is usually based upon information gained from known protein structures and refined by various energy minimization techniques.The procedure is useful for small changes but the accuracy of models based on regions of low homology is unknown. This work describes the modelling of four known proteins and thus gives an indication of the reliability of the procedure. The Cα coordinates were extracted from published data sets and the backbone structure generated by searching a database of known structures for regions of close structural homology (DGNL option of FRODO, A. Jones, 1986). Sidechains were first added according to observed interaction (aromatic, ionic and hydrogen bonding) distributions and 'template' criteria. Remaining sidechains were built in according to secondary structure subsets of the χ_1,χ_2 distribution profiles (after Janin, 1978). Clashes between sidechains were resolved by fixing the position of the sidechain with the highest statistical probability and allowing the other to seek an energetic minimum by scanning through all torsion angles (D. White,1986). Supersecondary structures were packed before the contact areas were optimized. Finally, a global energy minimization was applied.

The following parameters were assessed to determine the accuracy of the model: 1) the RMS deviation of the atomic coordinate positions for global and secondary structure domains, 2) the number and type of sidechain contacts, and 3) solvent accessiblity and contact area.

Results will be presented for representative proteins: myoglobin (α), immunmoglobulin (β), lysozyme (α+β), and flavodoxin (α/β).

N 326 STRUCTURAL CONSEQUENCES OF CLEAVAGE OF THE REACTIVE SITE PEPTIDE BOND IN TURKEY OVOMUCOID THIRD DOMAIN, Andrew D. Robertson, Gyung Ihm Rhyu, William M. Westler and John L. Markley, Dept. of Biochem., Coll. of Ag. and Life Sci., Univ. of Wisconsin-Madison, Madison, WI 53706. Turkey ovomucoid third domain (M_r 6000) is a potent serine proteinase inhibitor. In the presence of proteinase, virgin ovomucoid third domain is converted to a modified form through reversible cleavage at a single reactive site peptide bond (...Leu-18 - Glu-19...). Nuclear magnetic resonance (NMR) spectroscopic studies are being conducted to compare the structures of the virgin and modified third domain. ^1H NMR assignments have been made for nearly all of the backbone and side chain protons of both turkey ovomucoid third domain and turkey ovomucoid third domain in which the reactive site peptide bond has been hydrolyzed with proteinase B from Streptomyces griseus. Side chain spin systems were identified via $^1H\{^1H\}$ chemical shift correlation spectroscopy (COSY), relayed COSY, and two-dimensional homonuclear Hartmann-Hahn spectroscopy. COSY and two-dimensional nuclear Overhauser (NOESY) experiments were used for sequence specific assignments. Reactive site bond cleavage does not appear to affect secondary structure elements, as determined from NOESY data, although chemical shift changes for protons throughout the linear sequence indicate possible changes in the tertiary structure. Amide proton exchange studies are underway and will be used to assess the effects of reactive site cleavage on the dynamical properties of the virgin and modified third domain.

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N 327 ANTI-SENSE PEPTIDES - DESIGNING RECOGNITION MOLECULES, Yechiel Shai, Michael Flashner*, and Irwin Chaiken, National Institutes of Health, Bethesda, MD 20892 and *Triton Biosciences, Alameda, CA 94501

Recent observations of binding of synthetic anti-sense peptides (encoded in the anti-sense strand of DNA) to sense peptides (encoded in the corresponding sense DNA strand) have raised questions about the structural nature of anti-sense peptide interactions and about the possibility to use these for the design of macromolecular recognition molecules. To confirm the generality of sense/anti-sense peptide interaction and to obtain an understanding of its underlying mechanistic principles, we have used the experimental approach of analytical high performance affinity chromatography to determine equilibrium binding characteristics, with bovine pancreatic RNase S-peptide as the initial sense peptide test system. The data obtained so far demonstrate unambiguously that anti-sense peptides can interact with this sense peptide with substantial affinity and specificity and definable (but not necessarily 1:1) stoichiometry. Results with synthetic mutants and truncated forms suggest that contact of sense with anti-sense peptides may well be multi-site, dependent on the hydropathic characteristics of residues along the sequence. The results do not conform in an obvious way to the conventional view of native peptide and protein recognition by folding of the latter into compact, ordered conformations with interaction surfaces involving a relatively few residues. Nonetheless, anti-sense peptides may well provide a useful tool to help learn more about the forces that produce peptide and protein interaction in general and therein can act as a basis for recognition peptide design.

N 328 GENETIC SELECTIONS FOR THE GENE V PROTEIN OF BACTERIOPHAGE F1. Thomas C. Terwilliger, University of Chicago, Chicago, IL 60637 and Wilder Fulford, Rockefeller University, New York, NY 10021.

The gene V protein of bacteriophage f1 is a small dimeric protein which cooperatively binds to single-stranded DNA. Each subunit contains 87 amino acids and consists almost entirely of beta structure. As a first step in developing an enrichment procedure for obtaining gene V proteins defective in folding or stability, positive and negative genetic selections for the activity of the protein have been developed.

A plasmid which contains gene V of bacteriophage f1 under control of the tac promotor has been constructed. A positive genetic selection based on complementation of an amber (V) mutant of phage f1 was developed. Additionally, a negative selection was developed based on the observation that the gene V protein inhibits early stages of E. coli infection by single-stranded viruses. The positive and negative selections yield 300-fold and 2000-fold enrichments for and against active gene V protein, respectively.

N 329 STRUCTURAL STUDIES OF A SYNTHETIC HEPTADECAPEPTIDE DESIGNED AS A MONOMERIC HELIX IN SOLUTION, John F. Thomason, Erin K. Bradley, John D. Altman, Ruud M. Scheek†, and Irwin D. Kuntz, Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco CA 94143, †Department of Physical Chemistry, State University of Groningen, 7 Nyenburgh 16, 9749 AG Groningen, Netherlands

A peptide of seventeen amino acids was synthesized in order to explore the properties that might potentially stabilize a monomeric helix in solution. The most important design parameters for this peptide included: 1) formation of $i \rightarrow i+3$ side chain salt bridges between glutamate and lysine residues, 2) potential for helix dipole stabilization, 3) suitability for ^{1}H NMR spectroscopy, and 4) inhibition of aggregation. The sequence chosen was $^+NH_3$-E-T-G-K-T-A-E-L-L-K-A-T-E-A-T-H-K-COO^-. Circular dichroism studies, at 5° C, showed that the helix content is greatest at low pH, decreasing as the pH increases. When the amino-terminus was acetylated, the helicity increased. The maximum helicity for both peptides is induced by a 25% (v/v) trifluoroethanol solution at pH 5. Two dimensional ^{1}H NMR experiments show that at low pH the carboxy-terminus of the unblocked peptide is highly helical, while at the amino-terminus there is essentially no helix. Our initial results indicate that the helix dipole plays a great part in stabilization of the helix, whereas the role of stabilization *via* the salt bridges remains to be elucidated.

N 330 EFFECT OF SINGLE AMINO ACID REPLACEMENTS ON THE CATALYTIC AND REGULATORY PROPERTIES OF ASPARTATE TRANSCARBAMOYLASE. Susan R. Wente and H. K. Schachman, University of California, Berkeley, CA 94720. Aspartate transcarbamoylase (ATCase) from *E. coli* is composed of two catalytic trimers and three regulatory dimers and catalyzes the first committed step in pyrimidine biosynthesis. Site-directed mutagenesis has been used to replace specific amino acids thought to be implicated in interchain interactions affecting the catalytic mechanism and regulatory properties of the intact enzyme. *In vitro* complementation experiments utilizing hybrid catalytic trimers formed from virtually inactive mutants (Lys-84→Gln and Ser-52→His) and a mutant of low activity (His134→Ala) provide convincing evidence for shared active sites at the interface between adjoining catalytic chains. This conclusion is based upon two types of experiments. In one, hybrid trimers formed from two inactive mutants, Gln-84 and His-52, have greatly increased specific activities, more than 10^5-fold those of the parental proteins, indicating the defects in the two oligomers are on opposite sides of the interfaces between the adjacent polypeptide chains. Formation of active hybrids results from the assembly of a wild-type interface in each of the two purified hybrids which possess 33% the specific activity of wild-type trimers. Analogous hybrids between Gln-84 and Ala-134 provide comparable specific activities for the purified hybrids. Moreover, one hybrid with approximately 33% activity had one high-affinity binding site for a bisubstrate analog as compared to about three for wild-type trimer. In the second experiment, hybridization of the wild-type trimer and an oligomer defective on both interfaces (Gln-84/Ala-134) resulted in a loss of specific activity. The purified hybrid containing two chains with the double mutation and one wild-type chain had very little activity, and that composed of one double mutant and two wild-type chains had 32% the specific activity of wild-type trimers. This negative complementation experiment is in quantitative accord with the scheme based on a shared site trimer. Similar approaches involving site-directed replacements of amino acids in the regulatory chains are being used to investigate how CTP and ATP, though apparently binding to the same site, cause opposite effects on the enzyme activity of the holoenzyme.

N 331 EFFECTS OF AMINO ACID SUBSTITUTIONS ON THE STRUCTURE AND BIOLOGICAL ACTIVITY OF THE NEUROTOXIN ANTHOPLEURIN-B

J. W. West, J. R. Reasoner, and E. W. Radany, Battelle Northwest, Richland, WA 99352

Eight polypeptide neurotoxins of 46 to 49 amino acids have been isolated from several species of sea anemone. These polypeptides show marked conservation of primary sequence and presumably the three-dimensional structure is very similar. All exhibit biological activity by binding to Na^+ channel with the result of slowing inactivation. Coupling constants derived from two-dimensional NOE enhanced NMR of the toxin from Anemonia sulcata has led to the model containing four anti-parallel β sheets comprising a compact core, leaving at least one extended loop of 14 amino acids from Asp 7 to Gly 20. This 14 amino acid loop contains the amino acids, shown by chemical modification to be involved in channel binding (Arg 12, Arg 14) and Na^+ flux modification (Asp 7, Asp 9).

Armed with a model structure and specific amino acid involvement, we have synthesized a gene coding for the natural toxin molecule isolated from Anthopleura xanthogramica, as well as 15 specific mutants affecting the purported active region. The amino acid changes are primary sequence changes at Asp 9, Arg 14, amino acids directly involved in Na^+ flux modification, and channel binding, and at Phe 24 and Lys 48, amino acids indirectly involved by stabilization of the structure and ionic charge of the molecule. An attempt is made to correlate structural changes with altered Na^+ channel binding characteristics and effects on Na^+ flux.

N 332 SECONDARY STRUCTURE PREDICTIONS FOR BOTH TRANS-MEMBRANE AND GLOBULAR PROTEINS FROM HYDROPATHY DEPENDENT CONFORMATIONAL PREFERENCES. Robert W. Williams, Department of Biochemistry, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799.

Secondary structure predictions based on the conformational preferences of amino acids in globular proteins often indicate beta-strand for the hydrophobic sequences in trans-membrane proteins [Green & Flanagan (1976) Biochem. J. 153. 729]. The observation that known trans-membrane protein structures are helical has lead to methods for the prediction of trans-membrane structure based on the assumption that most very hydrophobic segments about 19 residues long are helical. This assumption is avoided in a statistical approach described here. The conformational preferences of the amino acids have been calculated as a function of the hydropathy of neighboring residues, using the known globular protein structures. These preferences show an approximately linear dependence on the average hydropathy of the four residues on either side of each amino acid. While preferences for helix and beta-strand tend to increase with the increasing hydrophobicity of neighboring residues, helix tends to dominate in hydrophobic segments. When these preferences are used in a modified Chou & Fasman [(1978) Adv. Enzymol. 47, 45.] prediction algorithm, putative trans-membrane segments are predicted to be helical. The accuracy of this method for the prediction of globular protein secondary structure is comparable to that of other commonly used methods. This work is supported by National Science Foundation grant PCM-8443154, and USUHS grant GM-7160.

Design and Structural Analysis

N 400 CORRELATION OF COORDINATED AMINO ACID SUBSTITUTIONS WITH FUNCTION IN TOBAMOVIRUSES. Danièle Altschuh, Arthur Lesk, Anne C. Bloomer and Aaron Klug, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

Tobacco mosaic virus (TMV) is the best studied example of a self aggregating system. Sequence data are available for the coat proteins of six tobamoviruses, with homologies ranging from 33% to 82% and atomic coordinates are known for tobacco mosaic virus wild type. The constraints on the overall size and shape of the protein subunit and on the character of those regions of the subunit surface involved in quaternary structure should be reflected in the nature and pattern of acceptable amino acid substitutions.
A significant spatial relationship has been found between groups of residues with identical amino acid substitution patterns. This strongly suggests that, after mutation, they have not become stabilised independently of each other, and that their location is linked to a particular function, at least in viruses identical to the wild type for these residues.
The most conserved feature of TMV is the RNA binding region. Core residues are conserved in all viruses or show mutations complementary in volume. The specificity of inter-subunit contacts is achieved in different ways in the three more distantly related viruses.
The strategy used here for detecting coordinated substitutions has worked well within the tobamovirus family where the protein has extensive quaternary structure. If this approach can be applied equally successfully to other families of proteins, it could contribute to the understanding of protein folding and interactions.

N 401 MONOCLONAL ANTIBODIES TO THE MAJOR VIRAL CAPSID PROTEIN OF SIMIAN VIRUS 40. Lilia M. Babe, Keith Brew and Walter A. Scott, Department of Biochemistry, University of Miami Medical School, Miami, FL 33101.

Viral chromatin isolated from simian virus 40 infected cells was used as immunogen for the preparation of monoclonal antibodies. These antibodies reacted with the major viral capsid protein (VP1) found associated with a subpopulation of chromatin. They also reacted with intact virions and with free VP1. A series of VP1 isoforms, generated by post-translational modification (e.g., phosphorylation and acetylation), was tested for antibody reactivity by Western blot analysis of two-dimensional polyacrylamide gels. The antibodies tested reacted with all of the isoforms suggesting that the epitopes recognized by these antibodies are not altered by the modifications. Several of the antibodies recognized discontinuous epitopes while others recognized continuous epitopes as determined by their ability to react with denatured protein. One continuous epitope has been mapped to a peptide fragment (Arg-312 to Pro-320) by immunoblot analysis of isolated peptides. This region of the VP1 molecule has a high level of hydrophilicity and coincides with the region of lowest hydropathy index in the entire molecule. One of the antibodies recognizing this epitope was capable of neutralizing viral infectivity. Synthetic peptides will be used to map this continuous epitope more precisely and to determine which amino acids are required for neutralization.

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N 402 DIFFERENTIAL SCANNING CALORIMETRY OF BACTERIORHODOPSIN IN DETERGENT MICELLES Christie G. Brouillette, Univ.of Alabama Med. Ctr., Birmingham, Alabama 35294

Bacteriorhodopsin (BR) has been extracted from the purple membrane of H. halobium and renatured in phospholipid-detergent (CHAPS) mixed micelles using a procedure modified from Liao et al (1). DSC and uv/visible spectroscopy were performed from pH 6.5 to pH 9.6 using a variety of buffers. Little pH dependence of the denaturation temperature was observed below pH 8, which correlates with the lack of titrable groups in this pH region. A systematic temperature dependence of denaturational enthalpy is also difficult to discern. On the other hand, the ratio of the van't Hoff to calorimetric enthalpy approaches 1 for denaturation and this ratio is directly proportional to the temperature of denaturation. Before denaturation, visible spectroscopy detects a shift in the maximum absorbance wavelength with increasing temperature, which often passes through an isobestic point, the wavelength of which is dependent on the pH. This transition does not appear to be reversible and is sometimes associated with an endotherm detectable by DSC. This spectral and calorimetric observation is very similar to that observed for BR in the purple membrane (2,3). A small endotherm, (which is reversible) is observed in the purple membrane prior to denaturation and is due to a cooperative change in the crystal lattice of the membrane. The small endotherm seen in purple membrane may be initiated by a protein conformational change, as this study suggests. REF: 1) Liao, London, and Khorana (1983) J. Biol. Chem. 258:9949; 2) Brouillette, Muccio, and Barton (1986)Biophys. J. 49:474a; 3) Jackson and Sturtevant (1978) Biochemistry 17:911. The advice given by H.G. Khorana and colleagues is gratefully acknowledged. This work is supported in part by NIH grant GM35474.

N 403 STRUCTURE-FUNCTION STUDIES OF LYMPHOKINES BY TOTAL CHEMICAL SYNTHESIS.

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Two hemopoietic growth factors, murine interleukin-3 (IL-3) (140 residues) and human granulocyte-macrophage colony stimulating factor (GM-CSF) (127 residues) have been chemically synthesized using automated solid-phase peptide synthesis. The optimized chemical methods used gave synthetic proteins in high yields with the expected physicochemical properties and correct primary structures. The synthetic molecules had the expected range of biological activities and, when purified, had similar specific activities to materials generated by recombinant DNA technology. Synthetic analogs of IL-3 in which alanines have been substituted for cysteines have allowed determination of the disulfide bridges that are essential for IL-3 activity. In the case of GM-CSF, experiments with synthetic fragments have defined a region essential for activity. The automated peptide synthesis approach is particularly useful for determining the minimal structure required for activity, and which amino acids are essential for activity. Furthermore, the availability of large quantities of these molecules will be useful for further structure determination and in vivo studies of native and engineered lymphokines.

N 404 KINETIC ANALYSIS BY DIFFERENTIAL PROGRESS CURVES

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Techniques such as cassette mutagenesis can produce many mutant enzymes whose structural alteration is best probed kinetically. A method of progress curve analysis is described which provides an accurate and rapid determination of the Vmax and Michaelis constant of an enzyme. Data from each time course measurement is derivatized and a plot of reaction velocity versus product concentration is fit to the differential form of the rate equation using a non-linear least squares fitting routine. This method of analysis has proved invaluable in the characterization of over 100 site-directed mutants of the serine protease subtilisin BPN'.

N 405 STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE VITAMIN B12 RECEPTOR PROTEIN (BtuB) IN THE OUTER MEMBRANE OF E.COLI BY MUTAGENESIS OF THE btuB GENE.

Agusta Gudmundsdottir and Robert J. Kadner, University of Virginia School of Medicine, Charlottesville, VA 22903.

The outer membrane protein BtuB functions as a receptor for vitamin B12, phage BF23 and the group E colicins. Deletion of the carboxy terminal six amino acids of the protein resulted in altered vitamin B12 binding and transport properties whereas a point mutation at the amino terminus showed defective energy coupling. To analyze the topology and functional domains of the BtuB protein a two codon TAB linker insertion experiment was performed. A plasmid containing the btuB gene was randomly linearized by partial digestion using the HpaII restriction enzyme and ethidium bromide. Hexameric TAB linkers were ligated to the HpaII linearized plasmids generating a unique BamHI restriction site in the plasmids. TAB linker insertions into various possible restriction sites in the btuB gene were obtained as shown by restriction mapping and the location of the mutations was confirmed by dideoxy sequencing of the btuB gene. The phenotypes of the insertion mutants generated were analyzed in a btuB mutant strain by various assays including growth on vitamin B12, affinity of BtuB for vitamin B12, transport of vitamin B12, sensitivity to phage BF23 and the group E colicins and protection against these agents by vitamin B12.

N 406 STRUCTURAL CORRESPONDENCE BETWEEN TWO TOXINS WITH NO ACCOMPANYING SEQUENCE HOMOLOGY, Susan K. Holtzman and Michael J. Holtzman, Molecular Architects, Sausalito,CA 94965 and UCSF, San Francisco, CA 94143.

An analysis of Exotoxin A from *Pseudomonas aeruginosa* (ExoA) and diptheria toxin (DT) using CASPAR (Computer-Aided Simulation of Protein Assembly using Rule-based logic) shows that there is no first-order sequence homology between DT and Exo A, but the two proteins are very similar in that the structural topology of both the ADP-ribosylating domain and the domain stabilizing the catalytic cleft have been conserved. The lack of obvious homology is due to the fact that the sequences have diverged considerably so the catalytic domain which is responsible for ADP-ribosylation is positioned differently in the two proteins. The catalytic domain in DT is comprised of residues from the amino-terminus of the protein, whereas the the catalytic domain of ExoA is comprised of residues from the carboxy-terminus. The structural correspondence between the two proteins is greatest in this region. The analysis also shows that the difference in structural arrangement between the two proteins extends to the components of the structures which stabilize the interactions between the catalytic domain and the rest of the protein. Because the catalytic domains of the two proteins are formed from different residues in the sequence, the residues which form the interface between the catalytic domain and the rest of the protein are also different in composition and structure. Finally, the domain of the DT protein which becomes membrane associated is the least well-conserved in terms of a one to one mapping between DT and Exo A. This is primarily due to the fact that the layering of this domain is its most prominent feature, and there is considerable structural leeway to accomplish this function. Our results suggest that it is possible to identify functional regions of proteins by structural homology in the absence of sequence homology.

N 407 EXPRESSION OF REDESIGNED MAMMALIAN PHOSPHORYLASES IN YEAST, Peter K. Hwang, Robert J. Fletterick, and Christopher B. Newgard, University of California, San Francisco, CA 94143.

Glycogen phosphorylase (GP) catalyzes the degradation of glycogen into glucose 1-phosphate, enabling cells/organisms to endure periods of metabolic stress. Among mammalian GPs, AMP is a principal allosteric activator of the muscle isozyme; however, it only weakly affects the liver isozyme. We are investigating the structural basis of this regulatory difference. The genes or cDNAs to muscle, liver, and yeast GP were previously cloned. To serve as an expression host, a yeast strain was constructed in which the endogenous GP gene was disabled. An expression vector was constructed from the yeast 2μ element plus 5' and 3' flanking regions of the yeast GP gene. The vector was able to direct high-level expression of yeast GP (1-3% of total protein) that could be isolated in good yield and purity. Using this system, we have expressed human liver GP in yeast, as confirmed by gel electrophoresis with activity staining; the expressed liver GP can be activated by rabbit muscle phosphorylase kinase. The results of expressing a muscle-liver hybrid GP and/or liver GP mutants (single residues replaced by muscle sequence counterparts) that directly address the coupling mechanism in AMP allosteric regulation will be presented.

N 408 THE CRYSTALLOGRAPHICALLY DETERMINED STRUCTURES OF ATYPICAL STRAINED DISULFIDES ENGINEERED INTO SUBTILISIN, Bradley A. Katz, Anthony Kossiakoff,Dept. of Pharmaceutical Chemistry, UCSF, San Francisco, CA, 94143; Dept. of Biocatalysis, Genentech Inc., South San Francisco, CA 94080; Genencor, Inc., South San Francisco, CA, 94080.

The geometries of 2 disulfide bridges (C24/C87, C22/C87), genetically engineered into subtilisin, have been characterized by X-ray crystallography to determine some of the structural and energetic contraints involved in introducing disulfide bonds into proteins. Both disulfides exhibit atypical sets of dihedral angles compared to those for other reported disulfide structures in proteins. Comparison of the disulfide containing mutant protein structures with the wild type structure shows that, for C24/C87 and for C22/C87, disulfide incorporation is accommodated by relatively minor changes in local main-chain conformation. Both disulfides produce short non-bonded contacts with the main-chain. In C22/C87, the cavity produced by changing Thr22 to Cys is filled by two new waters, one of which is hydrogen-bonded to the main-chain. The C22/C87 disulfide has two high energy dihedral angles. The crosslink has a higher calculated dihedral energy than that in C24/C87. The redox potentials of C24/C87 and of C22/C87, experimentally determined by Wells, confirm that the C22/C87 disulfide is the more strained. Since the disulfides in C24/C87 and in C22/C87 are in the same region of the molecule (on the surface) and share a common residue, these mutants comprise an ideal system for relating changes in disulfide structures to differences in redox potentials.

N 409 PREDICTION OF HELPER T-CELL ANTIGENIC SITES FROM THE PROTEIN SEQUENCE.
Hanah Margalit, John. L. Spouge, James L. Cornette, Kemp Cease, Charles DeLisi and Jay. A. Berzofsky. National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20892.
We have used a data base of 23 known immunodominant helper T-cell antigenic sites, located on 12 proteins, to systematically develop an optimized algorithm for predicting T-cell antigenic sites from the amino acid sequence. The algorithm is based on the amphipathic helix model, in which antigenic sites are postulated to be helices with one face predominantly polar and the opposite face predominantly apolar. Such amphipathic structures can form when the polarity of residues along the sequence varies with a regular period and hence, can be identified by methods that detect periodic variations in properties of a sequence. We examined two such methods: a Fourier transform and a least squares fit of a sinusoid. Different hydrophobicity scales and other model parameters were examined. An algorithm was tested by comparing the predicted amphipathic segments with the locations of the known T cell sites, and calculating the probability of getting this number of matches by chance alone. The optimum algorithm uses the Fauchere-Pliska hydrophobicity scale and a least squares fit of a sinusoid to detect periodic variation in the sequence of hydrophobicity values. By applying this algorithm, 18 of the 23 known sites are identified with a high degree of significance ($p<0.001$). The success of the algorithm supports the hypothesis that stable amphipathic helices are fundamentally important in determining immunodominance. This approach may be of practical value in designing synthetic vaccines aimed at T cells.

N 410 SITE-SPECIFIC ALTERATION OF POLIOVIRUS ANTIGENS. Guy S. Page and Marie Chow, MIT Cambridge, MA 02139
The poliovirus capsid is composed of four proteins, three of which are assembled on the external surface, the fourth lying internally in association with genomic RNA. As part of an effort to understand the interaction of the external proteins with the host immune system we have analyzed amino acid substitutions in the capsid proteins of 65 poliovirus variants that show resistance to neutralizing antibodies. Amino acid changes were found in all three of the external capsid proteins, and group into three limited areas. These groups define the locations and partial compositions of the Polio Type I antigens. To extend the description of the antigens and study antibody interactions in more detail, we are selectively altering the antigens and neighboring regions using oligonucleotide directed mutagenesis on infectious cloned viral cDNA.

N 411 SYNTHETIC ANTIBODIES WITH KNOWN 3-D STRUCTURE, Andreas Plückthun*,Rudi Glockshuber, Jörg Stadlmüller, and Arne Skerra, Genzentrum der Universität München, Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, W-GERMANY

The genes encoding the variable domains (V_H and V_L) of the phosphorylcholine binding antibody McPC603 were obtained by DNA synthesis. In addition, we constructed genes encoding the variable and the appropriate constant domains of each chain in order to directly express the exact F_{ab} fragment whose crystal structure is known. The design of the synthetic genes took into consideration the facile replacement of gene fragments (e.g. the hypervariable loops) as well as current knowledge about efficient expression. We have investigated purifications of the cloned gene products from bacterial expression systems and are comparing their efficiency in obtaining large amounts of protein. The essence of antibody architecture is a framework of fairly constant residues and hypervariable loops (complementary determining regions, CDR) that contain the antigen recognition sequences to a great variety of antigens. The particularly well studied antibody combining site of McPC603 is used by us as a model system for quantitatively investigating factors that contribute to efficient hapten binding, subunit interactions, as well as for the potential of stabilizing a transition state through the controlled modification of the protein.

N 412 PROTEIN-RNA INTERACTIONS IN AN AMINOACYL tRNA SYNTHETASE, Lynne Regan and Paul Schimmel, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA02139

We have identified regions of E.coli Ala-tRNA synthetase important in tRNA recognition. We studied the binding properties of several fragments of the enzyme, which were generated by in vitro manipulations of the cloned alaS gene. Small, monomeric fragments of the protein have been identified which are able to specifically bind $tRNA^{Ala}$. From these data we are able to locate a polypeptide sequence essential for specific tRNA recognition by this large (95kD monomer) tetrameric enzyme.

Point mutations which enhance or diminish affinity, or which alter the specificity of tRNA binding, are being pursued. These results add to our understanding of tRNA recognition by aminoacyl-tRNA synthetases.

N 413 ISOLATION AND CHARACTERIZATION OF A GENE FOR A SUBTILISIN-LIKE SERINE PROTEASE. Babru Samal, Barbara Karan, Thomas Boone, Kenneth Chen and Yitzhak Stabinsky, Amgen, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320.

Subtilisins are included in laundry detergent formulations for effective removal of protein-associated stains. In order to increase the stability of subtilisins, we and other groups have applied protein engineering techniques to introduce novel changes into these molecules. We have taken an additional approach, i.e., isolation of thermostable detergent-compatible proteases from natural sources. Two such proteases named TW3 and TW7 have been isolated. These are highly stable in the presence of SDS and in commercial detergent formulations. From a cDNA library we have isolated the gene for protease TW3. The gene has been completely sequenced. The amino acid sequence of TW3 as deduced from the nucleotide sequence will be compared with those of subtilisin and subtilisin-like enzymes. Thermal and detergent stability data will also be presented.

N 414 AN APPROACH TO A THREE-DIMENSIONAL SYNTHETIC VACCINE FOR MALARIA, Arnold C. Satterthwait, Robert A. Hagopian, H. Jane Dyson, Peter E. Wright, and Richard A. Lerner, Research Institute of Scripps Clinic, La Jolla, California 92037.

A repeating tetrapeptide, Asn-Ala-Asn-Pro, found on the surface of the infective malarial sporozoite is immunodominant and has been proposed for a synthetic vaccine. One possible method for enhancing synthetic vaccines might be to restrict the conformation of the synthetic peptide to that found in the native protein.

Two series of repeating tetrapeptides, $Ac-(NPNA)_n-NH_2$ (n=1-3) and $Ac-(NANP)_n-NH_2$ (n=1-3), were analyzed by NMR. These studies reveal that the NPNA sequence acts as the structural determinant in water. For each NPNA unit, the temperature coefficient for the alanine NH proton suggests the presence of a highly populated reverse turn in water. On the basis of this and other considerations, we have synthesized two series of repeating tetrapeptides, shaped by covalently linking asparagine side chains, for testing as potential vaccines.

N 415 CHARGE CLUSTERS IN THE CONTROL OF ASSEMBLY OF CUCUMBER GREEN MOTTLE MOSAIC VIRUS AND OTHER TOBAMOVIRUSES, Gerald Stubbs and Sharon Lobert, Vanderbilt University, Nashville, TN 37235

Carboxyl-carboxylate interactions are essential in controlling the assembly of many viruses, including the rod-shaped tobamoviruses. Despite their importance, these charged groups tend not to be conserved, even among relatively closely-related viruses. In tobacco mosaic virus, two charge clusters are found: one in a side-to-side protein subunit interface near the inner surface of the virus, and one in a top-to-bottom interface at higher radius. These clusters are believed to bind calcium, and to act as sensitive switches, active at physiological pH, controlling the assembly and disassembly of TMV (Namba and Stubbs, Science 231, 1401, 1986).

In cucumber green mottle mosaic virus, a distant relative of TMV, the inner charge cluster is conserved, but the outer is not. Lead binds to a site tentatively identified as corresponding to the inner charge cluster, and also to a site corresponding in radius to a major difference in the radial density distributions of TMV and CGMMV-W. Assuming that the protein fold of the two viruses is similar, a number of new carboxylate groups in CGMMV-W could form an alternative charge cluster. High resolution structure analysis of CGMMV-W by fiber diffraction is in progress.

In the U2 strain of TMV, the outer charge cluster is conserved, but several changes are introduced into the inner cluster. In this virus, the radial density distribution is significantly perturbed in the region near the inner surface, suggesting that here also, there is a redistribution of elecrostatic interactions.

N 416 DESIGN OF A pH TRIGGERED LIPOSOME DESTABILIZING PEPTIDE. Nanda K. Subbarao, Roberta A. Parente and Francis C. Szoka, Jr. Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA. Fusion proteins on the viral envelope are triggered by low pH conditions to induce virus fusion with the endocytotic membrane. We have attempted to study viral fusion proteins by designing model peptides which cause fusion of uncharged liposomes in a pH dependent manner. As a first step, we have designed a peptide-GALA-which destabilises neutral bilayers in a pH dependent manner. GALA has the following sequence: W E A A L A E A L A E A L A E H L A E A L A E A L E A L A A. The residues have strong α-helix forming tendencies. Glu and Leu residues are positioned so that they are aligned on opposite faces when the peptide is in an α-helical conformation. Helix formation is minimized at neutral pH by the charges on Glu and is expressed when the Glu are protonated at around pH 6. Protonation also increases the hydrophobicity of the sequence. These factors make the interaction of the peptide with the lipid much stronger at pH 5 than at neutral pH as indicated by the peptide induced leakage of liposome contents at low pH. Circular dichroism spectra indicate that GALA changes conformation at low pH and in the presence of divalent cations at neutral pH. The tryptophan fluorescence undergoes a blue shift at low pH in the presence of lipid. Truncated versions of GALA, with up to 16 residues, neither change conformation as pH is reduced nor effectively induce leakage from neutral liposomes. Peptides based on the GALA sequence should help in the study of the relationship between structure and function in fusogenic proteins. Supported by GM 29514 (FCS) and a Damon-Runyon Postdoctoral Fellowship to RAP.

N 417 SPECIFICITY AND MECHANISM OF ESTEROLYTIC ANTIBODIES, Alfonso Tramontano and Richard A. Lerner, Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037.

Antibodies to a transition state analog have been demonstrated to behave as enzymic catalysts.[1,2] New studies on the esterolytic activity of these monoclonal antibodies and their Fab fragments will be reported. These include observations on substrate specificity and kinetics that reflect on the probable mechanism of catalysis. The relevant kinetic parameters for these reactions will be reported. These may more accurately reveal the extent of catalysis intrinsic to antibodies elicited with the particular transition state analogs used. Studies on modification of the protein by affinity labelling will also be mentioned. The function and structure of these unusual proteins continues to be of interest.

1. A. Tramontano, K. D. Janda, and R. A. Lerner, Proc. Natl. Acad. Sci. (USA) 83 6736-6740 (1986).
2. A. Tramontano K. D. Janda, and R. A. Lerner, Science, in press (1986).

N 418 2-D ORGANIZATION OF POLYCLONAL ANTIBODIES ON PHOSPHOLIPID FILMS, E. E. Uzgiris, General Electric Company, Schenectady NY 12301.

The organization of monoclonal antibodies into 2-D crystals when bound to hapten derivatized phospholipid films is by now well known. Heretofore, it has been assumed that high homogeneity in antibody-hapten binding and in antibody-antibody contact characteristics were necessary conditions for the ordering to occur. This is not necessarily the case as I show in this work that rabbit IgG antibodies readily order to form 2-D lattices which have considerable long-range order accompanied by some local disorder. This phenomena is quite robust and occurs for a wide range of hapten density on the phospholipid bilayers, salt concentrations and incubation temperatures. It is insensitive to antibody source and antibody storage conditions and ordering can be achieved even from unpurified anti-serum or for different antibody-hapten systems (DNP and fluorescein). The 2-D ordering has been well characterized for rabbit antibodies but may occur less readily for other, perhaps more complex polyconal antibody sources. Initial attempts with goat anti-DNP antibodies have not yielded 2-D crystals. In this case, purification into a single IgG class may be required.

N 419 PARAMETERS AND MECHANISMS OF CALCIUM BINDING TO PEPTIDES AND PROTEINS, Harel Weinstein[1], Kenzi Hori[1] and Joseph N. Kushick [1,2], (1): Department of Physiology and Biophysics, Mount Sinai School of Medicine of CUNY, New York NY 10029; (2): Department of Chemistry, Amherst College, Amherst Mass 01002.
The characteristics of Ca^{2+} binding sites and of the structural reorganization induced by Ca^{2+} binding in storage proteins and ion carriers are being studied as models for molecular mechanisms in Ca^{2+} channels and in Ca^{2+}-dependent modulatory proteins. A first step in the study was the development of energy parameters for Ca^{2+}, compatible with those in the CHARMM package of programs for molecular mechanics and dynamics simulations. Such parameters were obtained from analytical fits to calculated potential surfaces for the interaction of Ca^{2+} with oxygen and carbonyl groups in molecular clusters. The interaction energies were obtained from ab-initio molecular orbital calculations with extended basis sets and correlation energy corrections. The resulting parametrization was tested by CHARMM calculations of structure and dynamics of Ca^{2+}-binding macromolecules for which data were available from experiments. The hexapeptide cyclo-(Pro-Gly)$_3$ [PG3], and a 75 residues long calcium binding protein from bovine intestine [ICBP] were used in these tests. The calculated geometrical parameters that characterize the structures of PG3 and of the complex of two PG3 molecules with one Ca^{2+} in between, were in good agreement with experimental data from crystallography and NMR. Similarly, structural comparisons of ICBP optimized with the new parameters for Ca^{2+} starting from x-ray coordinates showed good agreement both in the regions of the calcium-binding loops and in the organization of the flanking helices. The details of the dynamic rearrangement induced by calcium binding were studied with the new set of parameters.

Virus and Antibody Structure; Membrane Proteins

N 500 MULTIPLE SUBSTITUTIONS IN E. COLI THYMIDYLATE SYNTHETASE GENERATED BY NONSENSE SUPPRESSION, Catherine A. Ball and Jeffrey H. Miller, University of California, Los Angeles, CA 90024.
We have generated a series of nonsense mutations in the thyA locus of E. coli, which codes for thymidylate synthetase. Over 20 different nonsense sites have been defined. Using a set of nonsense suppressors that allows the insertion of 15 different amino acids at an amber (UAG) site, we have produced over 300 amino acid substitutions in this enzyme. The effects of these replacements will be described.

N 501 TWO CODON INSERTION MUTAGENESIS OF THE TAQI RESTRICTION ENDONUCLEASE,
Francis Barany, Cornell University Medical College, New York, N.Y. 10021.
Recently, the thermophilic TaqI restriction endonuclease and corresponding methylase (recognition sequence T↓CGA) have been cloned (1). The endonuclease gene has been subcloned under inducible control of an alkaline phosphatase promoter, in a small high copy plasmid. Surprisingly, E. coli cells overproducing TaqI endonuclease are still viable at 37°C, even in the absence of (protective) methylation. Indeed, neither transforming DNA nor infecting lambda phage are restricted by cells harboring TaqI. However, certain cells harboring this plasmid are barely viable at 42°C, and this phenotype has been exploited as a plate assay for in vivo TaqI endonuclease activity. Single stranded hexameric linkers were inserted into the endonuclease gene using biological selection (2), and the resultant two codon insertion mutants were assayed in vitro for TaqI endonuclease activity. A wide range of enzymatic activity was observed, from wild type activity (3 mutants), slight temperature dependency (2 mutants), moderate activity (2 mutants), greater activity in low salt buffers (2 mutants), nicking activity (1 mutant), to no activity (2 mutants, and 2 small in-frame deletions). These mutants are currently being characterized.
1. Slatko, B., Movan, L., Jager, T., Benner, J., Simcox, T., & Wilson, G. Ms in preparation 1986.
2. Barany, F. (1985) Proc. Natl. Acad. Sci. USA 82:4202-4206.

N 502 ALTERATION OF THE CATALYTIC PROPERTIES OF THE E. COLI recA PROTEIN BY SITE DIRECTED MUTAGENESIS, F. R. Bryant, Department of Biochemistry, Johns Hopkins University, Baltimore, MD 21205

RecA protein promotes the ATP-dependent pairing of DNA strands during homologous recombination in E. coli. We have been studying the mutant recA1 protein in an effort to determine the mechanistic role of ATP hydrolysis in various recA protein-promoted DNA pairing reactions. The recA1 mutation is a single point missense mutation in which glycine-160 of the recA polypeptide is replaced by an aspartic acid residue. Unlike the wild type protein, the recA1 protein does not function as a ssDNA-dependent ATPase at pH 7.5. Interestingly, the recA1 protein does exhibit a vigorous ATPase activity at pH 6.2. To explore the molecular basis of this pH activation, we have used oligonucleotide directed mutagenesis to replace aspartic acid-160 of the recA1 polypeptide with a sterically similar but nonionizing asparagine residue. The recA(asn-160) protein has been purified to homogeneity and preliminary experiments indicate that it has an ATPase that follows a pattern of pH activation that is similar to that found for the recA1 protein. These results suggest that the activation of the recA1 ssDNA-dependent ATPase activity at pH 6.2 may not be due simply to protonation of aspartic acid-160. The enzymatic properties of the recA(asn-160) protein and other new recA mutants will be presented in detail.

N 503 EXPRESSION AND MUTAGENESIS OF THE BOVINE TYPE I REGULATORY SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE. J. Bubis, L.D. Saraswat, P. Reynolds, and S.S. Taylor. Department of Chemistry. University of California, San Diego. La Jolla, CA 92093.
An expression vector for the regulatory (R) subunit of cAMP-dependent protein kinase was constructed in pUC7. Maximum expression (20 mg/L of culture) of soluble protein was achieved in E. coli 222 in the absence of IPTG. Directed mutagenesis of the R-subunit was initiated in order to better understand structural changes that are induced as a consequence of cAMP binding which promotes dissociation and thus activation of the catalytic subunit. The site that was first targeted for mutagenesis was identified initially by photoaffinity labeling with 8-N3-cAMP which leads to nearly stoichiometric modification of Tyr 371. Model building of R based on the crystal structure of the homologous E. coli cAMP-binding protein, the catabolite gene activator protein, is consistent with Tyr 371 being in close proximity to the adenine ring of cAMP. In order to determine whether polar or dipolar interactions exist between cAMP and Tyr371, this Tyr was changed to Phe. This mutation yields a stable protein with altered cAMP-binding properties: 1) the app. Kd(cAMP)was shifted from 16 nM to 60 nM; 2) Scatchard plots showed no positive cooperativity between the cAMP-binding sites in constrast to native R; and 3) the Hill coefficient was reduced from 1.6 to 0.99. Other mutations which have been introduced include changes in both cAMP-binding sites as well as a change that yields a trunkated protein which is missing the entire second cAMP-binding domain. Each mutation yields a stable protein which shows altered cAMP-binding properties, and each has been purified to homogeneity. Photolabeling methods also have been developed to screen rapidly for functional changes in cAMP-binding. Supported by USPHS Grant GM34921.

N 504 IN VITRO MUTAGENESIS OF CHLORAMPHENICOL ACETYL TRANSFERASE TO INVESTIGATE STRUCTURE-FUNCTION RELATIONSHIPS. Daniel K. Burns and Robert M. Crowl, Department of Molecular Genetics, Hoffmann-La Roche, Inc., Nutley, NJ 07110

We have chosen chloramphenicol acetyl transferase (CAT) as a model to investigate protein structure-function relationships for several reasons. First, the activity of this protein provides an easily discernible phenotype in E. coli, namely resistance to the protein synthesis inhibitor chloramphenicol. Second, the CAT protein has been extensively studied for several years providing a sound biochemical foundation for genetic analyses. Finally, the CAT protein is relatively small (219aa) and has been engineered for overexpression with yields of 30% of total cell protein - an important advantage when it becomes necessary to purify and study mutant forms of the protein. Random mutations were generated using hydroxylamine treatment of purified plasmid DNA. Mutants were initially classified into three groups: I) complete elimination, II) partial elimination, and III) increased resistance to chloramphenicol. Sequence analysis has identified several class I and class II mutations. Cys 31 — Tyr and His 193 — Tyr mutations completely eliminate resistance to chloramphenicol-corroborating previous biochemical data which implicated these regions in substrate binding. A Val 195 — Ile mutant exhibits reduced resistance to the antibiotic. Biochemical characterization of these mutants is currently underway and additional mutants are being identified and characterized. In addition, we are searching for second site revertants of the class I mutations to aid in the structure-function analysis.

N 505 A PREDICTED SUBSTRATE BINDING REGION OF AN AMINOACYL-tRNA SYNTHETASE EXAMINED BY RANDOM OLIGONUCLEOTIDE CASSETTE MUTAGENESIS, Neil D. Clarke, Donald C. Lien, and Paul R. Schimmel, Massachusetts Institute of Techonology, Cambridge, MA 02139

There are two significant regions of homology that have been found between the isoleucyl-tRNA synthetases of yeast and E. coli. One region (8/10 amino acids) of homology is not found in other aminoacyl-tRNA synthetase sequences. A second region (9/14 amino acids) does show homology to other synthetases, including the methionine and tyrosine enzymes. The methionine and tyrosine enzymes have been crystallized and much of each of the structures is solved. The two structures are remarkably similar, especially in the region which includes the homology to the isoleucine enzymes. We propose that the isoleucine-tRNA synthetases may share these structural features. If this is so, the 8/10 amino acid homology that is unique to the isoleucine enzymes is predicted to be in close proximity to the bound isoleucyl-adenylate (an enzymatic intermediate). Random mutations of this 10 amino acid region are being made by oligonucleotide cassette mutagenesis. The kinetic properties of the mutants will provide a test of the function of this sequence.

N 506 PHOTOAFFINITY LABELLING AND MUTAGENESIS OF A ACTIVE SITE RESIDUES IN BACTERIAL TOXINS, R. John Collier, Stephen F. Carroll, and Cameron M. Douglas, Harvard Medical School, Boston, MA 02115.

We have photoaffinity labelled active site residues of diphtheria toxin (DT) and exotoxin A (ETA) from Pseudomonas aeruginosa by UV irradiating complexes with substrate NAD. In both cases the nicotinamide moiety of NAD was efficiently and specifically transferred to single glutamic acid residue (Glu-148 in DT, Glu-553 in ETA) within an enzymically active fragment. The photoproduct at position 148 in DT was identified as an α-amino-γ-(6-nicotinamidyl) butyric acid residue, and the product was the same in ETA as judged by cochromatography in several systems. Substitution of Asp for Glu-148 of DT or Glu-553 of ETA by site-directed mutagenesis yielded products that had 10^{-2} to 10^{-4} the ADP-ribosyl-transferase activity of the wild-type proteins. Glu-553 projects into a cleft believed to correspond to the NAD site of the active domain of ETA. These studies offer a new approach to the production of vaccines, as well as providing a new means of locating active site residues of this class of proteins.

N 507 AMINO ACID REQUIREMENTS AT THE ACTIVE SITE IN β-GALACTOSIDASE FROM E. COLI
Claire G. Cupples and Jeffrey H. Miller, University of California at Los Angeles, Los Angeles, CA 90024

At least two functional groups are thought to be necessary for activity in the E. Coli enzyme β-galactosidase, an acidic group and a carboxylate group. Studies with chemical inhibitors have identified a glutamic acid at position 461 (Glu 461) as a possible candidate for the carboxylate group. We have investigated the effect of amino acid changes at position 461 on enzyme activity. Using site directed mutagenesis, we have changed the Glu 461 codon into an amber codon. The mutated gene has been introduced into a series of E. Coli amber suppressor strains developed in this lab. which allow insertion of fifteen alternate amino acids at the amber site. The results confirm that Glu 461 is essential for enzyme activity. Insertion of a glutamate at position 461 restores 60% of wild type activity; histidine at that position produces 2% activity, while all of the other amino acid substitutions allow less than 0.1% activity.

N 508 SITE SPECIFIC MUTAGENESIS OF YEAST CYTOCHROME C AT AN INVARIANT ARGININE RESIDUE.
Robert L. Cutler, A. Grant Mauk & Michael Smith, Department of Biochemistry, University of British Columbia, Vancouver, B.C., V6T 1W5, Canada.

Arginine-43 of yeast iso-1-cytochrome c is located at the bottom of the heme pocket, and is one of the invariant residues of eukaryotic cytochromes c . Electrochemical and NMR studies of cytochromes c from several species have led to the suggestion that this arginine may interact with the internal propionate of the heme to lower its pK and so maintain the reduction potential of the protein at physiological pH (Moore et al, BBA, 764, 331 (1984)).

Site specific mutagenesis has been used to alter the codon for Arg at position 43 of yeast iso-1-cytochrome c to that for Ala, His, Lys, Leu, Asn and Gln. The mutated genes have been expressed under regulation of the iso-1-cytochrome c promoter in a yeast strain which does not produce cytochrome c . All of the transformants grow on lactate indicating that the mutant cytochromes are at least partially functional.

All six substitutions at position 43 decrease the reduction potential of the protein compared to the wild-type (272 mV (pH 7, 25 °C)) in the order Lys (249 mV), His (245 mV), Asn (242 mV), Gln (238 mV), Leu (231 mV) and Ala (225 mV) . Increases in the pKs for the propionic acid are observed for the mutant proteins, and correlate with the decrease in reduction potential . In contrast to these effects on reduction potential, the mutant cytochromes show no differences from the wild-type protein in their reactions with yeast cytochrome c peroxidase or in their electronic spectra.

N 509 SITE DIRECTED MUTAGENESIS OF UBIQUITIN.
D. J. Ecker, T. R. Butt, N. A. Margolis, M. I. Khan, J. A. Marsh, E. J. Sternberg, S. Jonnalagadda, P. L. Weber, L. Mueller and S. T. Crooke. SmithKline & French Laboratories, Philadelphia, PA 19101.

Ubiquitin is a small (76 amino acids) highly conserved protein found in all eukaryotes either free in the cytoplasm or covalently attached to cytoplasmic, nuclear or membrane proteins. In the nucleus, ubiquitin is attached to histones H2A and H2B. In the membrane, ubiquitin is attached to cell surface receptors and may play a role in signal transduction or cell-cell recognition. In the cytoplasm, ubiquitin plays a central role in selective degradation of intracellular proteins. Apart from its role in the cell, ubiquitin is an interesting molecule for protein engineering studies because of its unique structure, heat and chemical stability and unprecedented sequence conservation.

To study these characteristics and functions of ubiquitin, we have chemically synthesized the ubiquitin gene. The gene was designed with eight unique restriction enzyme sites to facilitate site-directed mutagenesis and gene fusion studies. The crystal structure of ubiquitin was examined using computer modeling and graphics to identify important structural features. A series of site-specific mutations were chosen that selectively perturbed various regions of the molecule. Each of the mutant genes were constructed and expressed in E. coli under the heat inducible lambda PL promoter. Wild type and mutant forms of ubiquitin were purified and characterized by biochemical and biophysical techniques including 2D NMR. Preliminary biochemical studies have identified some of the amino acid side chains important to ubiquitin's biological activity.

N 510 STRUCTURE-FUNCTION STUDIES ON COMPLEMENT PROTEIN C4 USING SITE-DIRECTED MUTAGENESIS Dehmani Fathallah, David E. Isenman and Michael C. Carroll, Division of Immunology, Children's Hospital, Boston, MA 02115 and Department of Biochemistry, University of Toronto, Toronto, Canada M5S 1A8

The two isotypic forms, i.e. C4A and C4B, of the fourth component of the complement system in man show dramatic differences in their hemolytic function and in their preferences for covalent binding to hydroxyl or amino groups. These differences have been correlated with the known differences in the primary structure derived from cloned cDNA. The amino acids changes are clustered in the C4d region of the molecule relatively near to the covalent binding site. In order to determine the chemical basis for the observed differences in function, we are using a site-directed mutagenesis approach to modify existing C4 cDNA clones in the C4d region. The DAP, LTK (-) cell line, which was derived from murine fibroblasts, expresses an active C4 protein when transfected with the complete cDNA under the control of an SV40 promotor. A comparison of the functional properties of the expressed native and site-specific mutant proteins should provide the basis for understanding the hemolytic and covalent binding characteristics of the two C4 isotypes.

N 511 CRYSTAL STRUCTURES OF BACTERIAL GLUTAMINASE-ASPARAGINASES, Robert W. Harrison, Gary L. Gilliland, Irene T. Weber and Alexander Wlodawer, Center for Chemical Physics, National Bureau of Standards, Gaithersburg MD 20899; Herman Ammon, Department of Chemistry, University of Maryland, College Park MD 20742.

Asparaginases and glutaminase-asparaginases occur in many bacterial species and E. coli asparaginase is used to treat acute lymphoblastic leukemias. Crystals of four members of this family are being investigated by us. The enzymes come from four different species: Acinetobacter glutaminasificans (AGA), Pseudomonas 7A (PGA), Vibrio succinogenes and Escherichia coli. AGA crystallizes in space group I222 with one subunit in the asymmetric unit. A polypeptide backbone tracing has been fitted to a 3.2A electron density map of AGA which was obtained from low resolution MIR map, extended to higher resolution by two different and complementary techniques of density modification. The amino acid sequence for AGA is being determined and the refinement based on partial and predicted sequences is continuing. The AGA model has been used to determine the structure of PGA which crystallizes as a tetramer in space group $P2_12_12_1$. Diffraction data are available to 2.4A resolution and an averaged map of the PGA subunit has been calculated. Inhibitor binding is being used to mark the position of the active site. X-ray intensity data are also available for the enzymes from E. coli and Vibrio and the determination of these structures is underway. The comparison of several homologous asparaginases will give valuable information about the activity of this enzyme.

N 512 MUTATIONS IN THE ACTIVE SITE OF E. COLI PHOSPHOFRUCTOKINASE. H.W. Hellinga & P.R. Evans. MRC Laboratory for Molecular Biology, Cambridge, England.

Oligonucleotide-directed mutagenesis was used to construct mutations in three residues of E. coli PFK, which on the basis of X-ray structures of the closely related Bacillus stearothermophilus PFK are thought to be involved in the mechanism of catalysis (Asp 127, Arg 171), or cooperative binding of fructose 6-phosphate (Arg 243). Mutants were analyzed by steady-state kinetics. The (Asp -> Ser)127 mutation results in a large decrease of turnover number; (Arg -> Ser)171 has a much smaller effect. Analysis of the changes in the mutant Michaelis constants measured in both reaction directions indicate qualitatively which steps in the enzyme reaction pathway have been affected. Both Asp 127 and Arg 171 affect the transition state. In addition, Asp 127 is also involved in product release. The (Arg -> Ser)243 mutation results in a decreased homotopic cooperatively w.r.t. Fru6P. At low effector concentrations, heterotropic interactions between the inhibitor PEP or activator GDP with Fru6P result in an increased cooperativity w.r.t. Fru6P. Unlike the wild-type, this mutant does not obey a simple two-state model, but has at least four states.

N 513 PROBING PROTEIN-PROTEIN INTERACTIONS IN YEAST cAMPdPK BY OLIGONUCLEOTIDE DIRECTED MUTAGENESIS. Jeff Kuret, Karen Johnson, and Mark Zoller. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 11724.

In the yeast, Saccharomyces cerevisiae, the regulatory (R) subunit of cAMP-dependent protein kinase (cAMPdPK) is encoded by a single gene, BCY1. The function of the R subunit is to inhibit the catalytic (C) subunit encoded by three homologous genes, TPK1-3. Upon an increase in the cellular level of cAMP, the inactive holoenzyme (R_2C_2) dissocates into an R_2 dimer and two active C subunits. The amino acid sequences of the yeast C and R subunits are structurally and functionally homologous to their mammalian counterparts. We have expressed BCY1 and TPK1 genes in E. coli and have begun to study structure/function relationships by oligonucleotide directed mutagenesis. Yeast provides an excellent system for protein engineering by virtue of its ability to replace a wild type gene with a mutagenized gene into the proper place on the chromosome. Thus mutated proteins can be studied in vivo in the absence of their wild type counterparts.

Expression was accomplished using a T7 phage promoter vector developed by Studier and coworkers. BCY1 (yeast R subunit) was produced at a level of 5% of total cellular protein and could be easily purified. The expression vector contains the phage F1 origin so that single stranded DNA could be easily produced for mutagenesis experiments. This "all-in-one" vector facilitates mutagenesis and subsequent expression studies. Using this system we have begun to probe a region in the R subunit involved in the interaction between the R and C subunits, termed the hinge region. We will discuss amino acid changes in this region and their effect on complex formation.

N 514 EVIDENCE FOR AN ESSENTIAL HISTIDINE RESIDUE FOR A BIOACTIVE TUMOR NECROSIS FACTOR, Ralph Yamamoto, Alice Wang, Charles Vitt, Leo S. Lin, Cetus Corporation, Emeryville, CA 94608.

The role of the histidine residues of recombinant Tumor Necrosis Factor (TNF) was investigated by chemical modification and site-directed mutagenesis. At pH 6.5, diethylpyrocarbonate (DEP) inactivates the cytotoxic activity of this lymphokine in an in vitro cytotoxicity assay. Chemically inactive TNF partially competes with iodinated TNF for occupancy of receptors on a human breast carcinoma line- MCF-7. TNF contains three histidine residues, located at positions 15, 73, and 78. By site-directed mutagenesis, replacement of His15 with either asparagine, glutamine, lysine, or glycine the mutants are completely inactive in the assay described above. Whereas, replacement of His-73 and His-78 to Gln-73 and Val-78 gives a fully active TNF. Direct testing for the expression of inactive mutant TNF from E. coli was achieved by Western transfer of bacterial extracts. The data indicates an important involvement of His-15 in the cytotoxic activity of recombinant TNF.

N 515 DIRECTED MUTAGENESIS STUDIES TO PROBE THE SITES IN FIBRINOGEN THAT ARE IMPORTANT TO THROMBIN RECOGNITION. Susan T. Lord and Dana M. Fowlkes, University of North Carolina at Chapel Hill, NC 27514. A substantial accumulation of data indicates that the alpha chain residues of human fibrinogen which are important to thrombin recognition lie within amino acids 1-50. Thrombin cleaves the alpha chain between Arg16 and Gly17 releasing fibrinopeptide A (FPA). We have constructed plasmid clones which express a hybrid protein in E. coli. This hybrid consists of amino acids 1-50 of the alpha chain joined to a short segment of collagen followed by bacterial beta-galactosidase. Western blot analysis of E. coli lysates induced to synthesize this hibrid protein show a single high molecular weight band cross reactive with a monoclonal antibody, Y-18, which recognizes the alpha chain of fibrinogen but not the products of thrombin cleavage. When thrombin is incubated with these E. coli lysates, FPA is released as demostrated both by Western blot analysis and radioimmunoassay. We have constructed plasmids which encode specific amino acid substitutions within residues 1-23. One of these substitutions, Gly14 to val, significantly alters both cleavage by thrombin and recognition by Y-18. These data indicate that this single amino acid substitution alters the alpha peptide structure and thereby changes its interaction with thrombin and Y-18.

N 516 ENGINEERED DISULPHIDE BONDS IN SUBTILISIN. Colin Mitchinson and James A. Wells, Genentech Inc., South San Francisco, CA, 94080.

Previous work in this laboratory (Wells, J.A. and Powers, D.B., J. Biol. Chem. 261 6564-6570, 1986) has examined the in vivo formation and stability of two engineered disulphide bonds in Bacillus amyloliquefaciens subtilisin, a cysteine-free protein. Both disulphides were formed quantitatively following expression Bacillus subtilis but neither stabilised the enzyme against autolysis. Further disulphides have been designed by computer modelling and engineered into subtilisin, via. the single-cysteine mutants, including one positioned in an attempt to replace the calcium of a high-affinity binding site. The disulphide-bond formation and redox strength, the antolytic stability of the mutant subtilisins, and the interrelationship between these properties will be discussed.

N 517 ANALYSIS OF THE C-TERMINAL DNA-BINDING REGION OF MATa1 GENE PRODUCT BY IN VITRO MUTAGENESIS USING OLIGODEOXYNUCLEOTIDES WITH RANDOM MISMATCH BASE PAIRS, Sarbjit S. Ner and Michael Smith, Dept. of Biochemistry, University of British Columbia, Vancouver, B.C., Canada, V6T 1W5.

The MAT locus of S. cerevisiae codes for transcriptional regulators of cell-type. These proteins share homology with the 'homeo-box' sequences in eukaryotes and to the DNA binding helix-turn-helix sequences in prokaryotes. In the diploid cell type both the gene products of MATα2 and MATa1 are required to repress genes necessary for mating, to relieve repression of sporulation genes and to prevent switching at the MAT locus by turning off the HO endonuclease. This study is aimed at exploring the DNA-protein interactions that are presumed to occur between MATa1 and the upstream regulatory sequences recently reported for several genes. The C-terminal 30 residues of MATa1, which include the helix-turn-helix motif, have been mutagenised generating over 50 useful variants. A 1.8kb fragment that contains the MATa1 gene at the 5'-end was cloned into M13mp11, and uracil containing single-stranded DNA obtained by passage of this vector through E. coli RZ1032 (ung$^-$, dut$^-$). Three synthetic oligodeoxynucleotides were used to prime, in vitro, second strand synthesis. Each position of the oligodeoxynucleotide contained the wild-type sequence and a low level of the remaining three bases so as to statistically generate single or double point changes. On transformation of E. coli JM101 with the heteroduplex the in vitro synthesised strand is selected for, thus giving mutagenic frequencies of up to 60%. The variant constructs were analysed for their ability to repress a strain containing an HO promoter-LacZ fusion, and for their ability to sporulate, on transformation, a MATα/MATΔ diploid strain.

N 518 OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS OF A HUMAN DIHYDROFOLATE REDUCTASE (DHFR) cDNA, B.I. Schweitzer, S. Srimatkandada, S.K Dube and J.R. Bertino, Yale University, New Haven, Ct. 06510.

We have employed the method of oligonucleotide-directed mutagenesis to introduce several single-base substitutions into the cDNA of the human DHFR gene at residues that have been suggested to be involved in catalysis and/or ligand binding. Oligonucleotides were synthesized containing single-base changes corresponding to mutations of leucine 22 to arginine, glutamate 30 to lysine, phenylalanine 31 to serine, and arginine 70 to isoleucine. Mutagenesis efficiencies ranged from 10-20%. Putative mutants were sequenced by the Sanger method to confirm the mutation and to ensure that no additional mutations had taken place. One mutation, phenylalanine 31 to serine, had been previously characterized by us in a methotrexate (MTX) resistant human colon carcinoma cell line HCT8R; subsequently, site-directed mutagenesis was used to introduce this mutation into the wild-type human DHFR cDNA. This mutated cDNA was then cloned into bacterial and mammalian expression vectors in order to localize the MTX resistant phenotype to this mutation. Work is in progress to introduce the other mutated cDNAs into bacterial expression vectors so that the effects of the mutations on properties of DHFR such as turnover rate, cofactor affinity, substrate affinity, and sensitivity to inhibitors can be determined.

N 519 SITE-SPECIFIC MUTAGENESIS OF T4 GENE 32: THE ROLE OF AROMATIC AMINO ACIDS IN PROTEIN:NUCLEIC ACID INTERACTIONS. Yousif Shamoo, William Roberts, Richard Prigodich, Kenneth Williams, John Chase*, Joseph Coleman and William Konigsberg, Yale U., New Haven, CT 06510, *Albert Einstein College of Med., Bronx, NY 10461

T4 gene 32 protein (gp32) is a single-stranded DNA binding protein essential for T4 DNA replication, recombination and repair. Gp32 has served as a prototype for a biologically diverse family of proteins that bind non-specifically to single-stranded nucleic acids. Proton NMR studies on gp32 demonstrate that resonances corresponding to two phenylalanine and five tyrosine residues shift upon the addition of oligo-$d(pA)_8$ or $d(pT)_8$ thus implicating these amino acids in gp32:ssDNA complex formation. Since aromatic residues have also been shown to be involved in fd gp5:ssDNA complex formation, hydrophobic interactions between the side chains of aromatic amino acids and the bases of a polynucleotide may represent a common binding mechanism for this family of proteins.

To map the ssDNA binding surface of gp32 we are using *in vitro* mutagenesis to stepwise substitute non-aromatic amino acids for each of the eight tyrosines in gp32. Three of these mutant proteins (gp32ser73, gp32ser115, and gp32ala137) have been prepared and characterized. Limited proteolysis and fluorescence studies suggest that tyrosine 73 is essential for maintaining the native gp32 conformation and that tyrosine 115 is important for ssDNA binding. As expected, tyrosine 115 corresponds to one of the aromatic amino acids whose resonances shift upon oligonucleotide binding. In contrast, the alanine 137 mutation does not seem to have any major effect on either gp32 stability or binding to ssDNA.

N 520 SITE-SPECIFIC MUTAGENESIS OF T4 ENDONUCLEASE V-MUTANTS WHICH LOSE PYRIMIDINE DIMER SPECIFIC BINDING AND GLYCOSYLASE ACTIVITY, Donald G. Stump and R. Stephen Lloyd, Vanderbilt University, Nashville, TN 37232.

T4 endonuclease V incises DNA at the site of pyrimidine dimers through a two step mechanism. These breakage reactions are preceded by a scanning of nontarget DNA and a subsequent binding to pyrimidine dimers. In analogy with the synthetic tripeptides Lys-Trp-Lys and Lys-Tyr-Lys, which have been shown to be capable of producing single-strand scissions in DNA containing apurinic sites, endonuclease V has the amino acid sequence - Trp-Tyr-Lys-Tyr-Tyr (128-132). Site-directed mutagenesis of the endonuclease V gene, *denV*, was performed at the Tyr129 and Tyr129, Tyr131 positions in which the Tyr were converted to nonaromatic amino acids to test for their role in dimer-specific binding. The colony forming ability of repair-deficient (*uvrA rec*) *E. coli* cells containing either the wild type or mutant *denV* gene was examined. At a dose of .12 J/m^2 UV, the colony forming ability of cells containing *denV* Asn129 or *denV* Asn129,131 was decreased by 1.5 and 3.5 logs respectively as compared to wild type *denV*$^+$ cells. The mutant endonuclease V proteins were then characterized by 1) dimer specific nicking activity, 2) apurinic nicking activity, and 3) binding activity to UV irradiated DNA. Dimer specific nicking activity and binding activity of both *denV* Asn129 and Asn129,131 were abolished. The *denV* Asn129 cells had apurinic DNA nicking activity of approximately 60% that of the *denV*$^+$ cells, while *denV* Asn129,131 had no appreciable apurinic nicking activity. These data indicate that the Tyr129 and Tyr131 positions of endonuclease V are important in the binding and glycosylase nicking activity of pyrimidine dimers in DNA.
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N 521 DEFINITION OF THOSE REGIONS OF THE MOUSE INTERLEUKIN-2 PROTEIN THAT ARE EXPENDABLE FOR ACTIVITY, Sandra M. Zurawski, Timothy R. Mosmann and Gerard Zurawski, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 943004.

We have undertaken a molecular analysis of the structure-function relationships of the protein hormone mouse interleukin-2 (mIL-2). The analysis utilizes expression plasmids that direct synthesis of mIL-2 intracellularly in *E. coli*. Some of these plasmids have had their coding region synthetically reconstructed to include multiple unique restriction sites and preferred codons. The unique restriction sites have facilitated the construction of plasmids expressing mutant mIL-2 proteins that carry deletions that are N-terminal, C-terminal, or internal, or single amino acid replacements, or combinations of two classes. These mutant proteins are defined with respect to their relative specific activities utilizing western blot analysis to estimate protein amounts and HT-2 proliferation assays to measure activity. The results of this analysis define regions of the protein that are expendable for activity as well as regions that are sensitive to structural changes.