STRUCTURAL AND ORGANIZATIONAL ASPECTS OF METABOLIC REGULATION

Organizers: Paul Srere, Mary Ellen Jones and Christopher Mathews

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Structural and Organizational Aspects of Metabolic Regulation

Cell Growth

R 001 PROTEIN-TYROSINE KINASES AND THEIR TARGETS. Tony Hunter, Bill Boyle, Ellen Freed, Kathy Gould, Detlev Jähner, Rick Lindberg, Jill Meisenhelder, and John Pines. The Salk Institute, P.O. Box 85800, San Diego, CA 92138.

We have begun to identify novel protein-tyrosine kinases (PTK) by screening cDNA libraries with oligonucleotide probes to consensus sequences, and with anti-P.Tyr antibodies. Among the candidate PTK cDNAs we have isolated is a novel receptor-like PTK, *eck*, predominantly expressed in tissues containing cells of epithelial origin, which is closely related to *eph*, a newly described receptor-like PTK. We are currently attempting to prove that the *eck* protein has PTK activity. A second putative PTK, is closely related but distinct from the *trk* receptor-like PTK. This gene, which may encode another receptor-like PTK, is most highly expressed in the brain. Using anti-P.Tyr screening we have identified a cDNA clone for the *lyn* PTK, and at least two other putative PKs that are being characterized.

We have continued to analyse a number of PK substrates. p81, a substrate for both the EGF receptor and $pp60^{v\text{-}src}$ PTKs, is localized to the core of surface microvilli. With Tony Bretscher (Cornell), we have isolated a full length p81 cDNA clone. The predicted sequence of p81 is being used to elucidate its function and phosphorylation sites. In collaboration with Sue Goo Rhee (NIH) we have recently identified phospholipase C II (PLC-II) as a substrate for the PDGF and EGF receptors. PLC-II is rapidly (within 30 sec) phosphorylated on Tyr and Ser to a high stoichiometry when quiescent NIH 3T3 cells are treated with PDGF. The same Tyr sites are phosphorylated in vitro by purifed PDGF receptor. We are testing whether these phosphorylations stimulate PLC-II activity and thus account for increased PI turnover in PDGF-treated cells. Cyclin, is a highly conserved cell cycle regulated protein that is required in embryogenesis for entry into mitosis, and which may associate with the cdc2 protein-serine kinase. We have isolated a human cyclin B cDNA, and we are testing whether cyclin B associates with cdc2 and whether this association is regulated by phosphorylation.

To determine how PTKs activated at the cell surface induce nuclear events, we have started to examine the phosphorylation of nuclear regulatory proteins, which could be the target for a surface-initiated PK cascade. $p48^{v\text{-}myb}$, the AMV oncogene product, and its cellular counterpart, $p75^{c\text{-}myb}$, are phosphorylated at 5 clustered Ser near its C-terminus, and among several PKs tested only glycogen synthase kinase 3 (GSK3) can phosphorylate these sites in vitro. The transcription factor AP-1 and Fos, which form a tight complex, are both phosphoproteins and we are studying whether phosphorylation affects the ability of this complex to promote transcription. We have isolated 10 cDNAs for mRNAs whose synthesis is rapidly increased following shift of ts $pp60^{v\text{-}src}$ infected NRK cells. These include sequences shown by others to be mitogen-inducible (c-*fos*, KC, ODC, *Egr*-1) as well as some novel cDNAs.

R 002 CHIMERAS THAT ALTER THE NH_2- OR COOH-TERMINUS OF THE α-SUBUNIT OF $G_{s\alpha}$, THE ADENYLYL CYCLASE STIMULATORY G-PROTEIN, CONSTITUTIVELY ACTIVATE cAMP SYNTHESIS. Gary L. Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206. Chimeric Gα cDNAs have been constructed which have the NH_2-terminal 61 amino acids or the COOH-terminal 38 amino acids of $G\alpha_s$ replaced with the corresponding domain of $G\alpha_{i2}$, an inhibitory G-protein of adenylyl cyclase. The resulting chimeras have an altered charge distribution in the amphipathic helix at the NH_2- or COOH-terminus and constitutively activate cyclic AMP synthesis when expressed in fibroblasts. The chimeric Gα proteins couple to β-adrenergic receptors, are not ADP-ribosylation substrates for either cholera or pertussis toxin, and in reconstitution assays markedly enhance the Vmax of adenylyl cyclase activation by guanine nucleotides. In CHO cells the G-protein chimeras were also found to cause an increased rate of PI turnover and a markedly blunted thrombin-stimulated IP_3 generation. The effect of $G\alpha_s$ chimeras could not be mimicked by cAMP analogs. The chimeric G-proteins therefore not only stimulate adenylyl cyclase but also perturb receptor-coupled G-protein signalling pathways in fibroblasts. Neither truncation mutants or chimeras having nearly 60% of the $G\alpha_{i2}$ and 40% of the $G\alpha_s$ coding sequence were able to mimic the NH2- and COOH-terminal chimera phenotype. Several point mutations corresponding to changes in amino acids in ras that inhibit GTPase activity were shown to result in a $G\alpha_s$ polypeptide that markedly elevated cAMP synthesis in COS cell transient expression assays. However, only mutations in residues gln227 and val49 of $G\alpha_s$ gave significant elevation of cAMP synthesis in CHO cell stable transfectants, probably because of the ability of βγ-subunits to attenuate the $G\alpha_s$ activity in mutants that only partially inhibit the GTPase.

Structural and Organizational Aspects of Metabolic Regulation

Cellular Traffic and Organellar Biosynthesis

R 003 ANALYSIS OF METABOLIC AROUSAL DURING FERTILIZATION USING PERMEABILIZED OR PERFORATED CELLS, David Epel and Robert Swezey, Hopkins Marine Station, Department of Biological Sciences, Stanford University, Pacific Grove, CA 93950

The arousal of egg metabolism at fertilization follows a scenario similar to that seen in many cell-stimulation situations-a brief bout of phosphoinositide hydrolysis followed by a transient rise in intracellular calcium and a permanent rise in intracellular pH. Our work indicates that a change in cell structure has a major role in the metabolic activation of the egg. Specifically, we have been looking at activity of a number of enzymes in electrically permeabilized or perforated cells. The procedure allows addition of substrates directly to the cells and measurement of enzyme activity in the much more highly-structured situation of the organized cell as opposed to an homogenate. Our results indicate a remarkable restriction of enzyme activity in unfertilized eggs for most of the assayed enzymes. Following fertilization there is a partial relief of these restrictions.

Our experimental analysis indicates that the rise in calcium is a major causal factor. The system has also been used for looking at macromolecular synthesis, such as protein and DNA synthesis and also at changes in protein phosphorylation. Results of our current investigations on the nature of this structural change and the validity of these enzyme assays in vivo will be presented.

Metabolism of Genetic Elements

R 004 RIBONUCLEOPROTEIN ORGANIZATION: FROM CELL NUCLEUS TO RNP, H.C. Smith, Department of Pathology, Box 626, University of Rochester, Rochester, NY 14642

Complexes of protein and RNA (RNPs) constitute major structural and functional assemblies of the nonchromatin nuclear domain. Premessenger RNA and uridine-rich small nuclear RNAs are packaged as hnRNPs and U-snRNPs (respectively) by specific subsets of nuclear nonhistone proteins. Nuclear extracts, competent for in vitro premessenger RNA splicing, were chemically cross linked with thiol reversible reagents in order to study the organization of proteins within U-snRNPs. The distribution of select U-snRNP antigens within cross-linked complexes was determined by Western blotting of diagonal two dimensional gels. Based on calculations from the molecular weights of cross linked complexes containing U-snRNP common proteins B', B or D, it is proposed that each of these proteins was associated with U-snRNP common proteins E and G. In addition, D' is also proposed to be positioned close to D. The spatial distribution of U-snRNP common proteins was such that B' and B could not be cross linked to D. The data also suggested that the 63 kDa U1snRNP specific protein was cross linked to other U1 specific proteins, particularly C, but not to the UsnRNP common proteins. We propose that part of the U-snRNP core of common proteins contains at least two nonsymmetrical copies of B':B:D:D':E:G with stoichiometries of 2:1:1:1:1:1 and 1:2:1:1:1:1.

R 005 A GENERAL APPROACH TO DEFINING THE SPECIFICITY OF INTERACTIONS BETWEEN CYTOPLASMIC PROTEINS, Ronald L. Somerville, Jill H. Zeilstra-Ryalls and Tiee-Leou Shieh, Department of Biochemistry, Purdue University, West Lafayette, IN 47907.

Trp repressor protein of Escherichia coli is a small (108 amino acids) ligand-activatable protein whose role is to control transcription from promoters that contain some version of the trpO family of operator targets. Trp repressor protein functions in the form of a homodimer. E. coli cells that have been genetically engineered to hyperproduce Trp repressor exhibit a wide range of phenotypic abnormalities such as new nutritional requirements, slow rates of growth, and the cessation of transcription from promoters having no obvious internal trpO-related operators. The possibility that the cellular pathology associated with elevated levels of Trp repressor might involve interactions with other proteins was addressed by immunological procedures. Sera from outbred rabbits that had been repeatedly injected with electrophoretically purified Trp repressor were shown to contain anti-Trp repressor IgG and anti-anti-Trp repressor IgG. The latter category of antibodies reacted with a distinct subset of five E. coli cytoplasmic proteins (M_r 81 kD, 69 kD, 38 kD, 35 kD and 24 kD). The immunoreactivity of these proteins was blocked either by pretreatment of serum with mixed cytoplasmic proteins from a trpR deletion mutant or by allowing the cellular proteins to form complexes with purified Trp repressor. We conclude that a subset of the anti-idiotype antibodies in hyperimmune serum can function as positive stereochemical replicas of Trp repressor. It is predicted that some of the polyclonal anti-idiotype antibodies elicited in response to polyclonal primary antibodies should contain IgG molecules appropriate for the identification of proteins that interact with the primary antigen.

Protein separation systems of high resolving power, such as one- and two-dimensional gel electrophoresis and detection systems of high sensitivity, such as immunoblotting, are important supporting techniques in the characterization of such protein-protein interaction webs.

We established a phenotypic assay for identifying E. coli mutants defective in the ability to carry out subunit interchange between Trp repressor monomers. A collection of such subunit interchange defective mutants were all found to map to a single chromosomal locus, to have acquired a complex nutritional phenotype, and to have lost the ability to synthesize an anti-idiotype reactive cytoplasmic protein. This experiment and other genetic, biochemical and immunological approaches to the identification of the E. coli proteins that interact with Trp repressor will be discussed.

Protein and Nucleic Acid Biosynthesis

R 006 MEMBRANE ASSOCIATED REPLISOMES OF BACILLUS SUBTILIS AND PLASMID RK2, William Firshein, John Laffan, David Kostyal, Loretta Mele, Bethanie Wilkinson and Ann McCabe, Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, Connecticut 06457.

The involvement of the DNA/membrane complex in DNA replication has been strongly suggested by demonstrating the synthetic capabilities of such complexes in vitro. A variety of studies with several procaryotic systems have shown that initiation occurs and that membrane associated replication proteins are present and active in these complexes (1,2).

Further investigations with Bacillus subtilis and the broad host range plasmid RK2 cultured in Escherichia coli have shown the following: A 64 kDa membrane associated protein of B. subtilis which binds strongly to parts of the origin region including one possible initiation site may act as a partial repressor of initiation. This protein may also act in a cyclical manner and be part of a complex of at least 300 kDa. Another initiation gene locus of B. subtilis discovered by Sueoka and colleagues (3), the dnaB locus, is present and active in the DNA/membrane complex. Finally, a multienzyme complex involved in synthesizing DNA precursors has been found to copurify with the DNA/membrane extract. Both ribonucleoside and deoxyribonucleoside kinases as well as the ribonucleoside diphosphate reductase and nucleoside diphosphate kinase are present.

A DNA/membrane complex extracted from a miniplasmid derivative of plasmid RK2 cultured in a minicell mutant of E. coli exhibits the activity of at least one host encoded initiation protein, the dnaA gene product. This protein as well as a number of plasmid encoded proteins including an essential initiation protein of 32 kDa (and an overlapping protein of 43 kDa coded for by the same gene) have been localized to the inner, but not outer membrane fraction of E. coli. In addition, they resist procedures that dissociate peripheral membrane proteins. The inner membrane alone may be capable of carrying out the entire sequence of plasmid replication provided it is complemented by certain enzymes such as DNA gyrase.

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Prokaryotic Organization

R 007 **ON THE CONTROL OF MICROBIAL GROWTH RATES**

Douglas B. Kell, Department of Biological Sciences, University College of Wales, Aberystwyth, Dyfed SY23 3DA, U.K.

The metabolic control analysis (MCA) developed by Kacser, Burns, Heinrich & Rapoport allows one to approach two important general questions (in particular). The first concerns the quantitative contribution of a particular enzyme (or series of enzymes) to the control of a particular flux, as embodied in the so-called flux-control coefficient of the enzyme (or group of enzymes) of interest. To illustrate how, a brief review will be given of the main tenets, theorems and terminology of the MCA, together with examples of the experimental approaches which one may take to measure the flux-control coefficients. Several authors have considered the problem of whether the growth rate of microorganisms in "unrestricted" batch culture is controlled by catabolism or by anabolism. However, in view of the MCA it is clear that catabolism, anabolism and the 'energy leak' (imperfect coupling between them), should each contribute to the control of microbial growth rates. Inhibitor titrations of the growth and catabolic rates of batch cultures of Clostridium pasteurianum 6013-MR505 using iodoacetate and streptomycin indicate that both catabolism and anabolism contribute positively to the control of the growth rate. Similar titrations with tetrachlorosalicylanilide show that the energy leak exerts a negative and non-zero control on the growth rate. Further, glycolysis in this organism appears to be organised into a "metabolon" , as judged by the ability of electroporated cell suspensions of this organism to carry out the metabolism of G6P with no lag.

Finally, control analysis provides a rigorous means to detect whether metabolites are organised as "pools" or into "microcompartments". If the latter is the case, the flux-control summation theorem will appear to fail, when assessed using inhibitor titrations (Westerhoff & Kell, 1988). This is illustrated using inhibitor titrations of the photophosphorylative apparatus from Rhodobacter capsulatus.

R 008 THE BACTERIAL EQUIVALENT OF MITOSIS, Moselio Schaechter, Department of Molecular Biology and Microbiology, Tufts University Health Campus, Boston, MA 02111.

General introduction. We know a great deal about mitosis in higher cells and very little about the equivalent process in bacteria. For prokaryotes, we still have little insight into equivalent aspects of chromosome segregation. About all we know with certainty is that the bacterial chromosome segregates with fidelity. A popular notion of long standing, derived from the replicon model. I will first consider the similarities and differences in the timing of chromosome segregation between eukaryotes and prokaryotes, then the structural elements involved in bacteria. The questions at hand are: what are the bacterial equivalents of the centromere, kinetochore, spindle fibers, and spindle movement?

Recent experimental findings. Specific membrane-binding regions are contained within a 463 bp stretch of origin DNA between positions -46 and +417 on the *oriC* map[1]. This region of DNA contains an unusually high number of GATC sites, the recognition sequence for the *E. coli* DNA adenine methylase. We have shown that *oriC* DNA binds to membrane only when it is hemimethylated[2]. The *E. coli* chromosomal origin is hemimethylated for 8-10 minutes after initiation of replication, and origin DNA binds to membranes only during this time period. Based on these results, a speculative model for chromosome segregation in *E. coli.* has been proposed[3].

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Infrastructure of Organelles

R 009 MOLECULAR TOPOGRAPHY OF THE NUCLEAR MATRIX, R. Berezney, H. Nakayasu, D.J. Hakes and P. Belgrader, Department of Biological Sciences, State University of New York at Buffalo, Buffalo, NY 14260

While the nuclear matrix has been studied structurally and biochemically for over a decade, a clear definition of the individual proteins which constitute this isolated nuclear structure has yet to be demonstrated. As a step in this direction polyclonal antibodies have been generated to 10 of the 14 major proteins which comprise the isolated rat liver nuclear matrix. Indirect immunofluorescent microscopy demonstrated that all of the antibodies except anti-lamins A and C decorated an extensive extranucleolar fibrogranular network in the nuclear interior of whole cells or in the interior of the nuclear matrix structure following appropriate extraction of cells. During mitosis all of these major matrix proteins were dispersed in the cytoplasm with no particular concentration over the spindle apparatus or chromosomes. The internal matrix (IM) proteins have a broad range in apparent molecular mass (40->200 kD) and pI (5.5->8.5). Both immunoblotting and Cleveland peptide mapping suggested that the IM proteins are largely distinct from one another and from lamins A,B and C in protein sequence.The IM proteins do not appear to be closely related to intermediate filament proteins since none of them (unlike lamins A and C) recognized the Pruiss universal intermediate filament protein antibody on immunoblots. Moreover, only one cluster of the ten major IM proteins recognized antibodies to hnRNP proteins and none recognized antibodies to snRNP proteins.

We propose that IM proteins represent a unique but heterogeneous class of structural proteins in the nucleus which are assembled into a three-dimensional lattice in the interior of the interphase nucleus. This lattice, or the IM proteins which compose it, can then potentially interact with other RNA and DNA components in the nucleus. In this regard several of the IM proteins bind DNA on nitrocellulose. Sequencing of the c-DNA coding regions for several of the IM proteins is currently in progress and will be presented. Preliminary results for one of these proteins indicates that it is highly concentrated in hydrophobic amino acid residues.

R 010 DYNAMIC ASPECTS OF THE MACROMOLECULAR ORGANIZATION OF PHOTOSYNTHETIC MEMBRANES, L. Andrew Staehelin, Keith D. Allen and Rodney A. Sauer, Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309-0347.

Our work on photosynthetic membranes focuses on the functional organization of thylakoid membrane complexes and the way in which this organization responds to short- and long-term changes in light conditions. To this end we are characterizing the biochemical composition and spatial organization of the different classes of membrane complexes by a combination of non-denaturing "green gel" electrophoresis, membrane fractionation techniques, freeze-fracture and freeze-etch electron microscopy, and immunocytochemical localization methods. The presentation will start with an overview of the functional organization of the five major protein complexes (photosystems I and II [PSI/II], cytochrome b_6/f, peripheral light harvesting complex II [pLHCII], ATP synthase) in chloroplast membranes, and emphasize evidence correlating these complexes with morphological counterparts in freeze-fractured membranes. This will be followed by a description of studies in which we have correlated multimeric particles on the lumenal thylakoid membrane surface with the extrinsic proteins of the water-splitting apparatus. The next section will describe alterations in the spatial organization of the protein complexes during State 1 - State 2 transitions, which optimize the rates of turnover of PSII and PSI by regulating the distribution of excitation energy between the two photosystems. The mechanism involves membrane-bound kinase and phosphatase enzymes that phosphorylate/dephosphorylate a subset of pLHCII particles and thereby alter their distribution in the membrane. A new, high resolution, native green gel system for separating integral membrane protein complexes will be described in the final section of the lecture. When thylakoids of either *Chlamydomonas* or higher plant species are examined with this gel system, as many as eighteen chlorophyll-protein complexes can be resolved with very little release of free pigment. In *Chlamydomonas*, at least six bands are PSI-related, differing from each other with respect to chlorophyll a/b ratio, 77K fluorescence emission spectra and polypeptide composition. Three to four bands contain PSII complexes, about six bands pLHCII, and the remaining ones monomeric species related to CP29, CPII, CP27 and CP24 of higher plants. Supported by NIH grant GM 22912.

Structural and Organizational Aspects of Metabolic Regulation

Dissociable Complexes of Sequential Metabolic Enzymes

R 011 E. COLI PYRUVATE OXIDASE - AN ENZYME THAT "SHUTTLES" BETWEEN MEMBRANE AND CYTOSOL, John E. Cronan, Jr., Departments of Microbiology and Biochemistry, University of Illinois, Urbana, IL 61801.

Pyruvate oxidase is a tetrameric flavoprotein that catalyses the decarboxylation of pyruvate acetate. Oxidase activity is greatly stimulated (increased keat of 200 to 500-fold) by phospholipids and some detergents. This activation is accompanied by binding of the protein to lipid vesicles, but the lipid binding site is cryptic. Binding only occurs when the enzyme is functioning in the presence of pyruvate and the essential cofactor, TPP. We have isolated E. coli mutants encoding oxidases specifically defective in lipid binding and activation. All such mutants lack oxidase activity in vivo indicating that lipid binding is essential for the physiological function of the enzyme. Our data are consistent with a model in which the oxidase is membrane bound only during its decarboxylation of pyruvate. An interesting outgrowth of these studies is that pyruvate oxidase is closely related to the acetolactate synthases that catalyse the first steps of isoleucine-valine biosynthesis.

R 012 ATP-DEPENDENT PROTEOLYTIC ENZYMES IN BACTERIAL AND MAMMALIAN CELLS, Alfred L. Goldberg, James Driscoll, William Matthews, and A.Satish Menon, Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115.
Intracellular protein breakdown requires metabolic energy, due in part to the involvement of large multimeric proteases whose function is linked to ATP hydrolysis. In E.coli, the rate-limiting steps in the degradation of many polypeptides are catalyzed by the ATP-hydrolyzing protease La, the lon product. For each peptide bond cleaved, protease La utilizes two ATPs. This tetrameric enzyme binds an ATP on each subunit, and this step activates the proteolytic sites. ATP hydrolysis is necessary for the rapid degradation of proteins, but cleavage of peptides only requires ATP-binding. This enzyme's proteolytic capacity increases several fold when an unfolded protein interacts with an allosteric domain on the enzyme. This activation process helps determine the selectivity of proteolysis. ADP (an inhibitor of the protease) remains tightly associated with the enzyme in vivo and prevents excessive proteolysis. Protein substrates activate the enzyme in part by stimulating the release of this bound ADP and promoting the binding of new ATP molecules. The complete base sequence of the lon gene has been determined. It contains a heat-shock promoter which accounts for its induction when cells produce large amounts of abnormal proteins. The enzyme does not show sequence homology to other serine proteases. Mitochondria contain an ATP-dependent protease similar to protease La. E. coli also contain another ATP-dependent protease, called Ti. Its proteolytic and ATPase functions are present in different subunits which can be reconstituted to give ATP-dependent proteolysis. All mammalian tissues contain a 700kDa protease complex which we call the proteasome. These 19S particles are composed of multiple subunits which hydrolyze several types of peptides. After rapid purification from muscle, it shows a large activation by ATP. Nonhydrolyzable ATP analogs stimulate peptide hydrolysis, but do not support protein breakdown (as was found with protease La). The proteasome may represent a ubiquitin-independent, energy-requiring proteolytic pathway. In the mammalian cytosol, the breakdown of many proteins requires their covalent conjugation to ubiquitin. The subsequent breakdown of these conjugates also requires ATP, and is catalyzed by a very large (1500kDa) complex, UCDEN (Ubiquitin-Conjugate Degrading Enzyme). Antibodies against the proteasome block the breakdown of proteins conjugated to ubiquitin. Thus, the proteasome may be a component of UCDEN or these two complexes may work coordinately in the ubiquitin-dependent pathway.

R 013 **GLYCOLYTIC ENZYME-CYTOSKELETAL PROTEIN INTERACTIONS**, **Harvey Knull, Julie Walsh, and Tim Keith, Department of Biochemistry and Molecular Biology, Univ. of North Dakota, Grand Forks ND 58202**

Electron microscopy has revealed a network of proteins meandering through the cytoplasm which is called the microtrabecular lattice (1). Thelattice appears to be a polymeric structure composed of cytoplasmic proteins including those commonly considered cytosolic (1), such as the glycolytic enzymes (2). The nature and specificity of interactions between the proteins making up the lattice have not yet been established. Ourhypothesis is that the microtrabecular lattice and the cytoskeleton interact and that the cytoskeleton serves to anchor the microtrabeculae.

The structural components of the cytoskeleton include F-actin, microtubules and intermediate filaments. Much research suggests that several proteins of the glycolytic pathway associate with F-actin. However, one glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, hasbeen shown to complex with microtubules (3) and on the basis of co-electrophoresis in agarose gels we have found that aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and lactate dehydrogenase type-M interact with tubulin (4). In other studies we have found interactions of these same enzymes with microtubules and under special conditions have found that glucose-phosphate isomerase and phosphoglycerate kinase may also interact. The interactions have been demonstrated by cosedimentation, florescence anisotropy, and affinity chromatography. The K_D values for the interactions are in the micromolar range which approximates the cellular concentration of the enzymes.

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R 014 STUCTURAL AND METABOLIC CONSEQUENCES OF MITOCHONDRIAL PROTEIN-PROTEIN INTERACTIONS.
Balazs Sumegi. Institute of Biochemistry, University Medical School. 7624-Pecs, Hungary.

Previously, we have shown that enzymes catalizing consequtive reactions are organized in the mitochondrial matrix. These multienzyme complexes can be observed in gently disrupted mitochondria (metabolon). Here, we study the kinetic advantage of the organization of fatty acid β-oxidation enzymes. The respiration-linked oxidation of crotonyl-CoA progressed with 58 % of the rate of NADH oxidation in complete disrupted mitochondria. If we calculated the crotonyl-CoA oxidation for the amount of exposed enzymes, the metabolon catalyzed the oxidation approximately 7 times more effectively tha the disrupted mitochondria. The respiration-linked oxidation of 3-hydroxybutyryl-CoA was also studied in metabolon and, in accord with our previous observation, the metabolon oxidized this substrate much more effectively than the disrupted mitochondria. This suggests that the channaling of NADH between the 3-hydroxyacyl-CoA dehydrogenase and NADH: ubiquinone oxidoreuctase (Complex I) can contribute to the great kinetic advantage observed in metabolon.

Protein-protein interactions can be important not only in the catalysis but also in the assembly of mitochondrial inner membrane matrix compartment. The pyruvate dehydrogenase complex (PDC) is one of the largest multienzyme complex (MW.8,000 kD, diameter 45 nm), and it interacts with several functionally related mitochondrial enzymes. Electron microscopic evidences are given, herein, that this complex is much smaller "in situ" both in heart and in brain tissues than the isolated complex, as well as, it is shown that the complex starts to aggregate when the interaction between PDC and inner membrane is disrupted.

Structural and Organizational Aspects of Metabolic Regulation

Distributive Metabolic Control: Theoretical Approaches to Analysis of Metabolic Complexes

R 015 A HOLISTIC VIEW OF BIOCHEMISTRY EMERGES FROM THE STUDY OF BIOMOLECULES AND BIOCHEMICAL REACTIONS WITHIN A NATIVE OR NATIVE-LIKE ENVIRONMENT,
Allen P. Minton, Laboratory of Biochemical Pharmacology, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

The traditional reductionist approach to biochemistry leads to the study of biomolecules and their reactions in purified solutions that are almost invariably low in total macromolecular content. During the past decade evidence from several laboratories has shown that the presence of high concentrations of unrelated macromolecules (termed "background" species) in a solution can strongly influence the steady-state properties of biomolecules present at low as well as high concentration, as well as the equilibria and rates of biochemical reactions. Such influence is due to weak, nonspecific interactions between the background and target species, the importance of which becomes evident only when the total concentration of all background species exceeds several weight-percent.

Several different predicted and observed effects of environment upon biochemical equilibria and rates will be reviewed. The need to characterize the structural and functional properties of biomolecules in a native-like environment will be stressed.

Structural Basis of Protein Interactions

R 016 THE PHOTOSYNTHETIC REACTION CENTER FROM RHODOPSEUDOMONAS VIRIDIS,
J. Deisenhofer[1] and H. Michel[2], [1]Department of Biochemistry and Howard Hughes Medical Institute / University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75235-9050, [2]Max-Planck-Institut fuer Biophysik, Heinrich-Hoffmann-Str. 7, D-6700 Frankfurt 71, Federal Republic of Germany

Photosynthetic reaction centers (RCs) are membrane-bound complexes of proteins and pigments. They catalyze the primary reaction in photosynthesis: light driven charge separation across a membrane.

The RC from the purple bacterium Rhodopseudomonas viridis was one of the first membrane proteins for which well ordered 3-D crystals were obtained (1). The X-ray structure analysis at 3.0Å resolution of these crystals (2,3,4) allowed the construction of an atomic model including the RC's four protein subunits, and the major pigment cofactors. Crystallographic refinement at 2.3Å resolution (5) further improved the model, and led to the discovery of additional cofactors, and of localized solvent molecules.

The RC is an elongated complex whose surface is hydrophobic in the center, and polar at both ends. The central membrane spanning polypeptide chains are folded into 11 helices, connected by chains which contain shorter helices or peptides without regular secondary structure. The transmembrane helices vary in length between 21 and 28 amino acid residues.

The cofactors located in the membrane spanning region of the RC form two approximately symmetric pathways for electron transfer across the membrane. However, under most circumstances, only one of these pathways is actually used. This functional asymmetry in the presence of structural symmetry is one of the most surprising properties of the RC complex.

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R 017 THE THREE-DIMENSIONAL STRUCTURE OF TRYPTOPHAN SYNTHASE FROM *SALMONELLA TYPHIMURIUM*, C. Craig Hyde, Laboratory of Molecular Biology, Bldg. 2, Rm. 316, NIDDK, National Institutes of Health, Bethesda, MD 20892. Tryptophan synthase, which catalyzes the final two reactions in L-tryptophan biosynthesis, has been the subject of numerous biochemical and genetic investigations. It has become one of the classic examples of a multienzyme complex which "channels" an intermediate substrate (indole) between active centers. In bacteria, the enzyme is a bifunctional, bienzyme complex with an $\alpha_2\beta_2$ subunit composition (Mr= 143,000). The alpha subunits (Mr=29,000) catalyze the cleavage of indole 3-glycerol phosphate to indole and glyceraldehyde 3-phosphate. The beta subunits (Mr=43,000) contain the cofactor pyridoxal phosphate and catalyze the conversion of indole and L-serine to L-tryptophan. The three-dimensional structure of the enzyme has been determined by x-ray crystallography to a resolution of 2.5Å (1). The overall fold of the smaller alpha subunits is that of an eight-fold alpha/beta barrel. The binding site for pyridoxal phosphate is found deep within each beta subunit at the interface of two domains with similar folds. The active sites between neighboring alpha and beta subunits are separated by over 25Å. The mechanism of the indole channeling phenomenon can be explained in structural terms by the existence of a "tunnel" deep within the protein that connect the alpha and beta active sites. The tunnel is believed to facilitate the diffusion of indole from its point of production in the alpha subunit to the site of tryptophan synthesis in the beta subunit and thereby prevent its escape to the solvent during catalysis.

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Non-Invasive Measurements of Cell Metabolism

R 018 Use of enzymes and ion transporters as in situ probes of metabolite and ion gradients. Dean P. Jones and Tak Yee Aw, Department of Biochemistry and Winship Cancer Center, Emory University, Atlanta, GA 30322.
Absorption of endogenous chromophores such as myoglobin and mitochondrial cytochromes have been used for several decades to provide information on intracellular oxygenation. The approach to use endogenous proteins as in situ probes is applicable to many metabolites and ions, and with current genetic engineering techniques, one can target probes to specific sites. The requirements for obtaining useful information vary for different probes and metabolite or ion. This presentation will focus on the use of enzymes and ion transport systems to map intracellular gradients of [O_2], [ATP] and pH. The results from studies of O_2 dependence of various enzymes show that mitochondrial distribution within cells has a major effect on the local chemical environment (1, 2). Clustering of mitochondria creates zones that are relatively low in [O_2] while other regions of the cells, e.g. peroxisomes and endoplasmic reticulum, have relatively high [O_2]. Comparison of activities of ATPases with different locations in cells shows that when mitochondrial ATP production is limited, enzymes present at sites distant from the mitochondria are selectively vulnerable to ATP deficiency due to consumption-induced ATP gradients in the aqueous cytoplasm (3). Studies of the partitioning of pH indicators and endogenous anions between the cytoplasm and the matrix of kidney tubule cells show that the pH in the vicinity of mitochondria is low relative to the average pH in the aqueous cytoplasm (4). Anions that are transported by electroneutral, H^+-compensated systems are accumulated to a greater extent than that supported by the average cytosol/matrix pH gradient. Thus, endogenous enzymes and transport systems can be used to measure regional differences in metabolite and ion gradients in cells.

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R 019 ENERGETICS, FLUXES AND CONTROL IN INTACT TISSUE. George K. Radda, Department of Biochemistry, Oxford University, South Parks Road, Oxford OX1 3QU, U.K.
The control of energy supply in response to demand is critical to the function of organs like the brain, heart and skeletal muscle. While in each of these oxidative phosphorylation, glycolytic and creatine kinase activities are present, there are significant differences in the role of these pathways in the three organs. In skeletal muscle fluxes through creatine kinase remain constant in spite of a large change in ATP synthesis rate during isometric muscle contraction (both measured by NMR magnetisation transfer in animal and human muscle). During functional steady-state, the contribution from different metabolic processes is not constant and ADP and Pi are important in control of contraction and energy supply. In heart muscle the dynamic range is small and control is aimed at modulation of activities (smoothing function) rather than for rapid switching. The brain relies almost entirely on glucose as substrate but functional and cellular heterogeneity is a compounding problem. The study of these functions by 31P NMR in isolated organs and in animals and man in health and disease gives new insights into control, fluxes and cellular heterogeneity. For example, investigations of muscle energetics in patients with specific lesions in the glycolytic pathway (e.g. phosphorylase, phosphorylase kinase deficiency) in the mitochondrion (e.g. NADH-CoQ reductase, cytochrome oxidase defect) in the redox link between the two malate aspartate shuttle defect) and in other processes (e.g. Ca++/ATPase defect) allow us to assess the importance of such enzymes in normal muscle. The relation between ATP synthase flux and tension-time integral has been investigated in animal limbs responding to sciatic nerve stimulation, and the heterogeneity of creatine kinase activities in different muscle groups has also been studied by magnetization transfer. The distribution of creatine kinase activities in different parts of the human brain has also been observed.

Studies on Metabolic Regulation by Metabolism Engineering

R 020 CHARACTERIZATION OF MUTANT FORMS OF YEAST MITOCHONDRIAL MALATE DEHYDROGENASE, Lee McAlister-Henn, Joan S. Steffan, and Karyl Minard, Department of Biological Chemistry, College of Medicine, University of California, Irvine, CA 92717
The unfavorable equilibrium for oxaloacetate production in the reaction catalyzed by malate dehydrogenase is the basis for speculation that the mitochondrial enzyme must directly and alternately interact with other enzymes to efficiently route this metabolite through the tricarboxylic acid cycle or through the malate/aspartate shuttle cycle. Various methods have previously provided substantial evidence for specific physical interactions between malate dehydrogenase and appropriate mitochondrial components. To develop a molecular genetic system for examining the metabolic functions of interactions involving mitochondrial malate dehydrogenase *in vivo*, we have cloned the corresponding structural gene (*MDH*1) from *Saccharomyces cerevisiae* and have constructed a yeast strain containing a deletion in the coding region of the chromosomal *MDH*1 locus. The *MDH*1 deletion mutant is markedly impaired for growth on acetate as a carbon source. Using reversion of this growth phenotype as a test for malate dehydrogenase function *in vivo*, two approaches have been tested to determine the specificity of protein/protein interactions. First, functional complementation by heterologous forms of the enzyme was tested. This was accomplished by engineering and expressing genes encoding rat mitochondrial and *Escherichia coli* malate dehydrogenases to obtain mitochondrial localization of these enzymes in the *MDH*1 deletion mutant. Second, *in vitro* mutagenesis of *MDH*1 was conducted to alter the interaction of identical subunits in dimeric malate dehydrogenase. The subunit interface represents a defined model of a specific protein-protein interaction for this enzyme. Analyses of the resulting mutant forms of the yeast enzyme show a marked disparity between catalytic function as measured *in vitro* versus *in vivo* and thus underscore the necessity for such parallel tests.

R 021 THE KREBS TRICARBOXYLIC ACID CYCLE METABOLON: STUDIES WITH MOLECULARLY ENGINEERED YEAST, Paul A. Srere, Claudia Evans, Craig Malloy, and Gyula Kispal, VAMC and UT Southwestern Med. Ctr., Dallas, TX 75216. We and others have shown previously that there are specific interactions between metabolically sequential Krebs TCA cycle enzymes, and we have shown that the Krebs TCA cycle enzymes interact with protein components of the matrix surface of the inner membrane of the mitochondrion. These findings have led us to postulate the existence of a complex of interacting Krebs TCA cycle enzymes, termed a metabolon, which is bound to the inner membrane of the mitochondrion. Evidence for the metabolon has been obtained with briefly sonicated mitochondria in which the previously latent Krebs TCA cycle enzymes were now active in a large particle. These preparations oxidized citrate, isocitrate, and alpha-ketoglutarate faster than a completely sonicated system. In addition, they convert malate to citrate faster than the completely sonicated system. We have recently begun studying this metabolic pathway in yeast cells. Yeast contains two citrate synthases, a mitochondrial one, CS1, which functions in the Krebs TCA cycle, and a cytosolic one, CS2. Rosenkrantz and Guarente have prepared CS gene disruptions to produce $CS1^-$, $CS2^-$, and $CS1CS2^-$ cells. These cells not only do not contain the designated CS activity but lack the corresponding CS protein as well. Our results indicated that $CS2^-$ cells were essentially identical to the parental cells. $CS1^-$ cells grew normally on fermentable substrates but showed no growth on acetate. One explanation for the fact that the citrate supplied by CS2 cannot replace the mitochondrially generated citrate is that in the absence of CS1 protein a proper complex cannot be formed, and the operation of the Krebs TCA cycle with external citrate is inefficient thus preventing growth on acetate. To test this hypothesis we have engineered a yeast cell to contain CS1 which has been rendered enzymically inactive by site-directed mutagenesis. Such cells show the ability to grow on acetate. Thus, the inactive protein may have completed the metabolon to enable this phenotypic change in the organism. (Supported by grants from VA, NSF, and NIH).

R 022 INTRACELLULAR ORGANIZATION OF THE ENZYMES OF DEOXYRIBONUCLEOTIDE BIOSYNTHESIS, Christopher K. Mathews, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331-6503

Because the roles of deoxyribonucleoside triphosphates (dNTPs) are largely limited to their functions as DNA precursors, most cells have the potential to regulate DNA replication at the level of dNTP biosynthesis. Moreover, kinetic considerations of DNA metabolism make it important to understand how dNTPs are delivered to replication sites at rates sufficient to support V_{max} values observed for replication *in vivo*. For these reasons, a major objective of our laboratory is to understand how the enzymes of dNTP biosynthesis are organized within cells.

Using T4 bacteriophage-infected *Escherichia coli* as a model system, we have demonstrated that phage-coded and bacterial enzymes interact to form a multienzyme complex that carries out the kinetically facilitated synthesis of dNTPs *in vitro*. In current work, to be described, we are analyzing structural requirements for proper assembly of the complex, specific interactions among proteins in the complex, differences in kinetic and allosteric properties between complexed enzymes and their unbound counterparts, contributions of the complex toward fidelity of DNA replication, and structural and functional links between enzymes of dNTP biosynthesis and of DNA replication.

Using tissue culture systems, we are exploring the organization of dNTP metabolism in mammalian cells. Evidence from other laboratories, which apparently supports a direct coupling of dNTP synthesis to DNA replication can be interpreted differently. However, there is evidence supporting the existence of distinct pools of DNA precursors in mammalian cells, some of which are more readily utilized for DNA replication than others. Whether this involves the existence of cytoplasmic enzyme complexes for dNTP biosynthesis will be discussed. Finally, vaccinia virus encodes a novel form of ribonucleotide reductase, and this enzyme provides a probe, allowing us to ask whether dNTP biosynthetic complexes form during vaccinia infection and, if so, whether such complexes are linked to the viral DNA replication apparatus.

Structural and Organizational Aspects of Metabolic Regulation

Metabolism

R 100 MEASUREMENTS OF ENZYME KINETICS AND STRUCTURE IN VIVO USING NMR AND MOLECULAR GENETICS, Kevin Brindle, Peta Braddock, Corinne Spickett and Sandra Fulton, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U. K. The contribution of an enzyme to flux in a multi-enzyme pathway can be determined by genetically altering its intracellular concentration. We have used this approach, in conjunction with ^{31}P NMR, to study the kinetic properties of phosphoglycerate kinase (PGK) in the intact yeast cell (1). A similar approach is also being used to study the kinetics of the mitochondrial adenine nucleotide translocase in vivo. An important parameter to determine in these studies has been the intracellular free ADP concentration. This has been investigated by incorporating the gene for rabbit muscle creatine kinase into the yeast cell and using ^{31}P NMR to measure the near-equilibrium concentrations of the enzyme's substrates in cells incubated with creatine.

As well as being able to study enzyme kinetics in vivo, we have also been able to investigate aspects of enzyme structure in the intact cell by using ^{19}F NMR to monitor fluorine labelled PGK. The enzyme was fluorine labelled in vivo by using an inducible expression system and inducing enzyme synthesis in the presence of 5-fluorotryptophan. ^{19}F NMR spectra of these cells show two resonances from the two tryptophan residues in PGK. A comparison of the spectra obtained from the enzyme in the cell and in vitro will allow us to investigate the interaction of the enzyme with its intracellular environment.

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R 101 *IN VIVO* NMR SPECTROSCOPY STUDIES OF CEREBRAL METABOLISM: RATES OF GLUCOSE CARBON ENTRY INTO GLUTAMATE, GLUTAMINE AND GABA, S.M. Fitzpatrick, K.L. Behar[1] and R.G. Shulman, Departments of Molecular Biophysics and Biochemistry and [1]Neurology, Yale University School of Medicine, New Haven, CT 06510. Glutamate (GLU), glutamine (GLN), and γ-aminobutyric acid (GABA), amino acids present in millimolar concentrations in brain, are involved in the reactions of cerebral energy metabolism, in neurotransmission, and in the exchange of carbon between neurons and glia. We are using ^{1}H, ^{31}P and proton-observed, carbon-decoupled (POCD) NMR spectroscopy to investigate the metabolism of these amino acids in the rat brain *in vivo* and in protein-free extracts prepared from liquid-N_2 frozen rat brain. Male Sprague-Dawley rats (180-200g; fasted 24-hr), paralyzed and artifically ventilated (70% N_2O/30% O_2), received an i.v. infusion of [1-^{13}C]glucose sufficient to achieve a rapid (≈1-min) and constant ^{13}C-fractional enrichment of the blood of ≈60%. The entry of [1-^{13}C]glucose carbon into glycolysis and the TCA cycle labels the C-4 of α-KG and then the C-4 of GLU and GLN and the C-2 of GABA. Subsequent turns of the TCA cycle will yield GLU, GLN and GABA labeled in the C-3 position as well. The single exponential rate constant obtained *in vivo* for the incorporation of ^{13}C-label into the unresolved C-4 resonances of GLU and GLN was 0.130 ± 0.012 min^{-1} (n=6). The appearance of the ^{13}C-label in the unresolved C-3 resonances of GLU and GLN lagged that of the C-4 position and had an exponential rate constant of 0.026 ± 0.004 (n=5). In extracts, where the GLU, GLN and GABA resonances are more resolved, the labeling of the C-4 of GLN lagged the labeling of the C-4 of GLU and the C-2 of GABA. These studies provide information about the rate of entry of glucose carbon into glycolysis and the TCA cycle, the turnover rate of these amino acids, and their possible precursor-product relationships.

R 102 METABOLISM AND SURVIVAL OF *ESCHERICHIA COLI* DURING PHOSPHATE STARVATION, Steven F. Karel and Luke V. Schneider, Department of Chemical Engineering, Princeton University, Princeton, NJ 08544. *E. coli* cells were cultivated in batch or fed-batch reactors limited by phosphate supply. A temporal sequence of at least 3 sets of proteins were synthesized in progression during phosphate starvation, as determined by gel electrophoresis. The last set of these proteins was synthesized only after the synthesis of alkaline phosphatase, an enzyme characteristic of the short-time response to phosphate starvation, had abated. Both actual and potential rates of protein synthesis decline well in advance of losses in cell viability. The decline in an essential component for protein synthesis and cell growth can be inferred indirectly by measuring the duration of the shift-up lag that occurs on readdition of phosphate to the medium. A simple mathematical model based on the degradation and autocatalytic synthesis of this essential component predicts a linear relationship between the time of starvation and the shift-up lag, and a long lag-time before the onset of cell death, as experimentally observed.

R 103 REGULATION OF NITROGENASE ACTIVITY BY REVERSIBLE ADP-RIBOSYLATION. Paul W. Ludden and Gary Roberts, Departments of Biochemistry and Bacteriology and the Center for the Study of Nitrogen Fixation, University of Wisconsin, Madison, WI 53706.
Nitrogenase activity in the photosynthetic bacterium Rhodospirillum rubrum and several other bacteria is regulated by reversible ADP-ribosylation of the dinitrogenase reductase component of the nitrogenase enzyme system. Dinitrogenase reductase ADP-ribosyl transferase (DRAT) inactivates dinitrogenase reductase by the NAD-dependent modification of arginine 101 of the protein. DRAT activity is greatly stimulated by the presence of ADP and it is highly specific for dinitrogenase reductase. Removal of ADP-ribose is catalyzed by dinitrogenase reductase activating glycohydrolase (DRAG) in an Mn^{2+}- and MgATP-dependent reaction. Both DRAG and DRAT have been purified and characterized and the draT and draG genes encoding for DRAT and DRAG, respectively and have been identified and sequenced. The draTG region is near the nif (nitrogen fixation) structural genes on the R. rubrum genome even though nif and dra are not co-regulated in the cell. Nitrogenase activity can be monitored in vivo by the acetylene reduction assay and this allows monitoring of the modification status of dinitrogenase reductase without disrupting cells. Treatment of the cells with non-invasive stimulus (darkness) or fixed nitrogen sources such as ammonia or glutamine result in ADP-ribosylation of dinitrogenase reductase. It is not yet known how the signal is transduced in this organism, but it is known that DRAG and probably DRAT activities are regulated in vivo. The effect of ADP-ribosylation on dinitrogenase reductase appears to be the inhibition of its binding to its electron acceptor, dinitrogenase. A mutant dinitrogenase reductase with histidine in place of arg-101 has been isolated and characterized.

R 104 PURIFICATION AND TEMPORAL EXPRESSION OF A REPRESSOR OF DNA REPLICATION IN BACILLUS SUBTILIS. Loretta A. Mele, Bethanie E. Wilkinson, and William Firshein, Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06457.
A 64 kilodalton (kDa) protein binds specifically to double stranded DNA probes from the origin of replication in Bacillus subtilis (1). This protein can be extracted from both cytosol and membrane derived subcellular fractions. Antibodies raised against this protein enhance initiation activity in an in vitro replication system consisting of a DNA/membrane complex and soluble precursors (2). The observed enhancement suggests that in the absence of antibodies, the 64 kDa protein has a repressive effect on initiation. Attempts are underway to purify this protein in order to sequence it and eventually clone its gene. To do this, cytosol proteins are electrophoresed on SDS-polyacrylamide gels. The region containing the 64 kDa protein is excised and the proteins are electroeluted from it. FPLC on an anion exchange column is then used to further purify the protein. Isoelectric focusing has revealed that this protein has a pI of approximately 5.7. Gel permeation chromatography has indicated that it is part of a large complex (greater than 300 kDa) when isolated native from the cytosol.
The possible role of the 64 kDa protein has been studied throughout the life cycle of Bacillus subtilis by evaluating its relative concentration in vegetative cells, germinating spores, and disrupted spores. The data indicate that the 64 kDa protein dramatically decreases upon germination, while it is present in significant amounts in vegetative cells and disrupted spores. The distribution of the protein between cytosol and membrane fractions was examined. These results were obtained by quantitating the binding of the anti-64 kDa antibody (2) to immunoblots of the proteins from these three stage of the Bacillus life cycle. Such results support the role of this protein as a repressor of initiation.

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R 105 THE ROLE OF PYRROLINE 5-CARBOXYLATE IN THE TRANSFER OF REDOX POTENTIAL AND INTERCELLULAR COMMUNICATION, James M. Phang, Sylvia J. Downing, A. James Mixson, Marsha Merrill and G. Alexander Fleming, Endocrinology Section, Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892
Pyrroline 5-carboxylate (P5C), the obligate intermediate in the interconversions of proline, ornithine and glutamate, provides a novel mechanism for intercellular communication by the transfer of reducing-oxidizing (redox) potential. This molecule not only is an intracellular intermediate but also is a nutrition-dependent constituent of human plasma. Levels undergo diurnal fluctuations with peaks 10 to 15-fold baseline and these fluctuations are attenuated by fasting. The cellular uptake of P5C is mediated by its own saturable system which transfers oxidizing potential pari passu its entry into cells. The mechanism for this process may involve the physical association of the membrane carrier protein with P5C reductase, the enzyme which generates $NADP^+$ as it converts P5C to proline. The transplasma membrane transfer of oxidizing potential by P5C can be assessed by its inhibition of nitrobluetetrazolium reduction. More importantly, the oxidizing potential transferred by P5C markedly stimulates the activity of the pentose phosphate shunt and the production of PP-Rib-P. Although certain growth factors, e.g. platelet-derived growth factor (PDGF), can stimulate the production of PP-Rib-P during its mitogenic activation of quiescent cells, it acts synergistically with P5C in stimulating PP-Rib-P. Based on these findings we propose that P5C provides a mechanism for the integration of nutrient signals with those mediated by polypeptide communicators in regulating metabolism during cellular activation and differentiation.

R 106 THE POSSIBLE ROLE OF PYROPHOSPHATE LEVELS IN THE CONTROL OF CELLULAR METABOLISM WITH SPECIAL REFERENCE TO LEAF SENESCENCE.

S. Strother, Division of Biological and Health Sciences, Deakin University, Geelong, Victoria, 3217, Australia.

Leaves of terrestrial angiosperms are known to have their biosynthetic capacity correlated with alkaline pyrophosphatase activity. For example, during leaf senescence alkaline pyrophosphatase activity decreases irrespective of whether senescence takes place with the leaves attached to the plant or using detached leaves floating in a suitable medium. Acid pyrophosphatase levels are not consistently correlated with leaf senescence. These findings have led to the suggestion that during leaf senescence, decreased alkaline pyrophosphatase levels lead to increased cellular levels of inorganic pyrophosphate which results in a dampening effect on biosynthetic metabolism. This hypothesis will be examined in relation to recently published pyrophosphate levels and intracellular locations of alkaline pyrophosphatase activity and inorganic pyrophosphate. It will be shown that pyrophosphate levels in chloroplasts could be influenced by alkaline pyrophosphatase levels and hence control key biosynthetic reactions such as the involvement of ADPG pyrophosphorylase in starch synthesis. The hypothesis that cytosolic pyrophosphate levels are kept vanishingly small by pyrophosphatase activity does not appear to be tenable.

R 107 THE INFLUENCE OF PHAGE INFECTION ON STAPHYLOCOCCI METABOLISM,

Albert I.Vinnikov, Department of Microbiology Dniepropetrovsk State University, 320625, USSR

Transduction is the basic process which leads to spreading of genetic determinants of staphylococci resistance to antibiotics. One stage of this process is the transport of phage DNA trough staphylococci cytoplasmic membrane.

Injection DNA of transducing phage serology group B - 52A into staphylococci cells that contained plasmids of resistance to some antibiotic led to dissipation of transmembrane difference of electric potentials. At the same time the reversed stimulation of cell respiration processes was observed.

Moreover, the induction of ionic fluxes was observed during the transport of phage DNA. It led to K^+ going out and H^+ entrance to staphylococci cells. The effects mentioned above are commensurable in time with translocation of phage nucleic acid through cytoplasmic membrane of staphylococci strains investigated.

Channeling and Multi-Functional Proteins

R 200 YEAST OROTIDYLATE DECARBOXYLASE: A MODEL FOR THE BIFUNCTIONAL MAMMALIAN UMP SYNTHASE,

Juliette B. Barclay and Mary Ellen Jones, Department of Biochemistry, University of North Carolina-CH, Chapel Hill, NC 27599-7260.

Orotidine 5'-monophosphate decarboxylase (ODCase) catalyzes the decarboxylation of OMP to UMP in the last of six steps required for *de novo* pyrimidine biosynthesis of UMP. The amino acid sequence of yeast ODCase has 51% homology with the ODCase domain of the bifunctional UMP synthases isolated from mouse and human. Elucidation of structural and mechanistic features of the yeast ODCase could, therefore, provide important information applicable to the mammalian ODCase domain. We have used yeast cells transformed with a plasmid containing the inducible ODCase gene (engineered by Dr. Roger Kornberg and his colleagues at Stanford) as a source to obtain 100 mg quantities of ODCase. We have modified published procedures to obtain a protease-resistant ODCase with specific activity of 60-65 units/mg. Precrystallization studies using the incomplete factorial method have yielded crystals of ODCase in various liganded forms. X-ray diffraction quality crystals of ODCase complexed with the tight-binding inhibitor, 1-(5-phosphoribofuranosyl)barbituric acid, (BMP), have been grown. Single crystal precession photography will be used to determine the crystal space group. Studies on the mechanism of ODCase using nmr and protein modification are in progress. Preliminary results will be presented.

R 201 CLUSTERING OF SEQUENTIAL ENZYMES IN THE GLYCOLYTIC AND THE CITRIC ACID CYCLE PATHWAYS. Sonia Beeckmans* and Louis Kanarek, Laboratorium voor Chemie der Proteïnen, Vrije Universiteit Brussel, Paardenstraat 65, B-1640 Sint-Genesius-Rode, Belgium.

Accumulating evidence tends to indicate that metabolic pathways are organized *in vivo* as multienzyme clusters: consecutive enzymes are thought to be physically in contact with each other, so that metabolites can be transferred directly among enzymes without first equilibrating with the bulk phase. Such a strict organization of metabolic processes, both in space and time, allows a cell to interrupt, slow down or speed up very accurately a biochemical pathway according to its needs at any time.
In order to assess interactions between consecutive enzymes *in vitro* it is usually essential to modify the physical properties of water around the enzymes, *f.i.* by fixing the latter onto a solid support. Such immobilized enzyme preparations can be embedded in agarose gels and used in affinity electrophoresis. This technique allowed us to visualize specific interactions between the citric acid cycle enzymes fumarase, malate dehydrogenase and citrate synthase, and between the glycolytic enzymes aldolase, glyceraldehydephosphate dehydrogenase and triosephosphate isomerase. Moreover the effect of several metabolites on the respective associations could easily be investigated.
**Research Associate of the National Fund for Scientific Research, Belgium.*

R 202 REGULATION AND CONFORMATIONAL CHANGE IN THE PYRIMIDINE - BIOSYNTHETIC MULTIFUNCTIONAL POLYPEPTIDE CAD. Elizabeth A. Carrey, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

The mammalian carbamyl phosphate synthetase II (CPSII) activity, which is part of the 240 kDa multifunctional polypeptide CAD, is regulated *in vitro* and *in vivo* by the allosteric activator P-ribose-PP and the feedback inhibitor UTP. In addition, CAD is a substrate for phosphorylation *in vitro* by cAMP-dependent protein kinase. Phosphorylation at a site which maps to the C-terminus of the CPS domain, but which is not found in the CPSI sequence, activates CPSII by abolishing feedback inhibition by UTP. At physiological concentrations (0.1-0.2 mM), UTP protects the vulnerable interdomain regions in CAD, but not in phospho-CAD, against digestion by trypsin. Similarly, in the presence of 0.05-0.50 mM UTP the phosphorylation of CAD by cAMP-dependent protein kinase is specifically inhibited, while dephosphorylation of phospho-CAD by protein phosphatase 1 is not inhibited. I propose that the UTP-induced protection of the interdomain regions of the unmodified protein is caused by a generalised conformational change or aggregation of CAD. This interlocking of two regulatory mechanisms may be relevant *in vivo* when a fall in UTP concentrations, associated with a demand for pyrimidine biosynthesis, encourages the phosphorylation of CAD.

R 203 PHOSPHORYLATION OF THE YEAST ASPARTATE-TRANSCARBAMYLASE CARBAMYL-PHOSPHATE SYNTHETASE COMPLEX BY cAMP DEPENDENT PROTEIN KINASE, M. Denis-Duphil, Laboratoire d'Enzymologie, C.N.R.S, 91198 Gif-sur-Yvette, France and E.A. Carrey and G. Hardie, Biochemistry Department, Dundee University, Dundee DD14HN, U.K.

The multifunctional complex ATCase-CPSase coded for by the URA2 locus catalyses the first two steps of the pyrimidine biosynthesis in Saccharomyces cerevisiae. It is known to be regulated by pyrimidines : 1) at the protein level where both ATCase and CPSase activities are modulated by UTP feedback inhibition, 2) at the mRNA level where, UTP regulates mRNA transcription.
Possible regulation at the post-translational level by phosphorylation was investigated : analysis by SDS-PAGE of the partially purified complex shows a major polypeptide chain (240 kdaltons) which is the URA2 gene product. Phosphorylation of this species was found to occur in vivo. We have also found that, in vitro, this polypeptide is phosphorylated in the presence of c-AMP protein kinase catalytic subunit. However, in the standard conditions of assay, no significant modification of ATCase and CPSase activities was observed.
Further investigation of the possible modulation by phosphorylation of the 2 activities of the complex, as well as their respective sensitivity to UTP inhibition is presented; we also show the phosphorylated peptide sequence.

R 204 **EFFECTS ON THE ORGANIZATION, STRUCTURE AND FUNCTION OF THE TCA CYCLE BY SPECIFIC MUTATIONS IN CITRATE SYNTHASE.** Claudia T. Evans, Gyula Kispal, Curtis Small and Paul A. Srere. VAMC and UT Southwestern Med. Ctr, Dallas, TX 75216. The DNAs encoding the authentic and mutant forms of mitochondrial yeast citrate synthase (YCS1) were cloned into an expression system to determine the ability of these YCS1 proteins to complement a mutation in the YCS1 gene of S. cerevisiae. Oligonucleotide-directed mutagenesis was performed with a DNA template encoding YCS1 using primers (21-mer) that contained single or double base pair mismatches for the region of the known YCS1 DNA sequence encoding His313Gly and Asp414Gly, which are analogous to the catalytic amino acids determined for the pig heart enzyme. Mutant frequencies of greater than 50% routinely were obtained and were confirmed by [^{35}S] dideoxy sequencing techniques. YCS1$^-$ cells carrying the plasmid cloned non-mutant YCS1 gene grew on minimal acetate media and contained a level of YCS1 equivalent to that normally expressed by wild type yeast cells (1.0 - 2.0 U/mg protein). Mutant cells containing YCS1/Asp414Gly did not grow on minimal acetate media, while YCS1$^-$ cells were complemented by YCS1/His313Gly and grew on minimal acetate media. YCS1$^-$ cells carrying either YCS1/His313Gly or YCS1/Asp414Gly contained less than 0.005 U/mg protein and immunodetectable YCS1 protein in crude lysates. These results suggest that in YCS1$^-$ cells the mutant YCS1/His313Gly protein may form an intact TCA cycle complex. (Funded by the Veterans Administration)

R 205 MULTIPLE PARALLEL PATHWAYS IN PLANT AROMATIC METABOLISM. Geza Hrazdina[1] and Roy A. Jensen[2]. [1]Institute of Food Science, Cornell University, Geneva, NY 14456 and [2]Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611

The plant aromatic pathway consists of three segments that produce the aromatic amino acids phenylalanine, tyrosine and tryptophane; the phenylpropanoid segment that produces the cinnamic acid derivatives, which are precursors of flavonoids and the plant structural component lignin; and the flavonoid segment that produces the diverse flavonoid compounds. Key branchpoint enzymes of aromatic amino acid biosynthesis, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase and chorismate mutase have been shown to exist as separate compartmentalized isozymes in the chloroplasts and cytoplasm of various plants. While the chloroplastic pathway is under tight allosteric control, no such mechanism has been reported for the cytoplasmic segment. Key enzymes of the phenylpropanoid (phenylalanine ammonia-lyase) and flavonoid (chalcone synthase) segments of the plant aromatic pathway were reported to exist as multiple isozymes. Our hypothesis that the production of aromatic compounds by plants is the result of multiple parallel pathways which function as membrane associated enzyme complexes is based on data from our laboratories, and upon the large and growing list of higher plant species that possess isozymes in the aromatic amino acid, -phenylpropanoid and -flavonoid segments of the plant aromatic pathway.

R 206 **STEROIDOGENIC CYTOCHROMES P450 AS MODELS FOR THE HYDROPHOBIC CORES OF METABOLIC PATHWAYS: INTERMEDIATE CHANNELING AND MICRO-COMPARTMENTATION.** **Nikolaus Kühn-Velten, Institut für Physiologische Chemie II, Universität, Moorenstr. 5, D-4000 Düsseldorf, F.R.Germany.**

Two cytochromes P450 are the key enzymes and regulatory targets of gonadal androgen biosynthesis: The P450(cscc) catalyses the cholesterol to pregnenolone conversion in the mitochondrial membranes, and the P450(C17) catalyses the progesterone to androstenedione conversion in the endoplasmic reticulum membranes. In both reactions, a sequence of catalytic cycles firstly results in formation of hydroxylated intermediates prior to the final C-C-bond cleavage. The three cycles of the P450(cscc) system show a high degree of coupling with the consequence that the intermediates remain completely in an enzyme-bound state. The two cycles of the P450(C17) system, however, show a lower degree of coupling with the consequence that the intermediate (17α-hydroxyprogesterone (OHP)) dissociates from the enzyme to a significant degree. We can demonstrate in double-label/double-substrate experiments that OHP accumulates to a 3-fold higher degree in microsomal membranes when being in an "intermediate state" (endogenous formation) than in a "substrate state" (exogenous addition). These and further experiments using Van't Hoff plots and quantitation of steroid partition into the microsomal membranes clearly show that the P450(C17) accepts its substrate and the intermediate from the membrane phase, and that only the membrane-associated steroid pool is accessible to this enzyme. Furthermore, this system shows auto-regulatory behaviour in that it is able to respond to increasingly high progesterone concentrations with an over-proportional increase of free OHP "leakage" (which is reversible in a stationary but irreversible in a continuous-flow system) at the cost of product (androgen) formation efficiency. The cytochrome P450(C17) system therefore may represent a useful model of substrate/intermediate microcompartmentation and channeling by the hydrophobic cores of metabolic pathways.

R 207 A MULTIENZYME COMPLEX WHICH SYNTHESIZES DNA PRECURSORS CAN BE PURIFIED FROM A *BACILLUS SUBTILIS* DNA/MEMBRANE EXTRACT. **John J. Laffan, Deborah A. Hadley, Ira L. Skolnik, Michelle Bouyea, and William Firshein.**, Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06457.

A multienzyme complex which demonstrates nucleoside kinase activity has been shown to co-purify with initiation of DNA replication activity. This precursor synthesizing complex is contained within a *Bacillus subtilis* DNA/membrane extract originally shown to contain all the enzymes and template necessary for initiation of DNA replication (1). The complex incorporates deoxynucleoside triphosphates into DNA, however, deoxynucleosides are incorporated even faster, suggesting catalytic facilitation. Both ribonucleosides and deoxynucleosides have been shown via thin layer chromatography separation to be converted by the complex into their mono-, di-, and tri- phosphate derivatives. Ribonucleotides are incorporated into DNA via the action of ribonucleoside diphosphate reductase. Some regulatory mechanisms of the kinase system may also be retained by the complex.

1. Laffan, J., and W. Firshein. (1987). DNA replication by a DNA-membrane complex extracted from *Bacillus subtilis*: Site of initiation *in vitro* and initiation potential of subcomplexes. J. Bacteriol., 169: 2819-2827.

R 208 GLUTAMINE SYNTHETASE: EVOLUTION OF METABOLIC COMPARTMENTATION WITH MINIMAL PROTEIN SEQUENCE DIVERGENCE. D. D. Smith and J. W. Campbell, Dept. Biochem. & Cell Biol., Rice Univ., Houston TX 77251.
Inter- and intraspecies comparisons of compartmental isozyme protein sequences usually show substantial evolutionary divergence. Nuclear-encoded mitochondrial proteins appear to have arisen via gene duplication and an N-terminal fusion creating a transient targeting presequence whereas the cytosolic forms lack the latter. For aspartate aminotransferase, the mitochondrial and cytosolic proteins are now less than 50% identical. This is also true even for proteins where targeting of the mitochondrial isozyme is via an internal signal, e.g., adenylate kinase. Divergence of mammals and birds resulted in not only a difference in transcriptional regulation of hepatic glutamine synthetase (GS), but also in its compartmentation: it is cytosolic in the former and mitochondrial in the latter. Despite these differences, the primary sequence of GS is highly conserved. The sequence of chicken liver mitochondrial GS derived from a 1.6 kb cDNA clone and direct mRNA sequencing is 88% identical to that of cytosolic mammalian GS. Hydropathy plots for the two are very similar and both isozymes have N-termini which contain both positively charged and hydroxylated amino acids and are capable of forming amphipathic helices. Chicken GS contains two additional positive charges in its N-terminus which may possibly represent the mitochondrial targeting signal. (Supported by NSF DMB87-18402)

R 209 SUBSTRATE CHANNELING OF NADH AND BINDING OF DEHYDROGENASES TO COMPLEX I, H. Olin Spivey and Tetsuhito Fukushima, Department of Biochemistry, Oklahoma State University, Stillwater, OK 74078-0454 Our data show that NADH can substrate channel from donor dehydrogenases (E1) to beef heart complex I (NADH:ubiquinone oxidoreductase) (E2) by means of transient E1·NADH·E2 complexes (Srivastava, D.K. and Bernhard, S.A. (1985) *Biochemistry* 24, 623). These donor enzymes include mitochondrial and cytoplasmic malate dehydrogenases (MDH) and β-hydroxyacylCoA dehydrogenase (HAD). The K_m and V_m of the enzyme bound NADH is about two- and three-fold lower, respectively, than for the free NADH, which is similar to other donor-acceptor dehydrogenases (*op. cit.*). Since these donor enzymes have different chiral specificities for NADH, complex I appears more versatile in this regard than the previous donor-acceptor dehydrogenases tested (*op. cit.*). Excess apo-dehydrogenase causes little inhibition of the reaction (reduction of ubiquinone-1). Other mitochondrial enzymes are being tested for their ability as donor enzymes of NADH. Since mitochondrial matrix NADH should be predominantly enzyme bound, it is likely that this substrate channeling occurs *in vivo* , providing a dynamic microcompartmentation of NADH and several advantages therefrom. The binding of mitochondrial MDH and HAD to complex I is abolished by low concentrations of NAD and NADH, in contrast to that of α-ketoglutarate dehydrogenase complex. Mitochondrial MDH and HAD substantially compete for the same receptor site(s) on complex I. Photoaffinity labeling experiments with the ^{125}I-sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate (SASD) adduct of MDH indicate that the receptor site on complex I is either the 75 and/or 30 kDa transmembrane subunits. Supported by grants from NSF (RII-8610675) and American Heart Association, OK Affiliate (OK-88-G-12) and from the Oklahoma Agricultural Experiment Station.