COVALENT AND NON-COVALENT MODULATION OF PROTEIN FUNCTION

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Poster Sessions

Genetic Defects and Protein Function

001 ENZYME REPLACEMENT THERAPY, Ernest Beutler, George Dale and Wanda Kuhl, Department of Hematology, City of Hope Medical Center, 1500 E. Duarte Road, Duarte, California 91010.

Treatment of disorders due to hereditary deficiencies of enzymes has become an attractive possibility since the enzymatic basis of a large number of diseases has become recognized. Once the missing enzyme has been purified it is necessary to deliver it to its site of action and to have it perform its function for a sufficiently long time to be effective. The infusion of missing clotting factors, enzymes of blood coagulation, often suffices to provide dramatic albeit temporary, amelioration of clinical symptoms. In the case of enzymes which perform their vital functions intracellularly, however, the delivery of enzyme to site of action may be much more difficult. A number of model systems have been investigated to guide the clinical investigator in attempting to resolve this difficult problem. The entry of soluble enzyme into cells appears to be governed by a carbohydrate "code" on its surface. Encapsulation of enzyme in resealed erythrocytes and in liposomes has also been investigated in vivo and in vitro.

Among the most suitable candidates for enzyme replacement therapy are some of the lysosomal storage diseases. Those diseases which involve the central nervous system appear to defy presently available technology for therapy; on the other hand, diseases which affect primarily the reticuloendothelial system such as the adult type of Gaucher's disease, represent currently attractive targets for experimental enzyme replacement therapy. Experimental therapy of storage diseases such as Fabry's disease, Niemann-Pick diseases, Gaucher's disease, and Tay-Sachs disease has been attempted. The biochemical alterations which have been observed are of questionable significance, and the clinical benefit has been very modest, at best. We have now treated 6 patients with the adult type of Gaucher's disease with 49 courses of therapy, administering purified human placental glucocerebrosidase directly intravenously, encapsulated in red cells, and encapsulated in red cells coated with gamma globulin. The results of model systems suggest that the latter form of therapy may be most effective. However, any clinical benefit which has been achieved thus far is slight.

002 GENETIC DEFECTS OF THE HUMAN RED BLOOD CELL AND HEMOLYTIC ANEMIA. William N. Valentine, Department of Medicine and Donald E. Paglia, Department of Pathology, University of California, Los Angeles, CA 90024. (1, 2)

Lacking a nucleus, intracellular organelles, or any capacity for protein synthesis, the human red cell derives its small energy requirements from the production of lactate from glucose and from salvage pathways for adenine ribonucleotides. Severe heritable deficiencies of eight enzymes of anaerobic glycolysis cause hemolytic anemia presumably secondary to associated deficiencies in energy generation. Lactate dehydrogenase deficiency is recognized, but is not associated with anemia. Deficiencies of both enzymes of glutathione synthesis, of glutathione reductase and possibly glutathione peroxidase, and of glucose-6-phosphate dehydrogenase (G6PD) also produce hemolytic syndromes, primarily due to inability to protect hemoglobin from oxidative denaturation secondary to dysfunction of the hexosemonophosphate shunt. Severe deficiency of pyruvate kinase (PK) activity is second only to that of G6PD as a cause of hemolytic anemia in man. The deficient red cell isozyme is closely related to the "L" isozyme of liver, differs immunologically from the M_1 isozyme of muscle and in other ways from the M_2 isozyme of leukocytes and certain other tissues. Fructose-1, 6,-diphosphate is an allosteric modifier of red cell PK. Phosphoglycerate kinase and G6PD deficiency are X-chromosome linked; all other deficiencies are autosomally transmitted, hemolysis most often occurring only when two defective genes are inherited. Genetic polymorphism dictates that most affected subjects are doubly heterozygous for separate mutant genes except where consanguinity exists. An inherited hemolytic syndrome associated with a recessive autosomally transmitted deficiency of a unique pyrimidine-specific, 5^1-nucleotidase is defined and is associated with enormous, intracellular accumulations of normally nearly undetectable pyrimidine ribonucleotides. An acquired, lead induced deficiency of the same nucleotidase results in hemolysis in subjects with severe lead poisoning. Increased red cell adenosine deaminase activity (45-70 fold), inherited as a Mendelian dominant also causes a hemolytic syndrome. The associated diminished ATP is believed to result from inability of adenosine kinase to salvage adenosine for nucleotide replenishment in the face of the competing, massive deamination.

1) "Hereditary Enzymatic Deficiencies of Erythrocytes". Seminars in Hematology 8, No. 4, Oct. 1971, pp. 307-440. 2) Paglia, D.E., Valentine, W.N., Tartaglia, A.P., Gilsanz, F. and Sparkes, R.S.: "Control of red blood cell adenine nucleotide metabolism. Studies of adenosine deaminase". In: Progress in Clinical and Biological Research Vol 21, (The Red Cell): Alan R. Liss, Inc., N.Y., 1978, pps. 319-335..

Modulation of Enzymes of Intermediary Metabolism

003 MOLECULAR PROPERTIES OF PHOSPHOFRUCTOKINASE (PFK) RELEVANT TO MODULATION OF ITS FUNCTION. Tag E. Mansour, Glenda Choate, Litai Weng. Department of Pharmacology, Stanford University Medical School, Stanford, CA 94305

PFK is a rate limiting allosteric enzyme within the glycolytic pathway. ATP as well as other triphosphate nucleosides inhibits the enzyme at pH 6.9 while AMP, cAMP and ADP relieve ATP inhibition, "deinhibit". Both inhibition and deinhibition were explained on the basis of multimolecular nature of the kinetics at pH 6.9. In order to study the allosteric site we have labelled heart PFK with the affinity label 5'-p-fluorosulfonylbenzoyl adenosine (FSBA). The modified enzyme lost its allosteric regulatory properties as well as its ability to bind AMP, ADP and cAMP. Using ^{14}C-FSBA for enzyme modification followed by reduction, carboxymethylation and tryptic digestion, we were able to isolate a peptide with the highest radioactivity. The relationship between the amino acid labelled and the function of the allosteric site will be discussed.

Vanadate has been found to be a potent inhibitor of PFK. The species responsible for inhibition has been shown to be decavanadate. The inhibition by vanadate at pH 6.9 ([Van]50% inhibition$\sim$ 0.5 µM) has many of the properties of other inhibitors of PFK; inhibition is reversed by the allosteric activators cAMP and glucose 1,6-P_2 and the inhibition is synergistic with ATP. Unlike other inhibitors, vanadate inhibits PFK at pH 8.2 ([Van]50% inhibition$\sim$ 6 µM). Typically PFK has been characterized as displaying allosteric kinetics at pH 6.9 but Michaelis-Menten kinetics at pH 8.2. However, in the presence of vanadate, PFK has been shown to have allosteric properties at pH 8.2; namely, synergistic inhibition between vanadate and ATP and sigmoid kinetics with respect to fructose-6-P. The inhibitory effect of vanadate at pH 8.2 is also reversed by the allosteric activators, cAMP and glucose-1,6-P_2. PFK modified by FSBA is sensitive to vanadate inhibition but requires a higher concentration of vanadate than unmodified PFK. Vanadate has also been found to protect PFK from cold-inactivation at pH 6.5 in contrast to the idea that the sensitivity of PFK to inhibitors is related to an increase in its susceptibility to cold and low pH inactivation. Polyphosphate inhibits PFK in a way similar to that of polyvanadate. The relationship between these potent inhibitors and the kinetic properties of the enzyme will be discussed.

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004 MULTIMODULATION OF ENZYME ACTIVITY. PHYSIOLOGICAL SIGNIFICANCE AND EVOLUTIONARY ORIGIN. Alberto Sols, Instituto de Enzimología y Patología Molecular del C.S.I.C., Facultad de Medicina, Universidad Autónoma, Madrid 34, Spain.

Knowledge of specific mechanisms of regulation of enzyme activity has increased within less than 25 years from essentially zero to a rich variety, that in some cases reaches a bewildering multiplicity. The basic type of mechanism is heterotropic allosteric regulation, whose discovery opened a 3rd dimension in physiological enzymology. It is frequently accompanied by one or more of other types of regulatory mechanisms: sigmoidal cooperativity, metabolic interconversion, selective activation or inactivation, and control of enzyme synthesis. Moreover, several and occasionally many different allosteric effects have been reported for certain enzymes (up to more than 20!). Such multiplicity of presumptive regulatory effects makes particularly pressing the question of the physiological significance of multimodulation of enzyme activity, trying to sort out facts from artifacts, and biologically relevant mechanisms from in vitro curiosities. Protein chemistry, ligand specificity, metabolic behaviour, comparative biochemistry, molecular pathology and molecular evolution, if critically used, could contribute to the formulation of tentative principles and working hypotheses. Multiple heterotropic effects may range from essentially independent to strongly concerted or conditioned, with physiological activation being frequently based on counteraction of an allosteric hindrance. Possibilities concerning physiological significance of individual mechanisms in multimodulated enzymes include: convergent control, amplification, back up systems, alternative contingencies, significance in other tissues with the same isozyme but different metabolism, evolutionary relic, or no biological significance whatsoever. In general, multimodulated enzymes are highly sophisticated integrators of metabolic signals. The fact that most regulatory enzymes have a single kind of subunit, that some of them have up to at least half a dozen regulatory sites, and that in these cases the subunit size is not greater than that of the average non-regulatory enzymes, suggests that many, perhaps even most of the regulatory sites, originate from mutational development of a new specific site out of a neutral area of the enzyme.

005 ADENINE NUCLEOTIDE POOL MAINTENANCE DURING BACTERIAL GROWTH AND STARVATION, C. J. Knowles, Biological Laboratory, University of Kent, Canterbury, Great Britain.

Catabolism is a net producer of ATP from ADP whereas ATP is converted to ADP and AMP during biosynthesis. The adenine nucleotides are therefore ideally placed to act as overall regulators of metabolism. Thus, in addition to ATP being produced and consumed at many individual enzymatic steps of metabolism, these and other reactions are regulated by AMP, ADP or ATP. The total adenylate pools of bacteria, and the cytosol but not the mitochondrial matrix of eukaryotes are equilibrated by the action of adenylate kinase. Although the activities of many enzymes are regulated by ATP, ADP or AMP or the ratios of ATP:ADP or ATP:AMP, in the intracellular environment changes in concentration of any one of the adenine nucleotides will affect the content of all three of them. Atkinson (1) introduced the Adenylate Energy Charge concept. This is a scale from 0 (all AMP) to 1 (all ATP) of the proportion of the total adenylate pool that contains anhydride-bound phosphate of high free energy of hydrolysis. At intermediate energy charge (E.C.) values the concentrations of each of the nucleotides depend on the equilibrium position of the adenylate kinase reaction. Catabolic (ATP-regenerating) pathways are inhibited by high E.C. values, whereas biosynthetic (ATP-consuming) pathways are stimulated by increases in E.C. This results in a homeostatic mechanism, with the E.C. maintained at about 0.9. Unfortunately attempts to measure E.C. in intact cells are complicated by the high turnover of ATP and the need for rapid sampling techniques, and, in eukaryotes, metabolic compartmentation and the absence of adenylate kinase in the mitochondrial matrix. However, the adenine nucleotide pools of the bacteria, *Escherichia coli* and *Beneckea natriegens*, have been investigated in detail. The E.C. of both is about 0.9 during growth, but large differences in adenine nucleotide content and E.C. occur during substrate-sufficient non-growing conditions and during starvation.

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Photosynthesis and Polysaccharide Metabolism

006 REGULATION OF PHOTOSYNTHETIC CARBON METABOLISM AND PARTITIONING OF PHOTOSYNTHATE, James A. Bassham, Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720.

The Reductive Pentose Phosphate Cycle (RPP Cycle) is ubiquitous to all photoautotrophic green plants. The rate-limiting steps in the RPP cycle were identified by measurement of metabolite concentrations during steady-state free energy changes associated with all the steps in the cycle. The rate limiting, regulated steps are: the carboxylation of ribulose 1,5-bisphosphate (mediated by RuBPCase), the conversion of ribulose-5-phosphate to ribulose 1,5-bisphosphate with ATP (PR kinase) and the conversion of fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate to fructose-6-phosphate and sedoheptulose-7-phosphate, respectively (mediated by FBPase and SBPase). Examination of transient changes in metabolite concentrations in the dark following steady state photosynthesis disclosed that RuBPCase, RBPase, and SBPase become much less active during the first 30 seconds to 1 minute of darkness. As a result of many studies with isolated enzymes, reconstituted chloroplasts and chloroplasts, it is now clear that these enzymes plus PR kinase and to some extent triose phosphate dehydrogenase are inactivated in intact chloroplasts in the dark. The light activation of the above mentioned enzymes in chloroplasts involves specific changes in the metabolic environment which occur when the light is on. These include increased levels of reduced cofactors, especially reduced ferredoxin and NADPH, an increased level of magnesium ion, and an increase in pH. The regulatory mechanisms for PR kinase, SBPase and FBPase, and triose phosphate dehydrogenase apparently involve interaction of the enzymes with small protein molecules reduced in the light. RuBPCase-oxygenase is activated in the presence of Mg^{++} and CO_2, is further activated by 6-phosphogluconate or NADPH, and is inactivated by free RuBP (or an impurity formed spontaneously from RuBP). In the light, reduced carbon is exported from the chloroplast in the form of triose phosphate, but hexose monophosphates may be converted to starch within the chloroplast as a storage product. Since the ratio of these two types of carbon drain from the cycle can vary, a fine tuning of the activity of FBPase and SBPase compared to RuBPCase is required. With isolated chloroplasts, triose phosphate export increases with external inorganic phosphate (P_i) concentration, and this may be an important regulatory mechanism in vivo as well. Regulation of P_i concentration in the cytosol may be an important control mechanism. In developing cells, exported triose phosphate is mainly converted via phosphoenolpyruvic acid (PEPA) to amino acids, fatty acids, and other substances required for cell growth. In more mature cells, the triose phosphate is largely converted to sucrose which is translocated to other parts of the plant. Rate limiting steps for these branching pathways include pyruvate kinase and PEPA carboxylase as well as sucrose phosphate synthetase. The relative rates in isolated cells can be greatly influenced by addition of external 1 mM NH_4^+. This research was supported by the Division of Biomedical and Environmental Research of the U.S.D.O.E.

007 THIOREDOXIN AND ENZYME REGULATION IN PHOTOSYNTHESIS, Bob B. Buchanan, Department of Cell Physiology, University of California, Berkeley, CA 94720

We have recently described a regulatory mechanism whereby light governs the activity of certain pace-maker enzymes of chloroplasts. In this mechanism (named the "ferredoxin/thioredoxin system"), light modulates enzyme activity via protein-mediated oxidation-reduction reactions. In the light, electrons from chlorophyll are transferred to soluble ferredoxin--an iron-sulfur protein of chloroplasts--and then via the enzyme ferredoxin-thioredoxin reductase to thioredoxin--a hydrogen carrier protein discovered by others in studies on microbial DNA synthesis and sulfur metabolite transformations. Reduced thioredoxin, in turn, reduces and thereby activates four enzymes of the reductive pentose phosphate cycle of photosynthetic CO_2 assimilation (fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, NADP-glyceraldehyde 3-phosphate dehydrogenase and phosphoribulokinase) as well as two enzymes not associated with the carbon cycle (phenylalanine ammonia lyase and NADP-malate dehydrogenase). Reduced thioredoxin has also been shown to activate the ATPase associated with solubilized (heated) preparations of chloroplast coupling factor (CF_1) and, based on recent work by Wagner et al. (1), blue-green algal PAPS sulfotransferase. The importance of thioredoxins in photosynthesis is emphasized by our finding that leaves contain multiple forms of thioredoxin. Two different thioredoxins were found in chloroplasts (thioredoxins f and m) and a third thioredoxin was found outside chloroplasts, possibly in the cytoplasm (thioredoxin c). A unique feature of enzymes activated by the ferredoxin/thioredoxin system is that deactivation must occur in the dark. Current evidence indicates that unlike activation, the mechanism for the dark deactivation of thioredoxin-linked enzymes can differ as to the particular enzyme involved. Based on their deactivation properties, thioredoxin-linked enzymes are of three types: (i) Those that require for deactivation a soluble oxidant such as GSSG or dehydroascorbate (fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, phosphoribulokinase, and phenylalanine ammonia lyase); (ii) Those, of which NADP-malate dehydrogenase is the sole representative, that appear to be deactivated by an unidentified membrane-bound oxidant in the absence of soluble oxidants; and (iii) Those, such as NADP-glyceraldehyde 3-phosphate dehydrogenase that have no known mechanism of deactivation. It is noteworthy that the enzymes regulated by the ferredoxin/thioredoxin system show hysteretic properties--i.e., their rate of activation or deactivation is slow relative to their rate of catalysis.

In sum, thioredoxin seems to act in chloroplasts as a regulatory messenger between light and the enzymes of different biosynthetic processes that utilize the products formed by light--i.e., ATP and NADPH. We currently visualize that the ferredoxin/thioredoxin system operates in conjunction with other light-actuated regulatory mechanisms of chloroplasts.

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008 REGULATION OF ELECTRON TRANSPORT AND PHOSPHORYLATION IN PHOTOSYNTHESIS. Ulrich Heber, Institute of Botany, University of Düsseldorf, Düsseldorf, Germany.

On illumination, broken spinach chloroplasts phosphorylated added ADP until the phosphorylation potential $(ATP)/(ADP)(P_i)$ was about 80 000 (M^{-1}). Electron transport showed the phenomenon of photosynthetic control and was slowed down when ADP became unavailable. Electron transport was also controlled in intact chloroplasts, but even in the absence of ATP consumption the maximum phosphorylation potential was not much higher than 300 (M^{-1}) and significant ADP was always available. Adenylates were close to adenylate kinase equilibrium and the maximum energy charge was 0.85. Control of electron transport was released and the transthylakoid proton gradient was decreased after addition of amines, but the phosphorylation potential remained essentially unchanged. Also, the quantum yield of CO_2-dependent oxygen evolution was not decreased by amine concentrations that completely released control of electron flow. It appears that one of the factors controlling electron flow in intact chloroplasts is the intrathylakoid pH and not the phosphorylation potential.

Another factor is the redox state of the electron transport chain. During electron transport to NADP, insufficient ATP is synthesized by most chloroplast preparations to satisfy the ATP requirement of CO_2 reduction. The quantum yield of CO_2-dependent oxygen evolution differed in different chloroplast preparations and was generally lower than the quantum yield of phosphoglycerate-dependent oxygen evolution, which was often 0.125. Nonetheless,high light intensities produced high rates of CO_2 reduction even in chloroplasts which had a low quantum yield of photosynthesis. At the expense of light, electron transport is regulated so as to provide the additional ATP needed to satisfy the stoichiometric requirement for ATP and NADPH of the dark reactions of photosynthesis. Oxygen is an important factor in this regulation. Under anaerobic conditions, red light effectively inhibited far-red supported cyclic electron flow and chloroplast ATP decreased to the dark level. Admission of oxygen relieved the inhibition of electron flow and caused a rapid rise in chloroplast ATP levels. CO_2 reduction was also inhibited when oxygen was absent, and inhibition was relieved by admission of oxygen. Low oxygen concentrations were effective and a transient rise in oxygen was sufficient. Even in leaves, photosynthesis was inhibited under nitrogen and a low oxygen concentration greatly increased rates of CO_2 reduction. Oxygen appears to have a dual role. First, electron flow to oxygen is coupled and ATP is synthesized during oxygen reduction. Second, when NADP is largely reduced, the oxidation of electron carriers by oxygen minimizes inhibition of cyclic electron flow by over-reduction and permits cyclic photophosphorylation to contribute to ATP synthesis.

009 COMPARATIVE REGULATION OF α1,4-GLUCAN SYNTHESIS IN PHOTOSYNTHETIC AND NON-PHOTOSYNTHETIC SYSTEMS, J. Preiss, W.K. Kappel and E. Greenberg, Dept. Biochem. Biophys., University of California, Davis, CA 95616

ADPglucose (ADPG) pyrophosphorylase catalyzes the biosynthesis of ADPglucose from ATP and α-glucose-1-P and is the first unique enzyme in the bacterial glycogen and plant starch biosynthetic pathways. It is activated by glycolytic intermediates in most if not all bacterial and plant extracts and may be inhibited by 5'-AMP, ADP and/or P_i (see Preiss, J., in Adv. in Enzymol., Vol. 46, pp. 317-381 (1978)). The major activators are pyruvate (usually observed for the photosynthetic bacterial enzyme), 3-P-glycerate (the major activator for the higher plant, algal and blue green bacterial ADPG pyrophosphorylases), fructose-6-P and fructose-P_2. The last two activators are usually observed for ADPG pyrophosphorylases from bacteria that catabolize sugars via the Entner Doudoroff and glycolytic pathways, respectively. Thus, the activator specificity of the bacterial and plant ADPglucose pyrophosphorylases roughly correlates with the ability of the organism to metabolize various carbon sources for energy and growth. The activator specificity may also be used in certain classes of organisms as a taxonomic marker. Causal data with plants and bacteria correlating fluctuation of inhibitor and activator concentrations in vivo with rates of α-glucan synthesis strongly suggest that the activator and inhibitor effects observed in vitro are physiologically operative. Furthermore isolation of a class of mutants of E. coli and S. typhimurium altered in their ability to accumulate α-glucan can be correlated with altered affinities of the inhibitor AMP and the activator, fructose-P_2 for the organism's ADPglucose pyrophosphorylase. Covalent attachment of [^{3}H]pyridoxal-P (PLP) to the activator binding site of the E. coli enzyme has enabled us to determine the amino acid sequence around the activator site and determine it to be a lysyl residue 37 amino acid residues from the amino-terminus of the enzyme. The sequence is: NH_2-Val-Ser-Leu-Glu-Lys-Asn-Asp-His-Leu-Met-Leu-Ala-Arg-Gln-Leu-Pro-Leu-Lys-Ser-Val-Ala-Leu-Ile-Leu-Ala-Gly-Gly-Arg-Gly-Thr-Arg-Leu-Lys-Asp-Leu-Thr-Asn-Lys-(PLP)-Arg-Ala-Lys-Pro-Ala-Val-His-Phe-Gly-Gly-Lys..... (Supported by NIH Grant AI05520.)

Protein Phosphorylation

010 CYCLIC NUCLEOTIDE-INDEPENDENT PROTEIN KINASES FROM RABBIT RETICULOCYTES AND PHOSPHORYLATION OF TRANSLATIONAL INITIATION FACTORS, Jolinda A. Traugh, Department of Biochemistry, University of California, Riverside, CA 92521.

Three cyclic nucleotide-independent protein kinases have been obtained from rabbit reticulocytes in highly purified form by ion-exchange and molecular sieve chromatography. Casein kinase I (CK I) is a monomeric enzyme with an approximate molecular weight of 37,000. The K_m for ATP is 13 μM and 900 μM for GTP. Casein kinase II (CK II) is a multimeric enzyme (M_r 130,000) with an $\alpha, \alpha'\beta_2$ structure and subunits of 42,000, 38,000 and 24,000 daltons respectively. The enzyme utilizes both ATP and GTP in the phosphotransferase reaction with a K_m of 10 and 40 μM respectively. CK I and CK II are substrate specific and phosphorylate different sites in both α_{s1} and β-casein. CK I recognizes the primary sequence Glu-X-Ser and the recognition determinants for CK II are Thr(Ser)-Glu-Asp. The enzymes also exhibit substrate specificity with respect to initiation factors; CK I phosphorylates eIF-4B and eIF-5 while CK II modifies eIF-2β (M_r 53,000), eIF-3 (the 35,000, 69,000 and 130,000 dalton subunits), eIF-4B and eIF-5. Although neither enzyme is regulated by cyclic nucleotides, it has been observed that CK II is inhibited by deacylated tRNA from reticulocytes; 50% inhibition is observed with 50 nM tRNA. A protein kinase which specifically phosphorylates eIF-2α (M_r 38,000) has also been obtained in a highly purified form. The kinase activity is inhibited by hemin; 50% inhibition is observed at concentrations of 6 μM. The enzyme has a subunit molecular weight of 90,000 and is self-phosphorylated. The self-phosphorylation results in inhibition of the kinase activity. In addition, two protease-activated kinases (PAK) have been observed in reticulocytes. PAK I phosphorylates the 130,000 dalton subunit of eIF-3, eIF-4B and 40S ribosomal protein S15 (M_r 17,200). PAK II is activated by an endogenous Ca^{2+}-stimulated protease and modifies eIF-2 β and 40S ribosomal protein S13 (M_r 32,500). In reticulocyte lysates, under conditions of optimal protein synthesis, phosphorylation events mediated by CK I and CK II are observed. Under conditions of hemin deprivation, phosphorylation of two components, eIF-2α and a 55,000 dalton protein, is increased five-fold prior to inhibition of protein synthesis. (Supported by USPHS Grant GM 21424.)

011 REGULATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASES

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The type II cyclic AMP-dependent protein kinase purified from bovine cardiac muscle catalyzes the phosphorylation of its own cyclic AMP-binding protein component by an intramolecular reaction (1). Studies performed in reconstituted systems in vitro indicate that (a) the dephosphorylated cyclic AMP-binding protein regenerates the inactive protein kinase holoenzyme more readily than the phosphorylated protein (2-3) and (b) dephosphorylation occurs principally if not exclusively when the phosphorylated binding protein is dissociated from the holo-enzyme (4). We have begun to develop methods for evaluating the state of protein kinases under physiological conditions using the photoaffinity analog of cyclic AMP, 8-azido cyclic [^{32}P]AMP which reacts specifically and in stoichiometric amounts with cyclic AMP-binding components of protein kinases in tissue extracts (5-8). The phosphorylated and dephosphorylated forms of protein kinase II were resolved by isoelectric focusing and by sodium dodecylsulfate-electrophoresis in Tris-glycine buffer. The data obtained from one- and two-dimensional electrophoresis indicate that protein kinase II is present predominantly in the phosphorylated form in fresh extracts of bovine cardiac muscle (8). Analysis of the structural state of protein kinases is being pursued in two established murine cell lines amenable to hormonal and genetic manipulation: the insulin-sensitive 3T3-L1 preadipocyte and J774.2, a macrophage-like cell. In the latter system, stable variants in cyclic AMP synthesis and function have been developed (9). Protein kinases in variants resistant to the growth-inhibitory effects of cyclic AMP have been analyzed directly on polyacrylamide gels using the photoaffinity label described above. In this way information about molecular heterogeneity and size, peptide composition, sensitivity to proteolysis and affinity for cyclic AMP was obtained with unpurified cellular extracts and used to correlate protein kinase structure and function.

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012 THE ROLE OF PROTEIN PHOSPHORYLATION IN THE COORDINATED CONTROL OF GLYCOGEN AND INTERMEDIARY METABOLISM

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Glycogen metabolism in mammalian skeletal muscle is controlled by a network of phosphorylation-dephosphorylation reactions in which five protein kinases, two protein phosphatases and four thermostable "regulator" proteins determine the activities of glycogen phosphorylase and glycogen synthase, the rate limiting enzymes of the pathway

One of the protein kinases is cyclic AMP dependent protein kinase which mediates the control of glycogen metabolism by adrenaline. Three further protein kinases require the calcium dependent regulator protein (calmodulin) for activity and are likely to mediate the neural control of the system. Each of the dephosphorylation reactions which inhibit glycogenolysis or activate glycogen synthesis are catalysed by protein phosphatase-1. This enzyme is powerfully inhibited by a protein termed inhibitor-1, which is only an inhibitor after it has itself been phosphorylated by cyclic AMP dependent protein kinase (1,2).

It is already well established that cyclic AMP dependent protein kinase regulates the activities of many different enzymes and that it mediates most, if not all, of the intracellular actions of those hormones which work through cyclic AMP (3). Recent evidence now suggests that many of the other proteins involved in the regulation of glycogen metabolism are also involved in the regulation of other cellular processes, and that the major pathways of intermediary metabolism are cross-linked by a simple network of regulatory pathways which allow these processes to be controlled in a synchronous manner by neural and hormonal stimuli (4-6). Recent work from this laboratory which has demonstrated striking similarities between the regulation of glycogen synthesis and fatty acid synthesis (7,8) will be presented.

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Cyclic GMP and Cyclic CMP

013 THE PLEURIPOTENTIALITY OF THE cGMP SYSTEM. Nelson D. Goldberg, Gustav Graff and Janis Stephenson. Department of Pharmacology and Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455.

Cyclic GMP has been implicated as a biological regulatory component in the expression of a number of hormone actions, in mitogen action, and in a variety of altered physiological states which generally oppose, but in some instances compliment cyclic AMP-linked signals. The evidence in support of an involvement of cGMP in these processes remains indirect. It is based primarily on investigations demonstrating that the action of these agents or conditions is associated with enhanced cellular accumulation of cyclic GMP, and in some instances, that exogenous addition of cyclic GMP or derivatives (e.g., 8-bromo or dibutyryl) can mimic some of the cellular actions of these agents and conditions.

There is also evidence that in some systems cGMP may constitute only one of the components in the cellular regulatory system called into play by certain biologically active agents since an increase in cGMP levels alone does not always appear to be sufficient to trigger the event. This may be explained in part by our recent proposal that in some systems cGMP may not act as a classical mass action effector but instead serve as a source of protons and/or energy upon its hydrolysis by phosphodiesterases which are regulated and perhaps coupled to specific cellular events by macromolecular components such as "calcium dependent regulator," guanyl nucleotide binding components and others now being uncovered.

The suggestion that the cGMP system may be operationally different, and more diverse in function and exhibit a greater pleuropotential than that of cAMP is underscored by the strikingly different characteristics of the metabolic components comprising the cGMP and cAMP systems. These differences which are apparent with respect to the specific cyclases, phosphodiesterases and kinases in the two systems in addition to the increasing number of enzymes other than kinases that appear to be affected by cGMP (i.e., cAMP specific phosphodiesterases, phosphofructokinase, phosphoribosyl pyrophosphate synthetase, etc.).

For example, in the case of the cyclases that catalyze the generation of the two different cyclic nucleotides it is clear from their strikingly different properties that each subserves very different biological communication systems. Membrane associated adenyalte cyclase is more or less in direct communication with hormone receptors while guanylate cyclase activity a majority of which is soluble and associated with intracellular organelles is not affected directly by hormones but appears to be modulated by oxidizing equivalents (fatty acid hydroperoxides, prostaglandin endoperoxides, dehydroascorbate, ascorbate free radical) that may be generated within the cell by different stimuli or by a class of compounds represented by specific lipid components that may arise as a result of hormone action and orchestrate a complex sequence of coordinated cellular events that combine along with the cGMP generated to modify cellular function.

014 ENZYMATIC FORMATION OF CYCLIC CMP BY MAMMALIAN TISSUES, Louis J. Ignarro and Stella Y. Cech, Department of Pharmacology, Tulane University School of Medicine, New Orleans, LA 70112.

The natural occurrence of cyclic CMP in mammalian cells was first reported by Bloch (1), who also demonstrated that cyclic CMP abolishes the temperature-dependent lag phase and stimulates resumption of growth of leukemia L-1210 cells. Several years later, phosphodiesterase activity with specificity for cyclic CMP was identified in mammalian tissues (2). Studies from these laboratories revealed that cellular cyclic CMP levels were higher, and cyclic CMP phosphodiesterase activities were lower, in more rapidly proliferating than in quiescent cells. Studies from this laboratory demonstrated that mammalian tissues contain cytidylate cyclase and can therefore synthesize cyclic CMP from CTP (3). Cytidylate cyclase activity was determined by measuring the formation of [32-P] cyclic CMP from α-[32-P] CTP in 40 mM Tris HCl (pH 7.4) containing 1.0 mM CTP (3×10^5 cpm), 0.3 mM Mn^{2+} and 2-3 mg/ml of homogenate protein (10 min at 37°C). Incubations were terminated by addition of EDTA and samples were chromatographed on columns of neutral alumina. Cyclic CMP was formed from CTP in the presence of tissue homogenate and certain divalent cations ($Fe^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$). This reaction is enzymatic, in that product formation was pH-, temperature-, time- and substrate-dependent, and was inhibited by boiling the tissue. The Km and Vmax were 0.16 mM and 0.93 nmole/min, respectively. Neither ATP nor GTP served as substrates for cyclic CMP formation. Cyclic CMP was identified as one of the reaction products by comparison with authentic compound in several analytical systems including: column chromatography on Dowex 1-formate, and on PEI cellulose; thin layer chromatography; crystallization to constant specific activity; specific radioimmunoassay. It must be emphasized that the [32-P] reaction product could not be identified as cyclic CMP by column chromatography on Dowex-50(H^+), as the reaction product failed to elute from the column. Although this discrepancy has not yet been rectified, preliminary data suggest that cyclic CMP formed by tissue homogenate is protein bound and must first be released by treatment with proteases prior to chromatography on Dowex-50(H^+), but not on anion exchange resins. Only 5-10% of total homogenate cytidylate cyclase activity was present in the soluble fraction, the remainder being distributed in the total sedimentable fraction. The sedimentable fraction markedly lost activity upon washing but activity was completely restored by adding back the soluble fraction. These data suggest, but do not prove, that mammalian tissues possess the capacity (cytidylate cyclase activity) to synthesize cyclic CMP from CTP.

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015 CYCLIC CMP PHOSPHODIESTERASE: BIOLOGICAL INVOLVEMENT AND ITS REGULATION BY AGENTS, J. F. Kuo, Mamoru Shoji and David M. Helfman, Departments of Pharmacology and Medicine, Emory University School of Medicine, Atlanta, Georgia 30322.
Occurrence of cyclic CMP phosphodiesterase (C-PDE) was shown recently in a number of mammalian tissues (1) and leukemia L-1210 cells (2). Levels of C-PDE in tissues are generally 2-3 orders of magnitude lower than those of cyclic AMP phosphodiesterase (A-PDE) and cyclic GMP phosphodiesterase (G-PDE). Our experimental data indicate that decreased activity levels of C-PDE are invariably associated with tissues undergoing rapid cell proliferation, either physiologic or pathologic, as exemplified by the regenerating liver (3), the ontogenesis of a number of guinea pig and rat tissues (1, 3, 4) and the fast-growing Morris hepatoma 3924A (5). Changes in activity levels of A-PDE and G-PDE, on the other hand, were found to be variable and tissue-specific, suggesting that metabolism of cyclic CMP may be more closely related to cell proliferation than that of cyclic AMP or cyclic GMP, or both. C-PDE, which requires Fe^{2+} or Mg^{2+} for its maximal activity and has a Km for cyclic CMP of about 2 mM, was purified from pig and rat livers to remove most of the contaminating A-PDE and G-PDE. We noted that papaverine and 1-methyl-3-isobutylxanthine are without effect on C-PDE at a concentration (100 μM) inhibiting A-PDE and G-PDE 70-90% (6). 2'-Deoxy cyclic AMP (a specific A-PDE inhibitor) and 2'-deoxy cyclic GMP (a specific G-PDE inhibitor) are relatively poor and non-specific inhibitors of C-PDE (6). Imidazole (5 mM) stimulates both A-PDE and G-PDE but not C-PDE, whereas potassium phosphate (5 mM) greatly inhibits C-PDE without affecting A-PDE and G-PDE (6). 5'-Nucleotides (mono-, di-, tri-, and tetraphosphate) of adenosine, guanosine, inosine, cytidine or uridine, and their synthetic analogs, at 2 mM, inhibit C-PDE 60-80%; in comparison, they stimulate A-PDE 50-150%, with little or no effect on G-PDE. In summary, our studies provide evidence to suggest that the newly-identified C-PDE is a potential site of bioregulation particularly in cell proliferation, and of pharmacological intervention as well. (Supported by USPHS Grants HL-15696, CA-23391 and T32-GM-07594).

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Biological Cascade Systems

016 COVALENTLY INTERCONVERTIBLE ENZYME CASCADE AND METABOLIC REGULATION. P.B. Chock and E.R. Stadtman, NIH, Bethesda, MD 20014
The singular importance of covalent enzyme modification in cellular regulation is evident from the ever increasing number of reports demonstrating that the activities of key enzymes in metabolism are modulated by their interconversion between covalently modified and unmodified forms (1, 2). Because the modification and demodification reactions are catalyzed by so-called converter enzymes, they involve the action of one enzyme upon another and are therefore referred to as enzyme cascade systems. Enzyme interconversion is a cyclic process resulting from the coupling of two opposing cascades, one involves the covalent modification and the other the demodification of the interconvertible enzyme. A steady state analysis (3,4) of such cyclic cascade systems shows that dynamic coupling of the modifying and the demodifying cascades will lead to a steady state distribution between active and inactive forms of the last interconvertible enzyme in a cascade, and thereby determine its specific activity. The specific activity of this target enzyme is a function of multiple cascade parameters. Because each of these parameters can be varied independently, or several can be varied simultaneously, by a single or multiple allosteric interaction(s) of one or several effectors with one or more of the cascade enzymes, interconvertible enzymes can respond simultaneously to changes in the concentration of a large number of allosteric effectors; thus cyclic cascades provide high flexibility for metabolic regulation. It follows that the specific activity of the target enzyme can vary smoothly and continuously in response to ever-changing levels of multiple metabolites which are effectors of the cascade enzymes. Since the fractional modification of the target enzyme is a multiplicative function of various parameters in the cascade, interconvertible enzyme cascades are endowed with enormous signal and rate amplification potentials. When an allosteric effector acts on more than one converter enzyme, the enzyme cascade can generate a sigmoidal response in the target enzyme activity to increasing allosteric effector concentration. Results of the theoretical analysis reveal that interconvertible enzyme cascades provide an especially effective means of cellular regulation. However, it should be pointed out that for each complete cycle, one equivalent of ATP is consumed to provide the free energy needed to maintain the steady state activity of the modified enzyme at a metabolically required level. This is the price for such an effective regulatory mechanism.

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017 METABOLITE CONTROL OF THE GLUTAMINE SYNTHETASE CASCADE, E.R. Stadtman, P.B. Chock, and S.G. Rhee, *The National Institutes of Health, Bethesda, MD 20014*

The activity of glutamine synthetase in E. coli is regulated by a bicyclic cascade system (Fig. 1) (1, 2). In one cycle a regulatory protein, P_{II}, undergoes UTP-dependent interconversion between a uridylylated form (P_{IID}) and the unmodified form, P_{IIA}. This interconversion is catalyzed by a complex composed of a uridylyltransferase (UT) and a uridylyl-removing enzyme (UR). The second cycle is catalyzed by a single adenylyltransferase (AT) which catalyzes the P_{IIA}-dependent transfer of AMP from ATP to a tyrosyl residue in one or more of the 12 subunits of GS, and also the P_{IID}-dependent transfer of adenylyl groups from adenylylated enzyme (GSa) to Pi, thus forming ADP and regenerating the unmodified GS. Because adenylylated subunits are catalytically inactive under physiological conditions, the specific activity of GS is inversely proportional to the fraction of adenylylated subunits. A theoretical analysis (2) of the GS cascade shows that the steady state fraction of adenylylated subunits is a multiplicative function of numerous parameters including the activities and kinetic constants (α_n) of the converter enzymes AT, UR and UT, and the concentrations of metabolite effectors. By means of allosteric and substrate interactions with one or more of the converter enzymes, the GS cascade can sense simultaneously the changes in concentrations of at least 40 different metabolites. These multiple interactions lead automatically to adjustments in the steady state distribution of adenylylated and unadenylylated GS subunits. Thereby the cascade can integrate the vast amount of metabolic information needed to sustain fine control of GS activity. From detailed in vitro studies in which the allosteric effectors, α-ketoglutarate and glutamine, and the mole fractions of P_{IIA} and P_{IID} were varied experimentally, it was demonstrated that the AT-catalyzed adenylylation and deadenylylation cycle is quantitatively described by 10 binding constants, 12 synergistic/antagonistic coefficients and 6 rate constants (3). The experimental data are consistent with theoretical predictions of the cascade model and demonstrate the remarkable flexibility of such cascades to metabolic control as well as their enormous signal amplification capacity and their ability to generate sigmoidal responses to increasing concentrations of primary allosteric stimuli.

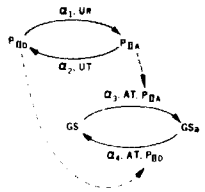

Fig. 1

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Methylation in Cellular Chemotaxis

018 METHYLATION AND DEMETHYLATION IN THE BACTERIAL CHEMOTACTIC SYSTEM, Sharon M. Panasenko, Daniel E. Koshland, Jr., Department of Biochemistry, University of California, Berkeley, CA 94720.

Covalent modification of proteins is a common regulatory mechanism in both prokaryotic and eukaryotic systems. A novel type of protein modification, the reversible formation of carboxy methyl esters is an essential feature of sensory transduction during bacterial chemotaxis. It has been demonstrated that methylation of glutamate residues in a class of membrane proteins in *E. coli* and *S. typhimurium* occurs during the chemotactic responses of these organisms. Physiological studies of the responses of cells in which this methylation reaction is blocked indicate that the methylation process plays a role in information processing and adaptation. The methylation reaction is catalyzed by a substrate-specific, SAM-dependent methyl transferase. The hydrolysis of the methyl esters is catalyzed by a protein methyl esterase. Both the methyl transferase and methyl esterase are found in the soluble fraction of extracts derived from wild type *Salmonella* or *E. coli* cells. Analysis of mutant strains indicates that the *cheR* gene product of *Salmonella* (*cheX* in *E. coli*) is necessary for the expression of the methyl transferase activity. The methyl esterase activity is associated with the *cheX* gene product of *Salmonella* (*cheB* in *E. coli*). The methyl accepting proteins have been shown to correspond to the *tar* and *tsr* gene products in *E. coli*. The level of methylation of the membrane-bound methyl accepting proteins is seen to be correlated with the presence of chemoattractants and repellents both *in vivo* and *in vitro*. The development of an *in vitro* methylation reaction as well as the isolation of mutants in all the components of the methylation system has allowed the biochemical dissection of the signal transmission process. Thus, it was possible to correlate changes in the pattern of protein methylation with alterations in physiological behavior, and to relate these changes to the time course of the response.

019 TRANSMETHYLATION REACTIONS AND THE CHEMOTAXIS OF EUKARYOTIC CELLS, Ralph Snyderman, Marilyn C. Pike and Nicholas M. Kredich, Duke Univ. Med. Ctr., Durham, N.C. 27710.
Chemotaxis by motile bacteria requires transmethylation reactions mediated by S-adenosyl-L-methionine(AdoMet). To see if methylation is required for the chemotaxis of eukaryotic cells, we studied the effects of inhibition of AdoMet-mediated transmethylation reactions on human monocyte chemotactic responsiveness. Methylation was inhibited by treating the cells with substances that elevated intracellular S-adenosyl-L-homocysteine(AdoHcy), a competitive inhibitor of AdoMet methylation. Incubation of monocytes with the adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine, plus adenosine and L-homocysteine thiolactone increased intracellular AdoHcy levels by as much as 1500-fold. Concomitant with increases in AdoHcy were a decrease in monocyte protein carboxy-O-methylation as well as inhibition of monocyte chemotactic responsiveness(1). Conditions that almost completely inhibited methylation and chemotaxis did not depress monocyte phagocytosis. Phagocytosis is therefore either independent of AdoMet-mediated methylation or is resistant to inhibition of such reactions by AdoHcy. Monocytes do not divide during the chemotaxis assay, and inhibition of protein synthesis by cycloheximide does not depress chemotaxis. Methylation of neither DNA nor RNA is therefore likely to be required for a chemotactic response. To determine which transmethylations were required for chemotaxis, we studied the effect of chemotactic factors on phospholipid methylation and protein carboxy-O-methylation in guinea pig macrophages. Neither C5a nor the N-formylated peptide chemoattractants altered protein carboxy-O-methylation. The same factors, however, had a marked effect on phospholipid methylation. Methylation of phospholipids was inhibited by up to 73% in g.p. mϕ incubated with chemotactic factors but not with chemically similar, non-chemotactic agents. The potency of chemotactic factors in inducing chemotaxis correlated with their ability to inhibit phospholipid methylation(r=0.99). The specificity of inhibition of phospholipid methylation for chemotaxis was evidenced by the failure of chemotactic factors to depress methylation in chemotactically unresponsive lymphocytes. Moreover, phagocytosis of opsonized erythrocytes by g.p. mϕ did not inhibit phospholipid methylation. While g.p. mϕ phospholipid methylation was depressed in the presence of chemotactic factors, total membrane phospholipid synthesis as measured by incorporation of $^{32}P_i$ into organic solvent soluble radioactivity was unaffected. Thus, chemotactic factors alter the composition of newly synthesized membrane of chemotactically responsive cells. For chemotaxis to occur, changes in phospholipid hydrophobicity produced by the methylation of phosphatidylethanolamine may be required for the alteration of membrane microviscosity or for the leukocyte membrane to interact favorably with external surfaces or internal cytoskeletal elements.

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020 ROLE FOR METHYLATION IN LEUKOCYTE CHEMOTAXIS. E. Schiffmann[1], R.F. O'Dea[2], P.C. Chiang[3], K. Venkatasubramanian[1], B. Corcoran[1], F. Hirata[3], and J. Axelrod[3]. [1]NIDR and [3]NIMH, NIH, Bethesda, MD 20014; [2]Univ. Minn. Med. Sch., Minneapolis, MN.
It has been shown previously that FMet peptides, related to chemotaxins from bacteria, are highly potent leukoattractants. These compounds bind to specific receptors on the cell and subsequently are hydrolyzed by a membrane-associated peptidase. The hydrolysis of the peptide probably frees receptors and allows the cell to respond continuously to a gradient of attractant. We have now studied subsequent events in leukotaxis, particularly a methylation reaction stimulated by FMet peptides. Formylated peptide attractants (FMet-Leu-Phe) stimulated the transfer of labelled methyl group from ^{3}H-methionine to protein carboxyl groups in the neutrophil. The increase in methylation was rapid but transient, reaching a peak within one minute and subsiding after five minutes. Neither the uptake of ^{3}H-methionine by the whole cell or its incorporation into protein was altered by the attractant. Antagonists (Z-Phe-Met) of binding of peptide attractants prevented the increased methylation. Both chemotaxis and methylation were inhibited by 5 mM EGTA, indicating a common requirement for Ca^{2+}. Neutrophil homogenates were found to contain protein carboxymethylase (PCM) and an acceptor substrate for the reaction, (MAP). Seventy percent of the PCM and thirty percent of the MAP were in the 30,000g pellet. As S-adenosylmethionine (AdoMet) is probably the methyl donor in such reactions, we determined the role of transmethylation in leukocyte chemotaxis. Here, we examined the effect of an inhibitor of S-adenosylhomoysteine (AdoHcy) hydrolase upon chemotaxis. It was found that 10 μM 3-deazaadenosine (DZA) markedly inhibited both cell motility and the hydrolase from neutrophil homogenates. A rapid increase of AdoHcy in the cell was also observed. The presence of both DZA and Hcy produced enhanced inhibition of chemotaxis as well as formation of DZAHcy, which like AdoHcy is an inhibitor of transmethylation. Compared to normal cells, DZA-inhibited cells did not have altered levels of AdoMet, suggesting rapid turnover of this intermediate. Since transmethylation is controlled by the ratio AdoMet/AdoHcy, the results imply a regulatory role for AdoHcy hydrolase in chemotaxis. Preliminary data suggest a role for methylation of membrane phospholipids in cell migration since both chemotaxis and lipid methylation were inhibited by 0.1 mM quinacrine, a phospholipase A_2 inhibitor. We conclude that the coupling between receptor and motile apparatus in the leucocyte is accomplished by the stimulation of enzymatic methylation of protein and perhaps lipids of the cell.

Regulation of Protein Synthesis I

021 EFFECT OF PHOSPHORYLATION ON eIF-2 FUNCTION, William C. Merrick, Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106.

During the last few years, several independent groups have purified most, if not all, of the eukaryotic protein synthesis initiation factors and have attempted to define the role of these proteins in the initiation process. Given below is a simplified composite pathway of 80S initiation complex formation.

1. $eIF\text{-}2 + GTP + Met\text{-}tRNA_f \longrightarrow [eIF\text{-}2 \cdot GTP \cdot Met\text{-}tRNA_f] \equiv 3°$

2. $80S \rightleftharpoons 40S + 60S \xrightarrow{eIF\text{-}3} 40S \cdot eIF\text{-}3 + 60S$

3. $40S \cdot eIF\text{-}3 + 3° \longrightarrow 40S \cdot eIF\text{-}3 \cdot 3°$

4. $40S \cdot eIF\text{-}3 \cdot 3° + mRNA \xrightarrow[(eIF\text{-}4A,4B)]{ATP} \xrightarrow{ADP,P_i} 40S \cdot eIF\text{-}3 \cdot 3° \cdot mRNA$

5. $40S \cdot eIF\text{-}3 \cdot 3° \cdot mRNA + 60S \xrightarrow{(eIF\text{-}4C,5)} 80S \cdot Met\text{-}tRNA_f \cdot mRNA + GDP + P_i + eIF\text{-}2,3$

In recent years there have been many reports that a protein termed HCR is capable of causing an inhibition of protein synthesis initiation, presumably via a direct phosphorylation of eIF-2, the only known substrate for this protein kinase. Studies in this laboratory on eIF-2 molecules which have been phosphorylated by HCR (to the level of 2 moles PO_4 per mole eIF-2) have indicated that phosphorylation of eIF-2 has not altered any of the following reactions: 1) the rate or extent of ternary complex formation (step 1); the extent of Met-$tRNA_f$ binding to 40S subunits (step 3); the rate or extent of 80S initiation complex formation (using AUG as mRNA, step 5); the rate of exchange of [^{3}H]GDP bound to eIF-2. These results indicate that phosphorylation of eIF-2 does not directly alter eIF-2 function.

022 REGULATION OF THE INITIATION OF EUKARYOTIC PROTEIN SYNTHESIS BY PROTEIN KINASES AND HEME. Irving M. London, Vivian Ernst, Daniel H. Levin, Raymond Petryshyn, Rajinder S. Ranu and Hans Trachsel, Massachusetts Institute of Technology, Cambridge, Mass.

Protein synthesis in reticulocytes and their lysates is regulated by heme. In heme-deficiency there is rapid activation of a heme-regulated translational inhibitor (HRI) which blocks the initiation of protein synthesis. HRI is a cAMP-independent protein kinase which phosphorylates the α-subunit (38,000 daltons) of eIF-2, the initiation factor which forms a ternary complex with initiator Met-$tRNA_f$ and GTP, and is required in the formation of the (40S, Met-$tRNA_f$) complex. The inhibitions produced by heme-deficiency or by the addition of HRI to hemin-supplemented lysates are similar, and are both overcome by the addition of eIF-2. The molecular mechanism of inhibition appears to be due to the diminished reactivity of eIF-2(P) with other initiation factors resulting in significant reduction in the rate of ternary complex and (40S, Met-$tRNA_f$) formation. The heme-reversible form of HRI has been purified to homogeneity and migrates as a single component of 95,000 daltons in SDS-acrylamide (BIS:15% acrylamide = 1:174) or 80,000 daltons (BIS:10% acrylamide = 1:38). HRI undergoes an autophosphorylation in the presence of ATP and this phenomenon is directly associated with its ability to phosphorylate eIF-2α. Pretreatment of HRI with heme blocks its ability to (a) autophosphorylate, (b) phosphorylate eIF-2α, and (c) inhibit protein synthesis in lysates. Similar inhibitory protein kinase activities are induced by physiological levels of dsRNA (1-20 ng/ml)(dsI) and by GSSG (50-500 mM). HRI and dsI appear to be different molecular entities based on physiological and immunological criteria. HRI is activated in high-speed supernates, whereas dsI is activated on the ribosomes. Antiserum prepared against purified HRI blocks its autophosphorylation, the phosphorylation of eIF-2α, and its inhibitory properties. However, this antiserum does not affect the protein kinase and inhibitory activities of dsI. The phosphorylations induced in lysates in response to these various conditions have been monitored directly after labelling for brief periods with a pulse of [γ-^{32}P]ATP. The <u>in situ</u> [^{32}P]phosphoprotein profiles were analyzed on one-dimensional SDS-PAGE under conditions in which HRI and eIF-2α are clearly distinguished. All three modes of inhibition produce a rapid phosphorylation of eIF-2α both before and after the onset of inhibition, compared to an uninhibited control lysate which displays little or no eIF-2α phosphorylation. Heme-deficient lysates also display a specific phosphorylation of the HRI component. The phosphorylations of eIF-2α and HRI in heme-deficient lysates are both rapidly diminished by the delayed addition of hemin. Induction of inhibition in lysates by dsRNA is accompanied by the phosphorylation of eIF-2α and of a 67,000-dalton polypeptide, but not of HRI. (Supported by NIH Grants AM 16272 and GM 24825).

023 REGULATION OF PROTEIN SYNTHESIS

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Globin synthesis in reticulocytes is controlled by the level of heme, the prosthetic group of hemoglobin. Protein synthesis in reticulocyte lysates is but briefly maintained in the absence of added hemin. As shown by Gross and Rabinovitz in 1972, hemin prevents the activation of an inhibitor of polypeptide chain initiation (hemin-controlled inhibitor, HCI). The inhibitor has been identified by the groups of Tim Hunt, Irving London and Boyd Hardesty, as a cAMP-independent protein kinase (eIF-2 Kinase) that phosphorylates the small (38 K) subunit of the initiation factor eIF-2. eIF-2 forms a ternary complex with GTP and Met-tRNA$_i$ which, on interaction with a 40S ribosome, gives rise to a 40S preinitiation complex. At the low concentration at which eIF-2 is present in lysates, it is virtually inactive in the absence of a protein (eIF-2 stimulating protein, ESP) which like eIF-2 is present in the high-salt ribosomal wash. This protein has now been extensively purified. Phosphorylation of the small (with eIF-2 kinase) but not of the middle (with casein kinase) subunit of eIF-2 blocks the interaction of eIF-2 with ESP and inhibits formation of the ternary complex.

At least one mechanism, possibly the physiological mechanism of activation of HCI involves phosphorylation catalyzed by a cAMP-dependent protein kinase. This would be in analogy to the activation of phosphorylase kinase, the cAMP-independent enzyme that catalyzes the phosphorylation (with ensuing activation) of glycogen phosphorylase. Addition of ATP and cAMP, or ATP and catalytic subunit from cAMP-dependent protein kinase, to crude preparations of inactive HCI (that are virtually free of cAMP and other nucleotides but contain a protein kinase highly dependent on cAMP for activity) activates HCI as measured by inhibition of ternary complex formation. At concentrations that maintain protein synthesis in reticulocyte lysates, hemin prevents the activation elicited by cAMP but not that produced by catalytic subunit, indicating that it blocks the activation of cAMP-dependent protein kinase by cAMP, i.e. the reaction R_2C_2 + 2 cAMP $\leftrightarrow$ 2 R-cAMP + 2 C. Hemin prevents the binding of [^{3}H] cAMP to cAMP-dependent protein kinase or its regulatory subunit (R). This effect is due to a specific binding of hemin to R. It binds to a site other than the cAMP binding site and allosterically blocks the binding of the cAMP. Our results are consistent with the view that protein synthesis in reticulocytes is regulated by cAMP and the action of cAMP is controlled by hemin. cAMP regulates translation also in other cells.

Regulation of Protein Synthesis II

024 CONTROL OF DNA STRUCTURE AND FUNCTION BY POST-SYNTHETIC MODIFICATIONS OF CHROMOSOMAL PROTEINS, Vincent G. Allfrey, Giorgio Vidali, Lidia C. Boffa and Richard Sterner, Rockefeller University, New York NY 1002].

Nuclear proteins are subject to a variety of post-synthetic modifications, some of which are rapidly reversible. The enzymatic transfer of acetyl groups from acetyl-Coenzyme A to the ε-amino groups of lysine residues in the key nucleosomal histones, H3 and H4, provides an example of multiple post-synthetic modifications in a very limited region of a polypeptide chain. Histones H3 and H4 may each contain 0 to 4 ε-N-acetyllysine residues in the highly basic NH_2-terminal regions. The charge-neutralization accompanying acetylation of these lysine residues weakens the electrostatic interactions between the positively-charged histones and the negatively-charged phosphate groups of the DNA strand enveloping the nucleosome core. The resulting release of constraints upon the DNA strand would be expected to alter DNA conformation and influence its template function in RNA synthesis. Evidence in support of this view will be presented. It includes correlations between increased histone acetylation and gene activation for RNA synthesis as induced by hormones, mitogens and developmental stimuli, as well as strong structural correlations between the intranuclear sites of acetylation and transcription. More direct evidence for causation - linking high levels of histone acetylation with an increased 'accessibility' of the associated DNA sequences - has recently been obtained. The addition of 5-7 mM Na butyrate to cell cultures leads to an accumulation of the multi-acetylated forms of histones H3 and H4 (1). We have shown that butyrate selectively inhibits histone deacetylase activity without affecting acetyl-transferase activity and that the inhibition is reversible within minutes after butyrate is removed from the medium (2). This has made it possible to vary levels of histone acetylation in vivo and compare the 'accessibilities' of the DNAs in control and hyper-acetylated chromatins, using DNase I as a probe. The results confirm the view that acetylation of the nucleosomal H3 and H4 releases constraints on the associated DNA sequences(3). Similar modifications have now been shown to occur in a class of non-histone DNA-binding proteins, called the high mobility group [HMG] proteins. All of the HMG proteins so-far examined contain ε-N-acetyllysine. It follows that the acetylation of lysine residues is not limited to histones but provides a more general mechanism for the modulation of interactions between chromosomal proteins and DNA.(4).

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025 PEPTIDE-CHAIN INITIATION IN HEART AND SKELETAL MUSCLE, L. S. Jefferson and H. E. Morgan, Dept. of Physiology, Pennsylvania State University, Hershey, Pa. 17033

Rates of peptide-chain initiation were estimated in heart and skeletal muscle by measurements of the rate of protein synthesis and levels of ribosomal subunits (1). During perfusion of isolated rat heart or hemicorpus with buffer that contained glucose and normal plasma levels of amino acids, polysomes decreased, levels of ribosomal subunits rose, and protein synthesis declined. These findings indicated development of a block in peptide-chain initiation in either tissue. In heart muscle, the block was reversed by addition of insulin, palmitate, octanoate, acetoacetate, propionate, acetate, leucine, α-ketoisocaproate, isovalerate or isobutyrate to the perfusion medium. In skeletal muscle of mixed fiber type, insulin and leucine were effective, but fatty acids, other non-carbohydrate substrates and metabolites of branched-chain amino acids were not effective in reversing the block. Faster rates of protein synthesis and decreased levels of ribosomal subunits were indicative of accelerated peptide-chain initiation. Activity of eIF-2 in postribosomal supernatants of heart or skeletal muscle homogenates that were prepared in 250 mM KCl to maximize eIF-2 recovery was unchanged by insulin when assayed by formation of the ternary complex of met-tRNA$_f$, GTP and eIF-2.

Differences in regulation of peptide-chain initiation in heart as compared to skeletal muscle were expressed in vivo. Starvation or induction of diabetes increased subunit levels and decreased synthesis in mixed skeletal muscle. In heart and soleus, a red skeletal muscle, levels of ribosomal subunits did not increase and synthesis was reduced to a lesser extent. Reduction in synthesis in mixed skeletal muscle was due to two factors: decreased number of ribosomes and efficiency of synthesis (activity/ribosome). Efficiency in heart was sustained due to maintenance of rates of initiation by fatty acids and other non-carbohydrate substrates. Provision of these substrates to heart, but not mixed skeletal muscle, increased tissue levels of glucose-6-phosphate, an intermediate that facilitates peptide-chain initiation in reticulocyte lysates (2). In skeletal muscle, efficiency also may be impaired by reduction in activity of eIF-2. Activity of the factor decreased in proportion to the reduction in levels of ribosomal RNA. These studies emphasize the importance of peptide-chain initiation as a rate-controlling step that is affected by availability of hormones and nutrients to heart and skeletal muscle.

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026 MODULATION OF EUCARYOTIC INITIATION FACTOR 2(eIF-2) FUNCTION AND PROTEIN SYNTHESIS DURING NUTRIENT DEPRIVAL IN THE EHRLICH ASCITES TUMOR CELL. Edgar C. Henshaw and Walter Mastropaolo, Cancer Center of the University of Rochester, Rochester, N.Y. 14642 and Alap R. Subramanian, Max-Planck-Institut fur Molekulare Genetik, Berlin 33 (Dahlem).

Deletion of an essential amino acid from the medium of Ehlich cell suspension cultures causes maximal inhibition of protein synthesis within 15 minutes. This inhibition is reversed within 15 minutes. This inhibition is reversed within 5 minutes by addition of the missing amino acid. A similar but more slowly developing effect is observed after omission of glucose. Nutritional deprivation inhibits principally the initiation steps of protein synthesis, specifically the binding of Met-tRNA$_f$ to the native 40s ribosomal subunit. An essential part of this binding is the formation of a ternary complex of Met-tRNA$_f$, GTP, and the initiation factor eIF-2. We have focused on modifications of eIF-2 that could regulate initiation and thus protein synthesis in response to the cell's nutritional state. Phosphorylation of eIF-2 is a possible modulator of its function since SDS PAGE analysis shows that two (38,000 and 48,000 daltons) of the factor's 3 subunits are phosphorylated in vivo within 2 hours after introduction of $^{32}PO_4$ into the medium. Preliminary experiments indicate that phosphorylation of eIF-2 bound to 40s subunits is much greater than that of eIF-2 in the soluble cellular fraction. This difference in phosphorylation could play a role in the cycling of eIF-2 between a ribosome-bound state and a free state during protein synthesis. Studies on nutritional effects on phosphorylation of eIF-2 are incomplete.

We have also examined the phosphorylation of ribosomal structural proteins and ribosomal associated proteins in fed and nutrient-deprived cells. A 40s subunit protein of MW=36,000 (presumably S6) is much more phosphorylated in well-fed cells than in cells deprived of glucose or an essential amino acid. Nutrition-dependent differences in phosphorylation in several acidic ribosomal proteins are also observed. The identity of these proteins and their possible role in the regulation of protein synthesis remain to be elucidate.

027 HORMONAL REGULATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE (GTP) SYNTHESIS, Richard W. Hanson, Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106.

The synthesis rate of hepatic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) is markedly stimulated by starvation and by diabetes. Refeeding starved animals a diet high in carbohydrate or the injection of insulin into diabetic animals causes a rapid deinduction of enzyme synthesis ($t_{\frac{1}{2}}$ = 40 minutes). The injection of cordycepin into starved animals also causes a deinduction of PEPCK in rat liver with a $t_{\frac{1}{2}}$ equal to that noted after refeeding or insulin injection. The induction of PEPCK synthesis in rat liver is regulated in part by cAMP, since the administration of this compound causes a ten-fold increase in enzyme synthesis within 90 minutes. This effect of cAMP can be blocked by the simultaneous administration of cordycepin, suggesting that some step in mRNA processing is involved in the response of PEPCK to cAMP administration. Work with Reuber H-35 hepatoma cells in culture has shown that cAMP is necessary to maintain enzyme synthesis, since its removal from the incubation medium results in a deinduction of enzyme synthesis equivalent to that noted in vivo when glucose is fed to starved animals. An analysis of PEPCK mRNA has shown that the levels of translatable mRNA as measured by the wheat germ protein synthesizing system, changes in parallel to alterations in enzyme synthesis. Thus, the administration of cAMP induces both PEPCK synthesis and PEPCK mRNA levels at a parallel rate. The same simultaneous change in mRNA and specific enzyme synthesis was noted during deinduction when starved rats were fed carbohydrate. Again, as was the case with enzyme synthesis, the administration of cordycepin blocked the induction of PEPCK mRNA caused by cAMP administration. We have noted parallel changes in the rates of enzyme synthesis and levels of translatable mRNA in all conditions noted to date. The only departure from this was our recent observation that the administration of cycloheximide, at levels which block protein synthesis, also caused a marked increase in PEPCK mRNA levels. The effect of cycloheximide was equal to the effect noted when cAMP is injected alone. In general, however, our results show rapid changes in the PEPCK mRNA pool corresponding to changes in enzyme synthesis and suggest a functional correlation between the two processes. Further studies are required to establish the mechanisms by which cAMP alter levels of PEPCK mRNA.

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Modulation and Inactivation

028 POLY(ADP-RIBOSE) AND ADP-RIBOSYLATION OF PROTEINS, Osamu Hayaishi, Kunihiro Ueda, Masashi Kawaichi, Norio Ogata, Kouichi Ikai, Jun Oka and Hiroto Okayama, Department of Medical Chemistry, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606, Japan

ADP-ribosylation is a unique group of posttranslational modifications of proteins using NAD as a donor (1). It comprises mono- and poly-ADP-ribosylation; the former is distributed among prokaryotes and eukaryotes, whereas the latter is restricted in eukaryotes. Biological significance of mono-ADP-ribosylation reactions, such as the inactivation of elongation factor 2 by diphtheria toxin has been well-documented, but the function of poly-ADP-ribosylation is not yet completely understood. This report mainly focusses on our recent progress in enzymology and biology of poly-ADP-ribosylation in chromatin.

Initiation and elongation of poly(ADP-ribose) are catalyzed by a single enzyme, poly(ADP-ribose) synthetase; we were able to reconstitute these two steps in vitro from highly purified synthetase, DNA, and histone or ADP-ribosyl histone, respectively (2). Degradation of poly-ADP-ribosyl protein proceeds in two ways, the splitting of ribosyl-ribose linkage by poly(ADP-ribose) glycohydrolase and that of ribosyl-protein linkage by a newly found enzyme " ADP-ribosyl histone hydrolase" (3). Regulatory mechanisms have been suggested to operate in all these reactions. The polymer is bound to various proteins including histones and nonhistone proteins. In the case of ADP-ribosyl histone H1 and H2B, glutamic aicd residues are ADP-ribosylated through an ester linkage.

Poly-ADP-ribosylation has been shown to occur in nature using a tracer method. Recently, we developed an immunohistochemical procedure, and studied the in situ distribution of poly(ADP-ribose). These studies suggested that poly-ADP-ribosylation is correlated to differentiation in some cells and to DNA repair in other cells. The results will be discussed in relation to possible roles played by this modification in nuclear events.

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029 SELECTIVE INACTIVATION AND DEGRADATION OF ENZYMES IN SPORULATING BACTERIA, Robert L. Switzer, Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

Enzyme regulation by selective inactivation and degradation has recently become appreciated as a general control mechanism in microbes (1). For example, several enzymes of de novo nucleotide and amino acid biosynthesis disappear from Bacillus subtilis cells during differentiation of vegetative cells into endospores, which is induced by nutrient starvation. The nature of the inactivation of two of these enzymes, aspartate transcarbamylase (ATCase) and glutamine PRPP amidotransferase, which are selectively and rapidly inactivated in the early stationary phase of growth, has been studied in detail. Both enzymes are stable in growing cells. The inactivation of ATCase requires metabolic energy in vivo, but not RNA or protein synthesis (2). Inactivation of the amidotransferase specifically requires O_2(3). Both enzymes have been purified to homogeneity (4,5). Immunochemical experiments indicate that both enzymes are probably degraded. Loss of cross-reactive protein and ATCase were simultaneous; a variety of immunochemical methods failed to detect inactive enzyme (6). On the other hand, inactive amidotransferase protein of native subunit molecular weight is transiently detectable during inactivation. Inactivation and degradation of both enzymes are normal under conditions that block most of the protein turnover normally seen in the stationary phase. The inactivation of purified amidotransferase by O_2 has been studied in vitro. Inactivation is associated with oxidation of a novel FeS center in the enzyme (5). The oxidized enzyme may be the inactive cross-reactive form transiently found during inactivation. Substrates and allosteric effectors of amidotransferase strongly affect the rate of inactivation by O_2. Preliminary results on the possible reconstruction of the inactivation of ATCase in vitro will also be discussed.

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030 ENDOGENOUS PROTEOLYTIC MODULATION AND INACTIVATION OF YEAST ENZYMES, Helmut Holzer, Biochemisches Institut, Universität Freiburg, D-7800 Freiburg, Bundesrepublik Deutschland.

Proteolytic processes have been shown to play a role in the regulation of yeast enzymes. Activation of yeast chitin synthase from an active zymogen by limited proteolysis was demonstrated by CABIB et al.. HASILIK and TANNER have obtained evidence that a precursor of carboxypeptidase Y is modified by proteinase B to the mature enzyme. Proteolytic activation of the inactive inhibitor complexes of proteinases A and B and of carboxypeptidase Y has been studied in the author's laboratory. The mechanism of the glucose induced inactivation of cytoplasmic malate dehydrogenase, called "catabolite inactivation", has been investigated by MECKE et al. It was shown that inactivation of malate dehydrogenase is caused by proteolytic degradation of the enzyme. There is also evidence for a proteolytic mechanism of the glucose induced inactivation of the maltose uptake system (GÖRTS) and the galactose uptake system (MATERN and HOLZER). Upon carbon starvation rapid proteolysis of NADP-dependent glutamate dehydrogenase in the yeast candida utilis occurs (HEMMINGS) and evidence for a proteolytic inactivation of this enzyme in S. cerevisiae has also been obtained (MAZON).

Modulation of Enzyme Activity

031 POLYAMINE-MEDIATED PHOSPHORYLATION OF A NUCLEOLAR PROTEIN FROM PHYSARUM POLYCEPHALUM WHICH STIMULATES rRNA SYNTHESIS. G.D. Kuehn, V.J. Atmar, H.U. Affolter, T. Seebeck, U. Gubler and R. Braun, Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003 and Institute of General Microbiology, University of Bern, Bern, Switzerland. An acidic nucleolar phosphoprotein of M_r 70,000 was purified from isolated nuclei of the slime mold Physarum polycephalum after its selective phosphorylation by a polyamine-mediated reaction. The phosphoprotein simulated rRNA synthesis 5-fold by RNA polymerase I within a ribosomal deoxyribonucleoprotein complex isolated from nucleoli. It was identified as a component of the complex. It bound with high affinity and specificity to the palindromic ribosomal DNA (rDNA) of 38 x 10^6 M_r from P. polycephalum which contained two coding sequences for 5.8 S, 19 S, and 26 S rRNA. It also bound to three fragments of rDNA of M_r 21.2 x 10^6, 17.1 x 10^6, and 8.1 x 10^6, prepared by cleavage with restriction endonucleases Hind III, Psp I, and BAMH I, respectively. All of the fragments included the symmetry axis of the palindromic rDNA. The phosphoprotein did not stimulate transcription and did not bind to rDNA or to the restriction fragments indicated after treatment with alkaline phosphatase-agarose. These findings suggest that this nucleolar phosphoprotein may specifically regulate functions of rDNA in a manner dependent on its degree of phosphorylation. (Supported by NIH Grant #GM 18538).

032 MODULATION OF ORNITHINE DECARBOXYLASE ACTIVITY: John L.A. Mitchell, Northern Illinois University, DeKalb, IL 60115.
Ornithine decarboxylase, which catalyzes the rate-limiting step in the synthesis of the polyamines essential for cell growth, is of intense interest due to its extreme sensitivity to hormones, tumor-promoting agents, and variations in media osmolarity and serum content. The quick response of this enzyme's activity is believed to be associated with its rapid turnover ($T_{1/2}$ of 10-30 min), considerably faster than that observed for other eukaryotic enzymes. We have investigated the regulation of ornithine decarboxylase activity in the eukaryote, Physarum polycephalum, and discovered that this enzyme is actually quite stable, and the observed rapid changes in activity are due to interconversion between active and relatively inactive forms of this enzyme. These two enzyme forms have been purified and found to be indistinguishable on non-denaturing, and SDS, acrylamide gels. They do possess distinct isoelectric points, differ greatly in their affinity for the coenzyme, pyridoxal 5'-phosphate, and are partially separated on weak anion exchanger columns. In responding to minor variations in media osmolarity this enzyme demonstrates both the speed and reversibility of this covalent protein modification. Within 4-6 min of a 25% increase, or decrease, in osmolarity the fraction of the total enzyme in the active form decreases from 50% to 20%, or increases to greater than 90%, respectively. These ratio changes are transitory with reversal beginning after about 10 min and completing within 1 hr. This enzyme modification is prevented by sodium azide or chilling to 4°C. Since similar rapid, but generally stable, variations in the ratios of these two enzyme forms are observed in response to inhibitors, growth promotors, hormones, etc., this appears to be a good model system for the study of the modulation of this key enzyme.

033 SPECIFIC EFFECTS OF CALCIUM ON THE PHOSPHORYLATION OF CYTOSOL AND MEMBRANE PROTEINS ISOLATED FROM 3T3 and SV3T3 CELLS. Marit Nilsen-Hamilton and Richard T. Hamilton, The Salk Institute, Box 1809, San Diego, CA 92112
In 3T3 and SV3T3 cells, the phosphorylation of a protein with an apparent molecular weight of approximately 100,000 daltons is dependent upon the presence of calcium. The calcium-dependent phosphorylation is demonstrated in an in vitro assay, which involves incubation of subcellular fractions, isolated by differential centrifugation, with [^{32}P]-γ-ATP and $MgCl_2$ (10 mM). The phosphorylated proteins are resolved by SDS gel electrophoresis and detected by autoradiography. The major calcium-dependent phosphorylation is of a protein (100,000 daltons) localized mainly in the cytosol (supernatant obtained from centrifugation at 100,000 xg for 60 min). The phosphorylation of two other proteins is increased by $CaCl_2$, but phosphorylation of most proteins in the cytosol is unaffected by concentrations of $CaCl_2$ causing maximal phosphorylation of the 100,000-dalton protein. Although there is a small quantity of the 100,000-dalton protein in the membrane fractions of the cell, the major effect of $CaCl_2$ on phosphorylation in these fractions is to specifically inhibit the phosphorylation of a group of three proteins with molecular weights ranging from 36,000 to 38,000 daltons. Neither the effects of $CaCl_2$ on cytosol nor on membrane protein phosphorylation are affected by cyclic AMP or cyclic GMP. The specific, cyclic-nucleotide-dependent phosphorylations of these subcellular fractions are unaffected by $CaCl_2$.

034 PURIFICATION OF RAT LIVER HMG CoA REDUCTASE KINASE (RK) AND RK KINASE, Thomas S. Ingebritsen, Rex A. Parker and David M. Gibson, Dept. of Biochemistry, Indiana University School of Medicine, Indianapolis, Indiana 46223

We have previously shown that liver HMG CoA reductase (R) is interconvertible between inactive and active states in vitro (BBRC 81, 1268, 1978; Life Sciences Review, Dec. 1978). Activation of R is catalyzed by phosphorylase phosphatase (P); and inactivation by ATP (Mg) in the presence of reductase kinase (RK). In the present studies we have characterized both microsomal RK (mRK) and cytosolic RK (cRK). RK activity was assayed using extracted microsomes as a substrate. Of the total RK activity in a 12,000 x g supernatant, 15% was in the microsomes while 85% was in the cytosol. RK from both sources was partially purified by chromatography on DEAE-Cellulose. Both cRK and mRK were bound to the column at low ionic strength and then eluted with a linear gradient from 0-0.6 M NaCl. Both activities were eluted between 0.1 and 0.2 M NaCl suggesting that cRK and mRK are identical enzymes. RK activity was unaffected by cyclic nucleotides. Incubation of RK with purified P at 37° resulted in a time and dose dependent inactivation of RK (blocked by 50 mM NaF). Inactive RK was reactivated by incubation with 2 mM MgATP in a low ionic strength medium (blocked in the high ionic strength medium used to assay both R and RK). Reactivation was not enhanced by cAMP. The RK activating enzyme was present both in the RK preparation and tightly bound to the particulate glycogen-protein complex. The latter preparation was devoid of RK activity. These data support the concept that R is regulated by a bicyclic system in which both R and RK are modulated by reversible phosphorylation. Research supported by grants from NIH (AM 21278, AM 19299) and the Showalter Foundation.

035 COVALENT AND NON-COVALENT REGULATION OF PYRUVATE DEHYDROGENASE IN THE PERFUSED RAT HEART. Merle S. Olson, Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas, 78284.

The pyruvate dehydrogenase multienzyme complex (PDC) is regulated both by feedback inhibition by its products, and by phosphorylation-dephosphorylation of the enzyme complex by a protein kinase-phosphoprotein phosphatase system. The phosphorylated enzyme complex is inactive. Studies were performed to elucidate the regulatory characteristics of this system in the isolated perfused rat heart. Metabolic flux through PDC was monitored by measuring the decarboxylation of infused [1-^{14}C] pyruvate and the proportion of PDC in its active form was determined by extraction and assay of the complex from freeze-clamped hearts under appropriate conditions. The metabolic flux through PDC and the interconversion of PDC between its active and inactive forms was determined as a function of changes in substrate (i.e., pyruvate, fatty acids, or ketone bodies) composition or concentration, the hormonal status of the tissue (i.e., epinephrine and insulin) and the presence of inhibitors of the PDC kinase and phosphatase and of mitochondrial pyruvate transport. Of primary concern was an assessment of the contribution of direct regulatory effects on the active form of PDC and the enzymatic interconversion of the active and inactive forms of PDC during physiological metabolic state changes of the heart. Measurements of various nucleotide species (i.e., NADH, NAD^+, acetyl-CoA, CoASH, ATP and ADP) in the freeze-clamped hearts were performed in order to obtain indications of the various mediators of the various regulatory changes seen in the pyruvate dehydrogenase complex in the present study.

(This work was supported by grant HL-20544 from the National Institutes of Health.)

036 ANALYSIS OF THE SURFACE TOPOGRAPHY OF GLYCOGEN PHOSPHORYLASE a: IMPLICATIONS FOR CONTROL OF METABOLIC INTERCONVERSION, N.B. Madsen, P.J. Kasvinsky, S. Sprang & R.J. Fletterick, Dept. of Biochem., Univ. of Alberta, Edmonton, Alberta, Canada

Computer drawings of the van der Waals contours of atoms on the surface represent the phosphorylase molecule at 2.5 Å resolution with color coding for acidic and basic residues, bound ligands or conformational changes. The assymmetry resulting from the two-fold axis of each dimer provides two faces which can be distinguished structurally and functionally. Thus a concave catalytic face contains the glycogen storage sites on its periphery with entrances to the glucose-1-P binding sites of the active centers, adjacent to the PLP moieties, near its center. On the opposite side of the dimer to the catalytic face is found a control face containing the binding sites for the allosteric activator, AMP, for which ATP also competes. Quite close to these sites are found the Ser-14-phosphates hydrogen bonded to Arg-69. Each Ser-14-P is surrounded by positive charges, including more than are found on the adjacent sequence. Thus comparative studies on peptides cannot describe fully the specificity and binding requirements of the kinase and phosphatase. The T→R conformational change causes the Ser-14-P to move 5 Å in from the surface, explaining why the phosphatase V_m is decreased 20 fold with unchanged K_m. This permits us to suggest which groups are not required for binding the phosphatase. The Ser-14-P residues are only 40 Å apart across the control face and could be bridged readily by the multimeric kinase ($\alpha_4\beta_4\gamma_4\delta_4$),1,340,000 daltons, or the large molecular weight form of the phosphatase. Therefore both Ser-14's on a dimer could be phosphorylated or dephosphorylated simultaneously. Since the control face is opposite to the catalytic face, this can occur while phosphorylase is bound to glycogen.

037 MODULATION OF THE ACTIVITY OF TREHALOSE-6-P SYNTHASE FROM DICTYOSTELIUM DISCOIDEUM, Kathleen A. Killick, Boston Biomedical Research Institute, Boston, MA 02114

A major product of development in the cellular slime mold, Dictyostelium discoideum, is the disaccharide, trehalose. Trehalose-6-P (T6P) synthase catalyzes the rate-limiting step in trehalose synthesis, i.e. the production of T6P from UDP-Glc and glucose-6-P (G6P). The in vivo activity of this enzyme may be regulated by: a) alterations in catalytic properties; b) changes in the levels and flux of substrates; and c) compartmentation phenomena. Evidence has recently been obtained for the existence of multiple, kinetically distinct forms of the enzyme and for the dual function of G6P as substrate and effector. Multiple forms of the synthase were detectable, though not resolvable during either molecular sieve chromatography or discontinuous sucrose density gradient ultracentrifugation. Both forms of the enzyme (i.e. A and B) were active at 23°C and double reciprocal plots of initial velocity data indicated complex bimodal kinetics. The Km values were (1) 0.2mM and 3.2mM G6P, and (2) 0.5mM and 2.2mM UDP-Glc. Comparison of the latter parameters with the cellular levels of the synthase substrates (i.e. 0.5mM UDP-Glc and 0.18mM G6P) suggests that activation of trehalose synthesis during development may provide a rationale for the presence of these isozymes. At 42°C, only synthase A was active and the Km values were 3.2mM G6P and 2.4mM UDP-Glc. Synthase A demonstrated positive cooperativity relative to G6P binding and a value for N_H equal to 1.6 at low G6P levels (i.e. 0.4mM to 1.0mM) was estimated from Hill plots. The Dictyostelium enzyme is the first T6P synthase which, after purification, has been shown to exist in multiple forms and to exhibit positive cooperativity relative to G6P binding. Supported by Grants AG00260 and AG00922 from the NIH.

038 HALF-SITES REACTIVITY IN UDP-GLUCOSE DEHYDROGENASE IS DUE TO INDUCED ASYMMETRY, James S. Franzen, James Ashcom, Paul Marchetti, and David S. Feingold, University of Pittsburgh, Pittsburgh, PA 15260.

The active site thiol group of bovine liver UDPGDH is attacked more rapidly than other thiols by a number of thiol specific reagents. These reactions exhibit a burst size of 3 moles of thiol reacted per mole of hexaprotomeric enzyme. Whether this behavior is a result of pre-existing or induced asymmetry can be decided by comparing inactivation versus incorporation profiles for a variety of reagents that block the catalytic site. If the subunits exist in two discrete conformations in the native enzyme, and these conformations are rigid such that alterations at the catalytic site of one does not affect the conformation of the other, then the catalytic activity of the half-sites blocked enzyme will be independent of the nature of the blocking agent. If, contrariwise, the catalytic activity of the half-site blocked enzyme varies with the identity of the blocking agent, induction can be inferred, since different derivatives on the thiol of the neighboring subunit could be expected to have different distorting effects. Half-sites reacted UDPGDH has 50%, 40% and 20% of initial activity when the blocking reagents are a bromoacetylaminophenyl-UDP, iodoacetate, and N-iodoacetyl amino ethyl-5-naphthylamine-1-sulfonic acid, respectively. These and other data relating to the pre-existing versus induced asymmetry question will be presented.

039 REGULATORY ROLE OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPD) IN HUMAN ERYTHROCYTE GLYCOLYSIS, Chi-Sun Wang and Petar Alaupovic, Laboratory of Lipid and Lipoprotein Studies, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Despite its high activity, as assayed in vitro, GAPD seems to play a crucial role in the regulation of glycolysis in human erythrocytes. Since the reactions catalyzed by GAPD and phosphoglycerate kinase result in the formation of ATP, we studied the effect of ATP as a possible feedback inhibitor of the GAPD activity. It was expected that, due to structural similarity, ATP would behave as a competitive inhibitor of NAD^+. The results showed, however, that ATP was a non-competitive inhibitor. This finding indicated that, most probably, ATP reacted with GAPD at the allosteric site. Further kinetic studies showed that the sequential addition of substrates NAD^+, GAP and Pi to the enzyme is an essential feature of the GAPD catalyzed reaction. Finally, a study on the inhibitory effect of GAP demonstrated that increased concentration ratios of GAP/NAD^+ caused the inhibition of GAPD (competitive substrate inhibition). Results of this study show that the GAPD catalyzed reaction is regulated by both ATP and GAP. The increased concentration of ATP will slow down the GAPD reaction. This, in turn, will result in increased concentration levels of GAP due to its decreased utilization. The increased level of GAP will cause further inhibition of GAPD. Thus, GAP serves as an "amplifier" for the inhibitory effect of ATP. Our results are consistent with the finding of Minakami (Metabolism and Membrane Permeability of Erythrocytes and Thrombocytes, First International Symposium, Vienna, pp. 10-15, 1968) that the increased levels of ATP resulting from the inhibitory effect of ouabain on Na^+-K^+-ATPase are accompanied by increased levels of GAP.

040 MODULATION OF THE ACTIVITY OF THIOSULFATE SULFUR TRANSFERASE BY LIMITED PROTEOLYSIS, Paul M. Horowitz, Robert Hampson, and Mark Gomillion. The University of Texas Health Science Center, San Antonio, Texas 78284.

The enzyme thiosulfate sulfurtransferase (TST; rhodanese; EC 2.8.1.1) can be proteolytically clipped by trypsin under controlled conditions to give rise to a product with a turnover number that is three times higher than that of the parent enzyme. Electrophoresis of trypsin treated TST under denaturing conditions shows that the parent protein is converted from a polypeptide of molecular weight 32,600 to two polypeptide chains having apparent molecular weights of 28,800 (DI) and 4,000 (DII). When trypsin-clipped TST is chromatographed on Sephadex G-100, the protein elutes as expected for the parent species. Electrophoresis under denaturing conditions of peak fractions from this gel filtration show the presence of both peptides DI and DII, indicating that they remain associated after the limited proteolysis. Fluorescence studies using the probe 1,8-anilino naphthalene sulfonate indicate that the proteolysis is accompanied by a decrease in the average degree of solute accessible apolarity on the surface of the TST molecule. When proteolytically clipped TST is incubated in the cold for long periods (up to three days) its activity is lost at a faster rate than that of the parent enzyme. The above results are interpreted in terms of the known structural flexibility of TST and the obligatory conformational changes that this enzyme undergoes during catalysis. It appears that the proteolytic clip relieves conformational constraints that not only allows for a more facile transition between the conformational states known to be important in the TST catalysis but also leads to a protein that has a high overall structural lability. (Supported by Grant #AQ 723 from the Welch Foundation and Grant #GM 25177 from the National Institutes of Health.)

041 A SINGLE GENE wyb AFFECTS L-SERINE DEAMINASE, PROLINE OXIDASE, AND THE PROTEIN-MODIFYING ENZYME, L-LEUCYL, L-PHENYLALNYL tRNA PROTEIN TRANSFERASE IN E. coli K12. E.B. Newman, J.F. Morris, F.Quetton and V. Kapoor, Concordia Univ., Montreal, Canada

The activities of several degradative enzymes in E. coli K12 are higher in cells grown with L-leucine than in cells grown without. Leucine is in this sense the 'inducer' of both L-serine deaminase and L-threonine dehydrogenase, neither of which is induced by its substrate.

R.L. Soffer has described a protein-modifying enzyme, L-leucyl, Lphenyl-alanyl tRNA protein transferase (Ann.Rev.Bioch.1975). This enzyme is reasonably well understood biochemically, but its regulatory significance is not known. The fact that leucine is a substrate of this enzyme suggested to us that the activity of the transferase may be involved in leucine "induction" i.e. that induction may actually be a protein modification.

This has been approached by genetic methods. A single locus, wyb, has been shown to be involved in the regulation of serine deaminase and proline oxidase, and in the expression of transferase (J.Bact.Sept.1978). The wyb mutation has been transferred to another genetic background where it retains only some of its phenotype. Some evidence on the physical nature of L-serine deaminase will also be given.

042 ROLE OF MICROTUBULES AND CA^{++} IN THE METHYLATION OF PHOSPHOLIPIDS(PL) BY CHEMOTACTICALLY RESPONSIVE CELLS, M.C.Pike, N.M.Kredich and R.Snyderman,Duke Univ.,Durham, N.C.

We have recently shown that macrophage(mϕ) chemotaxis requires S-adenosyl-methionine mediated methylation and that chemotactic factors inhibit the methylation of phosphatidylethanolamine in mϕ. The sensing of chemical gradients and/or cytoskeletal polarization by mϕ might require local membrane alterations in methylated PL composition. Chemotactic factors alter leukocyte microtubule assembly as well as Ca^{++} fluxes, so we determined the effects on PL methylation of other agents which affect both these cellular processes and chemotaxis. The antitubulins colchicine(10^{-5}M) and vincristine(10^{-5}M) inhibited guinea pig mϕ PL methylation by ca. 60%. The EC_{50}s for inhibition of PL methylation correlated well with the EC_{50}s for inhibition of chemotaxis. PL methylation was not affected by antitubulins in nonchemotactically responsive lymphocytes. Ionophore A23187(10^{-5}M) also inhibited guinea pig PL methylation by as much as 67%. Inhibition was dependent upon the extracellular Ca^{++} concentration and could be reversed by exogenous Mg^{++}. Cytochalasin b, which prevents assembly of actin filaments, produced no alteration in mϕ methylation. Total PL synthesis by mϕ was not inhibited by either the antitubulins nor by A23187, indicating that these substances cause changes in PL composition. The inhibition of PL methylation in mϕ treated with chemotactic factor, colchicine and A23187 was similar in that these substances prevented the addition of the first methyl group to phosphatidylethanolamine. Thus, Ca^{++} and agents disrupting microtubular structure affect PL methylation in chemotactically responsive cells. By altering Ca^{++} flux and microtubule assembly, chemotactic factors might induce rapid alterations in the local hydrophobic properties of the mϕ membrane.

043 MALONYL-COENZYME A - DEPENDENT REGULATION OF RAT LIVER FATTY ACID SYNTHETASE BY NADP, Rene A. Frenkel and Michael J. Stark, The University of Texas Health Science Center at Dallas, Dallas, Tx. 75235

Studies with homogeneous preparations of fatty acid synthetase from rat liver have revealed a strong inhibition of the enzymic activity by low concentrations of NADP. The inhibition is totally dependent on the presence of malonyl-coenzyme A and is specific for the dinucleotide phosphate of its etheno analog (ε NADP); NAD is totally inactive in causing enzyme inhibition. Exposure of the inhibited enzyme to NADPH restores the synthetic capability at different rates depending on temperature. The kinetics of the reactivation indicate that there are pronounced conformational changes associated with the inhibition and that the observed loss of activity may be due either to competitive binding of NADP at the NADPH binding site with an extremely low K_D or to conformational changes that show a hysteretic response. Supported by Grants AM 17983 from NIH and I-385 from the Robert A. Welch Foundation.

044 THE ROLE OF E. COLI THIOREDOXIN IN PHOSPHOTRANSFERASE REACTIONS, Vincent Pigiet, Edward B. Skolnick and Charles Lunn, The Johns Hopkins University, Baltimore, MD 21218

Thioredoxin has functional roles demonstrated in several redox reactions, including ribonucleotide reduction, (reductive) sulfate assimilation, sulfoxide reduction and in the regulation of chloroplast fructose-6-phosphatase. In each case, the mode of action is presumed to involve oxidation/reduction of the single disulfide on thioredoxin. Recently, thioredoxin was demonstrated to play an obligatory role in DNA replication of T-7 phage.

Using immunoadsorbents, we have isolated a phosphorylated species ("phosphothioredoxin") with cysteine S-P linkage from E-coli W3110 grown in [^{32}P] media. Two isomers of the phosphorylated form account for at least 96% of all thioredoxin, including 86% as the Cys_{32}-P, and 10% as the Cys_{35}-P isomers. In vivo labeling studies show a distinctive time course of phosphorylation with respect to growth, with low though detectable levels during log phase to a transitory peak at the onset of stationary phase. In vivo studies compare phosphorylation levels with the rate of DNA synthesis, effect of metabolic inhibitors, and with the T-7 phage cycle. In vitro, an enzyme activity transfers phosphate from [γ-^{32}P] ATP to thioredoxin. These bacterial extracts also catalyze phosphate transfer from phosphothioredoxin to available acceptors, including nucleotides. Oxidizing agents, including I_2, GSSG, and thioredoxin reductase (+NADP(H)), also catalyze transfer by the mechanism of "oxidative activation of phosphate transfer".

Current research is directed towards elucidating the mechanism of phosphate transfer as a model system and on the in vivo role of thioredoxin phosphorylation.

045 INTERACTION OF THE PHOSPHATASE, ESTERASE AND CO_2 HYDRATASE ACTIVITIES OF MUSCLE CARBONIC ANHYDRASE III. Ernst A. Noltmann and Martha K. Koester, University of California, Riverside, CA 92521.

A low ionic strength extract from rabbit muscle was found to contain a basic protein of unknown function which was subsequently characterized as a third major isoenzyme of carbonic anhydrase (CA), with some unique properties which distinguish it from the erythrocyte CA's I and II. Like the other carbonic anhydrases, CA III is a zinc metalloprotein. It has a molecular weight of 29,000, and posesses extensive amino acid homology with them. It is distinct, however, with respect to its immunochemical properties, its peptide composition, and by virtue of the resistance of its CO_2 hydratase activity to the highly specific CA inhibitor acetazolamide. Also, it is the only CA isoenzyme which hydrolyzes p-nitrophenyl phosphate. This phosphomonoesterase activity has an acidic pH optimum and is competitively inhibited by the general acid phosphatase inhibitors phosphate (K_i = 1.22 x 10^{-3} M), arsenate (K_i = 1.17 x 10^{-3} M), and molybdate (K_i = 1.34 x 10^{-7} M), with these inhibitors having no effect on the CO_2 hydratase or the p-nitrophenyl acetate esterase activities of CA III. The latter activity, although low when compared with those found for CA's I and II, has the sigmoidal pH profile with an inflection at neutral pH typical of CA's for all their substrates, and is inhibitable by acetazolamide to the same degree as CO_2 hydration. In contrast, the acid phosphatase-like activity of CA III is inhibited only slightly by acetazolamide. This evidence suggests that the phosphatase proceeds by a mechanism different from that of the other two activities.

046 PROTEIN MODULATION AND RECOGNITION OF MODIFIED COLLAGEN SURFACES IN VASCULAR GRAFTS
Philip N. Sawyer, Boguslaw Stanczewski, Electrochemical and Biophysical Labs., SUNY, Downstate Medical Center, Brooklyn, New York 11203

Vascular prostheses were prepared by ficin digestion of human umbilical cords or bovine carotid arteries. Each resulting tubular collagen matrix was then modified chemically. With various procedures a net positive, neutral, or negative intimal surface charge was produced. Cross-linking was specific with side chain end terminal amino acids. These modified grafts of glutaraldehyde fixed or fresh arterial segments were implanted into arteries, or veins of mongrel dogs for periods of 1 sec., 15, 30, or 120 min., 24 hr, 3,6, 12, 24, and 36 mos. The grafts were subsequently examined macroscopically, histologically and by scanning electron microscopy for type, presence and degree of thrombus formation. Atypical occlusions resulted when an atypical surface signal from modified collagen precipitated a peculiar thrombus of one protein or cell type, i.e. RBC's, or single leucocyte class. From these studies it was found that by increasing the net negative charge on the pseudo-intimal surface, thrombotic occlusion could be substantially prevented. Incrementally reducing or reversing residual negativity led to an increasingly rapid and often atypical replicative thrombotic event. Other variations included a pure protein thrombus. These results support the hypothesis that the various cellular elements and blood proteins involved in thrombosis respond to specific electrochemical signals from the blood vessels. Blood cells and protein may or may not respond to other abnormal collagen surface modifications in thrombus formation of atypical type.

047 HORMONAL REGULATION OF HEPATIC ACETYP-CoA CARBOXYLASE, Lee A. Witters, Doreen Moriarity, Joseph Avruch and Donald B. Martin, Massachusetts General Hospital, Boston, MA 02114

The direct short-term regulation by insulin and glucagon of hepatic fatty acid and sterol biosynthesis and of the activities of the key regulatory enzymes of this cascade has been studied in hepatocytes isolated from fed and fasted/refed rats. Insulin increases and glucagon decreases the rate of fatty acid synthesis, while neither hormone affects sterol synthesis. In both fed and fasted/refed hepatocytes, insulin stimulates, and glucagon inhibits the activity of the acetyl-CoA carboxylase (ACC), while the activity of fatty acid synthetase is unaffected. The demonstration of insulin stimulation of ACC requires cell homogenization at room temperature, short assay times and the avoidance of prior activation of the enzyme by incubation with Mg++ and/or citrate. Insulin-stimulated ACC displays an enhanced sensitivity to the positive allosteric effector, citrate, and a relative insensitivity to the negative allosteric effector, long-chain acyl-CoA, while the glucagon-inhibited enzyme has the opposite kinetic characteristics. Immunoprecipitation studies of ^{32}p-labeled cytoplasm from hepatocytes and adiposytes have demonstrated that the enzyme is covalently phosphorylated within cells. Epinephrine in fat cells and glucagon in hepatocytes stimulate ^{32}p incorporation into the enzyme coincident with enzyme inactivation. Comparison of the kinetic characteristics of the hormonally modified enzyme from the whole cell with that of isolated enzyme incubated in vitro under conditions favoring phosphorylation or dephosphorylation suggests that the insulin-stimulated enzyme is dephosphorylated, and the glucagon-inhibited one is phosphurylated. Hormone regulation of hepatic ACC may involve allosteric modulation and changes in enzyme phosphorlyation

Cyclic Nucleotides

048 PARTIAL CHARACTERIZATION OF RAT BRAIN cAMP BINDING PROTEINS: CELLULAR LOCATION MOLECULAR WEIGHTS, AND NUCLEOTIDE EFFECTS. Patricia B. Hoyer, Bruce W. Culver, and Boyd E. Haley, Dept Biochem, Univ. of Wyoming, Laramie, WY. 82071. Using the cAMP photoaffinity probe [^{32}P]8-N_3cAMP (8-azidoadenosine-3',5'-monophosphate) the cAMP binding proteins of rat brain have been identified. Five different proteins were photolabeled by [^{32}P]8-N_3cAMP. The approximate molecular weights were 127,500; 89,500; 58,000; 54,000; and 48,000 ± 5% as determined by SDS-PAG electrophoresis. Cell fractionation procedures showed that the 89,500 MW protein (protein B) was found only in the soluble fraction and that the 127,500 MW protein (protein A) was found only in the particulate fraction. Proteins A and B are of unknown function. The three smaller proteins in order of decreasing MW have been tentatively identified as R_{II}-phosphorylated (R_{II}-P), R_{II} and R_I where R is the regulatory protein of type II and type I protein kinases. R_I, R_{II} and R_{II}-P were found in both fractions. R_I saturates with 8-N_3cAMP (at ≈90nm) before R_{II} and R_{II}-P which saturate at approximately the same concentrations (at ≈150nm). Addition of cAMP protects all 5 proteins from photoincorporating 8-N_3cAMP. MgATP(300μM) decreased photoincorporation in protein A by ≈15%, protein B by 100%, R_{II} by 40% and R_I by 60%. MgATP had very little if any effect on R_{II}-P but definitely decreased photoincorporation into the total R_{II} and R_{II}-P subunits. Dibutryl-cAMP (150μM) did not protect against photoincorporation in any of the bands. The data suggest that:(1)cAMP binding by both types I and II protein kinases is under the regulation of MgATP,(2)that cAMP probably exerts its effects through additional proteins other than types I and II protein kinases,(3)and that the effects of Dibutryl cAMP do not appear to be mediated by its ability to mimic cAMP binding to types I and II protein kinases.

049 KINETIC EVIDENCE FOR GUANYL NUCLEOTIDE REGULATION OF ADENYLYL CYCLASE (AC) BY MODULATION OF STEADY STATE LEVELS OF AN ACTIVE STATE WITHOUT INVOLVEMENT OF COVALENT ENZYME INTERCONVERSION, R. Iyengar, T.L. Swartz, J. Abramowitz and L. Birnbaumer, Department of Cell Biology, Baylor College of Medicine, Houston TX 77030.
The non-hydrolyzable analogs of GTP, guanyl-5'-yl-imidodiphosphate and guanosine-5'-O-(3-thiotriphosphate) activated the liver plasma membrane AC system with characteristic lags. Cholera toxin (CT) treatment did not alter the rate of activation of AC by these analogs. GTP activated AC much less than the analogs and without a measurable lag. After CT treatment GTP activated AC as well as its analogs, still without a lag. Competitive reversal of analog mediated activation by excess GTP showed that approach to steady state activation by analogs is associated with a decrease in the degree and rate of reversal. Data show: (1) AC is an hysteretic enzyme with slow time transients for non-hydrolyzable GTP analogs, and (2) rates of activation of AC by nucleotide depend on the nature of the nucleotide used, are unaffected by CT and hence independent of the "GTPase" activity of the system. The results were analyzed using mathematical modeling of AC as a system in which guanyl nucleotides stabilize an active form having an ATP to cAMP cyclizing activity and a CT inhibitable GTPase activity. The data support the view: (1) AC may exist in only 2 states: an inactive (E°) state and an active (E') state, with E° being the preferred conformation in the absence of allosteric ligand (L); and (2) the degree of activation of AC by nucleotide depends both on the rate of isomerization of a liganded inactive state (EL°) to a liganded active state (EL') [rapid with GTP, slow with analogs], and on rates of ligand dissociation [slow for GTP and analogs], and degradation [rapid for GTP, zero for analogs] to give E', which then isomerizes back to E°. Supported by AM-19318 & HL-19423.

050 ALTERATIONS IN POLYAMINE BIOSYNTHESIS AND CYCLIC AMP-DEPENDENT PROTEIN KINASE DURING HYPERTROPHY AND HYPERPLASIA OF THE THYROID, Wendell L. Combest, Robert B. Chiasson and Diane Haddock Russell, University of Arizona Health Sciences Center, Tucson, AZ 85724.
A close temporal correlation has been established between cAMP-dependent protein kinase (cAMP-PK) activation and ornithine decarboxylase (ODC) induction in a variety of growth systems. We studied alterations in polyamine biosynthesis, cAMP-PK activity ratio, and cAMP-PK isozyme patterns during hypertrophy and hyperplasia of the thyroid. After 14 days of goitrogen treatment (propylthiouracil [PTU] in drinking water), the thyroid of rats increased to 284% of control wet weight, 226% of dry weight and 250% of control DNA content with each group containing at least 10 rats. The cAMP-PK activity ratio (-cAMP/+cAMP) showed a biphasic pattern during the 2-week thyroid growth period in rats with maxima at day 1 (132% of controls) and day 6 (148% of controls). ODC, the initial enzyme in polyamine biosynthesis, showed a similar biphasic pattern with a 6- to 7-fold elevation in activity at 2-3 days and a 4-fold elevation at 6 days. S-adenosylmethionine decarboxylase, the enzyme which catalyzes spermidine synthesis, was elevated 4-fold at 9 days of treatment. The thyroid in both chickens and rats had about 10% type I isozyme and 90% type II isozyme of cAMP-PK. The specific activity of both type I and type II isozymes increased to 150% of controls within 4 days in rats. Only type I isozyme increased in chickens to 197% of controls within 7 days. Type I isozyme remained increased above control levels throughout the 14 days of PTU treatment in rats with a maximal specific activity of 180% of controls at 9 days. Total supernatant cAMP-PK (+cAMP) activity increased to a value 171% of controls within 14 days. Changes in cAMP-PK activity ratio as well as isozyme ratios underline the complexity of a cAMP-mediated response.

051 COVALENT MODIFICATION OF THE NUCLEOTIDE BINDING SITE OF THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE USING p-FLUOROSULFONYL-(^{14}C)-BENZOYL-5'-ADENOSINE, Mark J. Zoller and Susan S. Taylor, University of California, San Diego, La Jolla, CA 92093
Treatment of the catalytic subunit of cAMP-dependent protein kinase II from porcine skeletal muscle with p-fluorosulfonyl-(^{14}C)-benzoyl-5'-adenosine (FSBA) resulted in 100% inhibition of ATP:phosphotransferase activity. Mg-Adenosine 5'-triphosphate protected against this inhibition. Approximately 0.97 moles of FSBA were incorporated per mole of catalytic subunit. Following tryptic digestion of the covalently modified protein, a single covalently labeled peptide was isolated. This peptide was purified according to a modification of the procedure of Esch and Allison (J. Biol. Chem. (1978) 253, 6100), and its amino acid sequence is presently being elucidated.

052 THE RELATIONSHIP DURING GROWTH BETWEEN CHANGES IN CELL SIZE, CAMP-DEPENDENT PROTEIN KINASES AND THE POLYAMINE BIOSYNTHETIC PATHWAY, David J.M. Fuller and Eugene W. Gerner, University of Arizona Health Sciences Center, Department of Radiology, Radiation Oncology Division, Tucson, AZ 85724

The use of mitotic selection to study cell cycle parameters often has the disadvantage of temporal dispersion and the use of inhibitors. This study has employed centrifugal elutriation of exponentially growing Chinese hamster ovary fibroblasts, enabling the study of late S and G_2 phase phenomena without having to use metabolic inhibitors. Flow microfluorometry and cell-size distribution analysis were used to identify the populations of cells examined. The cyclic AMP-dependent protein kinase type I isozyme pool was shown to correlate with cell size and protein content. The type II pool, in contrast, showed a biphasic distribution, peaking first in fractions corresponding to late G_1/early S phase cells, confirming data derived from mitotic selection, and rising again to highest levels in G_2 phase. Ornithine decarboxylase (ODC) activity rose steadily from G_1 to G_2 phase, increasing by a factor of 4 over this interval. The ratio of ODC to protein kinase type I activity suggested that the increase was in late S to G_2 phase in agreement with other studies. S-adenosyl methionine decarboxylase activity displayed a similar pattern to ODC. The high enzyme activities in late S/G_2 phase cells were matched by increased polyamine levels in those fractions. In summary, polyamine biosynthesis and cAMP-dependent protein kinase type I correlate with cell size, whereas the type II isozyme displays a specific pattern of activity, peaking in fractions containing late G_1/early S phase cells and again in fractions containing G_2 cells.

053 CYCLIC AMP DEPENDENT PROTEIN KINASES AND THE INDUCTION OF ODC DURING AND AFTER A REVERSIBLE BLOCK OF DNA SYNTHESIS, Anne E. Cress and Eugene W. Gerner, University of Arizona, Tucson, AZ 85724

Previous studies have shown a temporal map of late G_1 phase consisting in part of an increase in amount and activity of cyclic AMP dependent protein kinase type II, (PK-II) and an increase in ornithine decarboxylase (ODC) activity just prior to S phase. If G_1 traversing CHO cells are treated with 2 mM hydroxyurea (HU) for 13 hours, only 1-5% of the cellular DNA is allowed to replicate. This treatment results in a reversible, noncytotoxic block of cell progression through S phase. When bulk DNA synthesis is prevented using HU, PK-II, as measured by DEAE column chromotography, increases four fold and remains elevated while the activation ratio fluctuates similar to control cultures. When HU is removed, and 20% of the cellular DNA has been replicated, PK-II decreases in amount, concurrent with an increase in the activation ratio. These data suggest that PK-II accumulates in the inactive holoenzyme form during a block of DNA synthesis and that as DNA synthesis resumes, the activation ratio increases primarily due to changes in PK-II. ODC activity is reduced by 60% during the block of DNA synthesis and remains low after HU is removed. After 50% of the cellular DNA is replicated, ODC activity is observed to rise, corresponding to two hours after the increase in the protein kinase activation ratio. These data suggest that in this system, ODC activity is important only for the continuation of S phase and that the increase in the activity of protein kinases is necessary but not sufficient for the induction of ODC.

054 LYSINE VASOPRESSIN, ADENOSINE AND SERUM STIMULATE INTRACELLULAR cAMP LEVELS OF FETAL RAT CHONDROCYTES IN MONOLAYER CULTURE. R.Paul Miller & Susan Lohin, St. Mary's Hospital & University of Rochester School of Medicine & Dentistry, Rochester, New York 14611

Evidence has accumulated that intracellular cyclic 3',5' adenosine monophosphate (cAMP) levels are involved in cellular replication. Basal intracellular cAMP was higher in chondrocyte monolayers developed to early exponential growth than in cultures developed to late exponential growth where cell division was attenuated by the density of cells. Lysine vasopressin (LVP) and serum have been shown to stimulate cell division of chondrocytes grown in monolayer culture. The effects of LVP on intracellular cAMP of chondrocytes during the first 10 minutes of hormone stimulation was examined and contrasted to the effects of serum and adenosine. In serum free medium 0.1 to 0.5 ng/ml of LVP failed to influence cAMP while 100 ng/ml of the hormone within 2 minutes significantly elevated intracellular cAMP. This response was dissipated by 10 minutes. In the presence of spent growth medium, which supports viability but fails to stimulate cell division, LVP concentrations as low as 0.1 ng/ml stimulated a rise of cAMP over the first 2 minutes and enhanced cell division. Addition of fresh serum (final concentration=11 ml/dl) had a similar effect but intracellular cAMP was invariably lower than that generated by 100 ng/ml of LVP. Adenosine caused a rapid and more sustained rise of cAMP but failed to enhance cell division. No direct relationship between the rise of cAMP and the enhancement of cell division was noted. With these additives we failed to demonstrate an acute drop in cAMP which is believed to be the initial intracellular signal for mitosis.

055 HORMONE SPECIFIC KIDNEY PROTEIN PHOSPHORYLATION, J.A. Near, F.C. Szoka, M. A. Acara and M.J. Ettinger, Dept. Biochemistry, SUNY-Buffalo, Buffalo, N.Y. 14214.
Electrophoretic patterns exhibit cAMP-dependent protein phosphorylations in porcine renal subcellular fractions which are both subcellular-fraction and kidney-region specific (J. Biol. Chem. 253, 6536, 1978). These experiments have now been extended to tissue-slice and perfused-organ systems. Parathyroid hormone (PTH)-specific phosphorylations are observed in both cytosol and membrane fractions from rat kidney slices. After labeling intracellular ATP pools with ^{32}P-orthophosphate, subcellular fractions are isolated under conditions designed to inhibit protein kinase and phosphoprotein phosphatase activities. PTH treatment leads to enhanced phosphorylation of at least three membrane components with molecular weights in the 70,000-90,000 dalton range, and a 57,000 dalton component in cytosol. Analogous studies using isolated perfused rat kidney pre-labeled with ^{32}P-orthophosphate yield protein phosphorylation patterns similar to those observed in tissue slices. Increases in urine pH and phosphate excretion are also observed in response to PTH treatment, confirming the presence of a physiological response to the hormone. These results are quite similar to the porcine subcellular fraction results, but fewer cytosolic components are detected in slices and perfused kidney. Antidiuretic hormone mediated specific phosphorylations remain difficult to detect in these systems.
Supported by NIH - AM-17911.

056 STUDIES ON THE PHOSPHORYLATION OF PHOSPHOFRUCTOKINASE AND FRUCTOSE BISPHOSPHATASE BY THE CATALYTIC SUBUNIT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE, Frank Marcus, M.Marlene Hosey, Patricio Riquelme and Robert G. Kemp. Department of Biochemistry, University of Health Sciences/The Chicago Medical School, Chicago, IL.
Physiological concentrations (0.2 to 1.0 μM) of bovine heart or liver catalytic subunit of cyclic AMP-dependent protein kinase catalyze the phosphorylation of rabbit skeletal muscle phosphofructokinase (isozyme A), liver phosphofructokinase (isozyme B), and the C isozyme found in brain. The reaction with muscle phosphofructokinase is inhibited by the specific inhibition of protein kinase and proceeds at about 2% the rate observed with phosphorylase kinase but more rapidly than with rat liver fructose bisphosphatase as substrate. Maximum extent of incorporation (0.43 to 0.85 moles per mole of protomer) plus the covalently-bound phosphate present in the isolated muscle enzyme (0.20 to 0.34 moles per mole) approaches one mole per mole. Phosphorylation of either muscle or liver phosphofructokinase does not change the maximum activity measured at high pH or ATP inhibition and the affinity of the enzyme for fructose-6-P at neutral pH. In the presence of concentrations of protein kinase indicated above the stoichiometric phosphorylation of rat liver fructose bisphosphatase was observed in confirmation of the results of Riou *et al.* (PNAS 74: 4615). On the other hand, neither mouse liver nor pig kidney fructose bisphosphatase was phosphorylated under identical conditions.
(This research was supported by U.S. Public Health Service Grants AM 19912 and AM 21167.)

057 PURIFICATION AND CHARACTERIZATION OF CYCLIC AMP PHOSPHODIESTERASE FROM DICTYOSTELIUM DISCOIDEUM, Richard Kessin, Seth Orlow, Renée Shapiro, and Forrest Spencer, The Biological Laboratories, Harvard University, Cambridge, Mass. 02138.
An extracellular and a membrane bound cyclic AMP phosphodiesterase are produced during the growth and aggregation phases of the life cycle of the cellular slime mold D. discoideum. Gerisch and his colleagues have shown that the activity of the extracellular cAMP phosphodiesterase is masked by the presence of a protein inhibitor. We find that by treating this enzyme-inhibitor complex with dithiothreitol the enzyme activity can be stimulated 50-100 fold. This is because the sulfhydryl reagents permanently inactivate and presumably remove the inhibitor. In cell suspensions treated with pulses of cyclic AMP, the production of the inhibitor is reduced and in this case, very little further activation by dithiothreitol is observed. The use of dithiothreitol allows the production of the enzyme to be measured directly and thus simplifies the study of its regulation during development. Preparations treated with DTT were used for purification of the cyclic AMP phosphodiesterase. After ammonium sulfate fractionation and concanavalin A affinity chromatography, the enzyme was subjected to preparative isoelectric focusing. The major peak of activity had an isoelectric point of 4.2. A minor peak, containing 7% of the total activity, had a pI of 3.8. The enzyme in the major peak has been purified 40-50 fold and constitutes the majority of the protein remaining. The widely varying Km's reported for this enzyme are explained by a model relating the synthesis of the cAMP phosphodiesterase and its inhibitor.

058 MECHANISM OF ACTIVATION OF PROTEIN KINASE BY cAMP, Builder, S.E., Beavo, J.A., Krebs, E.G., Department of Pharmacology, University of Washington, Seattle, WA 98195.
The general (overall) scheme by which cAMP activates protein kinase has been known for several years. However, the mechanism of this activation has not been firmly established, although there have been several proposals. The current study shows that activation occurs almost exclusively via a pathway involving formation of an RCcAMP ternary complex, followed by dissociation of the complex to RcAMP and C. Both Peak 1 and Peak 2 isozymes from bovine skeletal muscle and heart respectively follow the same mechanism. We have determined the kinetic constants for the interaction of the regulatory (R) and catalytic (C) subunits of protein kinase at 4°C, namely $RC \rightleftharpoons R+C$. They are, for Peak 1 and Peak 2 respectively, K_D (nM) 0.6, 0.1; k_{-1} (10^{-4} sec^{-1}) 24, 6; k_{+1} (10^6 M^{-1} sec^{-1}) 3, 6. From these data, it is clear that activation in the presence of cAMP, which occurs in less than 2 seconds, cannot be accounted for by the slow dissociation of the subunits followed by cAMP binding to R. During characterization of the R used in these studies it was found that 2 moles of cAMP are bound per mole of R subunit, in agreement with the work of Corbin et al. (JBC 253, 3997-4003, 1978). We have observed that when equilibrium methods such as ethenocAMP fluorescence quenching and equilibrium dialysis are used, 2 moles of cAMP per mole of R subunit are bound. But when non-equilibrium methods are used such as millipore filtration and optical absorbance following dialysis then less than 2 but significantly more than 1 mole per mole is found. Protein concentration has been determined by AA analysis, Lowry, Biuret, Bradford and A280. Both isozymes have the same saturation stoichiometry.

059 INTERACTIONS BETWEEN NON-COVALENT AND COVALENT CONTROL OF CARDIAC MYOFIBRILS. R.J. Solaro, M.J. Holroyde and E. Howe, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267.

Contractile activity of cardiac myofibrils is triggered by non-covalent adsorption of Ca to the thin filament receptor, troponin C. We have evidence that this action of Ca is modified by the state of covalent phosphorylation of troponin I, the inhibitory component of the troponin complex. Phosphorylation of myofibrillar troponin I (TnI) by 3',5' cAMP dependent protein kinase caused a rightward shift of the relation between free Ca and myofibrillar ATPase, while maximum myofibrillar ATPase activity was unaffected. Our data show that addition of 1 mole P/mole of myofibrillar troponin I shifts the free Ca required to half-maximally activate myofibrils from 0.2 μM to 1.0 μM. The mechanism responsible for this shift in calcium sensitivity is an inhibition of myofibrillar Ca binding. We found that cardiac myofibrils bound less Ca over the range of activating free Ca concentrations when TnI was fully phosphorylated. In previous work (Solaro et al. Nature 262:615, 1976) it was shown that 1 mole P/mole is covalently attached to troponin I in response to perfusion of rabbit hearts with epinephrine. Moreover, the site of phosphorylation was shown to be in a region of 26 extra amino acids unique to cardiac troponin I and not present in other types of TnI. Myofibrils extracted from hearts perfused with epinephrine had a reduced sensitivity to calcium when compared to myofibrils extracted from control hearts. We propose that phosphorylation of myofibrillar troponin is responsible, in part, for the increased rate of cardiac relaxation during the inotropic response. Supported grants from the NIH and AHA.

060 INTRACELLULAR MOVEMENT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE. Craig V. Byus and William H. Fletcher, University of California, Riverside, CA 92521.
The cellular location of the free catalytic subunit of cAMP dependent protein kinase was determined using a specific fluorescinated probe. The heat stable inhibitor of the catalytic subunit was purified to homogeneity and reacted with fluorescein isothiocyanate. This fluorescinated inhibitor (FI) retained its high affinity for the catalytic subunit in vitro, bound to the catalytic subunits of fixed cells stoichiometrically and did not bind nonspecifically to cell proteins other than the catalytic subunit. Using this staining procedure we were able to determine the cellular location of the catalytic subunit freed by the activation and dissociation of the cyclic AMP dependent protein kinase holoenzyme. Reuber H-35 rat hepatoma cells grown in monolayer were incubated in culture medium containing 0.1 mM DBcAMP (stimulated) or in culture medium alone (unstimulated) for 5 min. to 4 hours. Following incubation cells were rapidly fixed in acetone. Unstimulated cells stained with FI did occasionally exhibit cytoplasmic fluoresence, indicating some intrinsic activation of the protein kinase. 15 min. incubation with DBcAMP resulted in the activation of protein kinase in ca. 20% of the cells while after one hour incubation ca. 50% of the cells were extremely fluorescent. At both these times fluoresence was confined to the nucleolus and to the cytoplasm: small, spherical cytoplasmic organelles staining even more intensely than cytoplasm in general. Following 2 hours of DBcAMP incubation ca. 75% of the cells showed intense fluoresence. At this time nucleoplasmic staining became evident though it was less intense than that of the nucleolus and the cytoplasm. By 4 hours cytoplasmic staining, including that of organelles, was diminished and only the nucleus was brightly fluorescent. These observations suggest that cAMP-dependent protein kinase is first activated within the cytoplasm and then translocated to the nucleus, perhaps with the help of a yet to be identified organelle.

Modulation of Protein Synthesis and Degradation

061 HSV PHOSPHORYLATED PROTEINS. AN INVESTIGATION OF THEIR STABILITY, CELLULAR LOCATION, AND DNA BINDING AFFINITY - Kent Wilcox, Alex Kohn, Elena Sklyanskaya, and Bernard Roizman. University of Chicago, Chicago, Il 60637.
Phosphorylated species of Herpes Simplex Virus infected cell proteins were identified by electrophoresis through polyacrylamide gels (PAG) of extracts from cells labeled with ^{35}S methionine and ^{32}P. Extracts from cells fractionated into cytoplasmic and nuclear components revealed that although many of the nuclear proteins were highly phosphorylated, certain phosphoproteins remained largely in the cytoplasm while some nonphosphorylated species were transported to the nucleus. Pulse-chase studies indicated that the phosphate moiety on virus-specific polypeptides had a relatively low turnover rate in comparison to certain host phosphoproteins. Analysis by chromatography on duplex DNA-cellulose yielded two populations of virus-specific proteins, namely the Nonbound proteins, which passed straight through the column, and the Bound proteins which were eluted with 2M NaCl. In general, the Nonbound fraction was enriched for phosphoproteins. Addition of heparin to the column buffers further diminished the DNA affinity of phosphoproteins, whereas the addition of spermine resulted in no change from the control. The DNA-affinity of specific HSV phosphoproteins was determined by co-chromatography of ^{35}S and ^{32}P-labeled polypeptides on DNA-cellulose, followed by electrophoresis on PAG of the proteins in the Nonbound and Bound fractions. Quantitation of the ^{35}S and ^{32}P radioactivity in individual virus-specific polypeptides indicated that in most cases the phosphorylated forms were enriched in the Nonbound fraction.

062 PARTIAL PURIFICATION AND CHARACTERIZATION OF INITIATION FACTOR eIF-2 FROM MUSCLE, Jeanne B. Li, Robert H. Sahms, D. Eugene Rannels, Howard E. Morgan, and Leonard S. Jefferson, The Pennsylvania State University, College of Medicine, Hershey, PA 17033
Regulation of protein synthesis in rat cardiac and skeletal muscle by insulin and nutrients involves alterations in the rate of peptide-chain initiation. The proposed first step in initiation is the formation of a ternary complex of met-tRNA$_f$, GTP and eIF-2. The activity of eIF-2 in muscle, assayed by binding of [^{35}S]met-tRNA to nitrocellulose filters in the presence of GTP and postribosomal supernatant, fell in association with a drop in protein synthesis in insulin-deficient animals. Since eIF-2 may be involved in the regulation of initiation in muscle, purification of this factor from bovine heart was begun. Muscle was homogenized in buffer containing 250 mM KCl to maximize eIF-2 recovery. eIF-2 activity was precipitated from the postribosomal supernatant with 50% saturated ammonium sulfate. Chromatography of this fraction on heparin-Sepharose, DEAE-cellulose, hydroxylapatite, and phosphocellulose resulted in a 220-fold increase in eIF-2 specific activity over that in the postribosomal supernatant. Polyacrylamide gel electrophoresis (in SDS and urea) of the peak fraction from phosphocellulose yielded three major bands with molecular weights of 62,500, 53,500, 39,000 daltons. These corresponded closely to the molecular weights of the subunits of rabbit reticulocyte eIF-2 and constituted 30% of the total protein in the fraction. The molecular weight of eIF-2 determined by chromatography on Sephadex G-200 was 150,000. In hemin-deficient reticulocyte lysates, where protein synthesis was inhibited, the addition of eIF-2 from muscle partially restored synthesis. Thus eIF-2 from muscle shares some characteristics with eIF-2 from reticulocytes. (Supported by NIH grants HL-20388, HL-00294, and AM-15658.)

063 REGULATION OF PROTEIN BIOSYNTHESIS BY GLUCOCORTICOID HORMONES AND ENERGY-PROVIDING SUBSTRATES MEDIATED BY AN INFLUENCE RELATED TO THE ENERGY CHARGE, ACTING AT THE LEVEL OF INITIATION. Donald A. Young, Wm. A. Guyette, Stephen L. Mendelsohn, Steven K. Nordeen, Bruce P. Voris and Robert T. Lyons, Univ. of Rochester, Rochester, N.Y. 14642.
In these studies we continue to explore the relationships between influences on cellular energy metabolism and rates of protein biosynthesis in normal mammalian cells that are responsive to physiologically relevant controls. Depriving thymic lymphocytes of energy-providing substrates for 2 hrs results in a 5% drop in the adenylate energy charge, along with 80% decline in protein-synthetic activity. Glucose prevents both changes, or when added later rapidly (within 5 min) restores them. A variety of other manipulations that include marginal substrates, oxygen deprivation, rotenone, and hormones, establish a consistent relationship between small changes in the adenylate energy charge and large changes in the rates of protein biosynthesis. Examination (via 2-D gel electrophoresis) reveals that the synthetic rates for about 500 individual proteins change roughly in parallel; however, some distinct exceptions are also noted. The rapid redistribution of the numbers of initiated ribosomes and nascent peptide chains that accompany small changes in AMP suggests that mechanisms responsive to the latter exert regulation at the level of the initiation action. Within this context, the classical overall suppressive actions of adrenal glucocorticoid hormones are consistent with their known actions on energy metabolism (cf Young, J. Biol. Chem. 244:2210, 1969; and Nordeen and Young, J. Biol. Chem. 251:7295, 1976). Supported by NIH Grants 5 T32 GM 07136, 5 F32 CA-5381 and AM 16177 and an award from the United Cancer Council of Monroe County.

064 CONTROL OF PROTEIN SYNTHESIS BY HEMIN, Martin Gross, The University of Chicago, Dept. of Pathology, Chicago, Illinois 60637.

The control of protein synthesis by hemin in rabbit reticulocyte lysates is mediated by the formation of a high molecular weight protein inhibitor of polypeptide chain initiation termed the hemin-controlled translational repressor (HCR), which acts by phosphorylating the initiation factor (eIF-2) responsible for binding Met-tRNA$_f$ to 40 s ribosomal subunits. We have studied the effect of HCR on the binding of [^{35}S]Met-tRNA$_f$ to ribosomal components isolated from reticulocyte lysate on Sepharose 6B. Following a 1 minute lag, HCR reduced the rate of formation of 80 s initiation complexes to about 40% of the control, and, when the formation of 80 s complexes was blocked by edeine or Guanosine 5'-(β,γ-methylene) triphosphate (GDPCP), HCR slowed the rate of binding of Met-tRNA$_f$ to 40 s subunits to about 35% of the control. When Met-tRNA$_f$ was allowed to accumulate on 40 s subunits in the presence of GDPCP and then allowed to shift to 80 s complexes by adding excess GTP, HCR had no direct inhibitory effect on this shift reaction. In addition, no deacylation of Met-tRNA$_f$ due to HCR could be detected in this system. These results indicate that HCR inhibits the binding of Met-tRNA$_f$ to 40 s subunits, isolated by gel-filtration, directly. In contrast, other studies have revealed that HCR has a greater and more complex inhibitory effect in the lysate, where it not only inhibits binding of Met-tRNA$_f$ to 40 s subunits but also causes accumulation of a 48 s initiation complex, containing a 40 s ribosomal subunit, tRNA$_f^{Met}$ (that is largely deacylated), and mRNA.

065 PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6 DURING TRANSITION OF QUIESCENT CELLS INTO G$_1$. George Thomas, Michel Siegmann and Julian Gordon, Friedrich Miescher-Institut, P.O.Box 273, CH-4002 Basel, Switzerland

One of the earliest events affected by the serum-induced transition of quiescent animal cells into G$_1$ phase of the cell cycle is a rapid increase in protein synthesis. We have been investigating whether the phosphorylation of ribosomal proteins may serve as a regulatory signal for this event. The results show that within minutes of induction there is a large increase in the amount of labeled phosphate incorporated into 40S ribosomal protein S6. This change is not accounted for by changes in phosphate transport or specific activities of phosphate pools, but to a quantitative increase in the amount of phosphate incorporated into S6 as measured by its complete electrophoretic shift on two-dimensional polyacrylamide gels. Pulse-chase experiments show that as cells progress into G$_1$, there is a net increase in S6 phosphorylation with no turnover, and that the S6-phosphate-donor must be in a separate compartment from that of total cellular ATP. Cycloheximide does not block S6 phosphorylation, however, theophylline strongly inhibits S6 phosphorylation and in turn polysome assembly. In addition, serum withdrawal immediately triggers dephosphorylation and disaggregation of polysomes. All the results in this system show a negative correlation between S6 phosphorylation and cAMP levels and a strong correlation between S6 phosphorylation and increased translation.

066 PHOSPHORYLATION OF 40S RIBOSOMAL SUBUNITS BY cAMP-DEPENDENT AND PROTEASE-ACTIVATED PROTEIN KINASES, R. Del Grande & J. A. Traugh, Univ. of Calif., Riverside, CA 92521

The phosphorylation of ribosomes has been described in a variety of eukaryotic tissues. Numerous studies have shown that one ribosomal protein, known as S13 (M_r 32,500) in rabbit reticulocytes or S6 in rat liver, is the major phosphate acceptor. This protein exists in multiple phosphorylated states and may contain as many as five phosphates. The phosphorylation of reticulocyte ribosomes has been studied *in vitro* in an attempt to reconstitute the characteristics of ribosome phosphorylation observed *in vivo*. Purified 40S ribosomal subunits contain endogenous phosphoryl groups. Under optimal conditions, the cAMP-dependent protein kinases incorporate one to two phosphates into S13. Following phosphorylation with the cAMP-dependent protein kinase, the total number of phosphates per S13 was found to be two to three. 40S ribosomal subunits are also phosphorylated by two cAMP-independent protein kinases that are activated by proteolysis. These protease-activated kinases (PAK), designated PAK I and PAK II according to their elution from DEAE-cellulose, utilize only ATP to differentially phosphorylate two 40S ribosomal proteins. PAK I incorporates up to one phosphate into 40S ribosomal protein S15 (M_r 17,200). Conditions necessary to activate this protein kinase *in vivo* are being investigated. PAK II phosphorylates S13, the same ribosomal protein phosphorylated by the cAMP-dependent protein kinases. Since two to three phosphate groups on S13 may be attributed to the cAMP-dependent protein kinases, PAK II may be involved in the maximal phosphorylation of S13 observed *in vivo*. Supported by USPHS Grant GM 21424.

067 PHOSPHORYLATION OF TRANSLATIONAL INITIATION FACTORS FROM RABBIT RETICULOCYTES BY TWO PROTEOLYTICALLY ACTIVATED PROTEIN KINASES, Stanley M. Tahara and Jolinda A. Traugh, University of California, Riverside, CA 92521

Two cyclic nucleotide-independent protein kinases have been isolated from the post-ribosomal supernate of rabbit reticulocyte lysates in a catalytically inactive "proenzyme" form; conversion of the enzymes to the active state requires mild proteolysis. At pH 8.5 the two protein kinases are eluted at 0.05 and 0.1 M NaCl from DEAE-cellulose; from this elution order the enzymes have been designated Proteolytically Activated Kinase (PAK) I and II respectively. PAK I and II preferentially phosphorylate basic proteins such as histones and ribosomal proteins and use only ATP in the phosphotransferase reaction. They differ from the cAMP-dependent protein kinases and casein kinases based on a lack of Ca^{2+} stimulation by cAMP (cGMP, cCMP or cUMP), a difference in substrate specificity and a high Mg^{2+} requirement (40-50 mM) when histone is used as substrate. As a further criterion, PAK I and II are not inhibited by the heat-stable inhibitor protein of the cAMP-dependent enzymes. PAK I specifically phosphorylates the 130,000 dalton subunit of eIF-3 and eIF-4B; PAK II phosphorylates the 53,000 dalton subunit of eIF-2. Phosphorylation of these initiation factors has been observed in vivo; thus, the phosphorylation observed in vitro may be physiologically relevant. Although PAK I and II can be activated with trypsin, an endogenous Ca^{2+}-stimulated protease has been partially purified from the post-ribosomal supernate of reticulocytes which specifically activates PAK II. The role of this Ca^{2+}-mediated process in the phosphorylation of initiation factors is currently under investigation. (Supported by USPHS grant GM-21424).

068 PURIFICATION AND PROPERTIES OF A TRANSLATIONAL INHIBITOR FROM WHEAT GERM, Walden K. Roberts and Thomas S. Stewart, The University of Colorado Medical Center, Denver, Colorado 80262.

A protein from wheat germ has been purified more than 400-fold to apparent homogeneity. This protein efficiently inhibits translation in cell-free extracts from animal cells, but not in homologous wheat germ extracts. The wheat germ inhibitor is effective at an inhibitor to ribosome ratio of 1:100, suggesting an enzymic mechanism of action.

The translation of endogenous mRNA, exogenous mRNA, and polyuridylic acid are all inhibited by the wheat germ protein. Inhibition is at the level of polypeptide chain elongation, with the mRNA becoming "frozen" on the polyribosomes. The inhibitor does not affect mRNA stability nor the aminoacylation of tRNA.

An interesting feature of the inhibition reaction is that it requires, in addition to the wheat germ inhibitor, both ATP and tRNA. The functions of ATP and tRNA in the reaction are not known at the present time. However, ATP can be replaced in the reaction by relatively high concentrations of the analog AMP-PCP, suggesting that this is not another example of a protein-kinase mediated inhibition.

069 STEROID INDUCTION OF THE SYNTHESIS OF SPECIFIC PROTEINS IN BRAIN AND PITUITARY, Margery C. Beinfeld and Paul M. Packman, Washington University, St. Louis, 63110

Corticosterone and estrogen are retained in areas of the brain and pituitary. The possibility that the synthesis of specific soluble proteins follows this uptake has been investigated using Sprague-Dawley rats. The experiment was performed by the procedure used by Notides and Gorski to demonstrate an estrogen-induced uterine protein.

In estrogen-treated immature female rats, a protein of about 50,000 daltons is induced in the hypothamus. This protein easily separates from uterine-estrogen induced protein on SDS-polyacrylamide gels. In pituitary of estrogen-treated animals, several high molecular weight proteins are indueed. No estrogen-induced proteins were observed in liver, cortex, or cerebellum.

Corticosterone induced the synthesis of proteins of 24,000 and 70,000 daltons in pituitaries of immature and mature adrenalectomized male rats. No corticosterone-induced proteins were found in hypothalamus, hippocampus, cortex, or cerebellum.

Other features of steroid-induced proteins synthesis in these tissues and their possible significance will be discussed.

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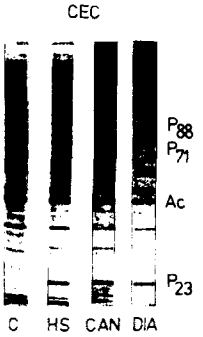
070 PURIFICATION OF TWO ANDROGEN DEPENDENT RAT PROSTATE POLY(A+)-MESSENGER RNAs AND CHARACTERIZATION OF THEIR SYNTHETIC GENES PRODUCED USING REVERSE TRANSCRIPTASE, Stephen E. Harris, John J. Monahan*, Per-Erik Mansson, and Alan B. Silverberg, Laboratory of Environmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709 and Roche Institute for Molecular Biology, Nutley, NJ

Two poly(A+)-messenger RNA (mRNA) have been purified from rat ventral prostate using oligo dT-cellulose chromatography followed by a series of sucrose gradient in either 1% SDS or in 70% formamide. These mRNAs (γ-mRNA and β-mRNA) apparently code for subunits of a larger protein (51,000 daltons) known as prostate binding protein and is under androgen control (1) and constitutes up to 50% of the total protein secreted by the prostate. The γ-mRNA and β-mRNA have sizes of 560 NT and 700 NT respectively, as estimated from sucrose gradients and neutral agarose gels. Full length single stranded complementary DNAs were prepared to both mRNAs using AMV reverse transcriptase. The purified single-stranded ^{32}P-cDNAs were subsequently used in a second reaction with reverse transcriptase to generate double stranded cDNAs to these mRNAs. The material binding to HAP (35-50%) has a Tm of 88°C in 0.14 M PB and after dialysis and reannealing to a Cot of 10^{-3} is 95% resistant to S1 nuclease. The rate constant for the reannealing of the γ-d.S. cDNA and the β-d.S. cDNA are both approximately 1600 (Cot ½ = 6-8 X 10^{-4}) indicating a complexity of 500-700 nucleotide pairs (NTP). We are presently mapping these two synthetic genes with a variety of restriction enzymes and are tailing the two genes with dA's using terminal transferase for subsequent cloning in a modified pBR322 plasmid. 1. Heyns, W., Peeters, B., Mous, J., Rombauts, W., and DeMoore, P. (1978) Eur. J. Biochem. 89, 181-186.

071 ABNORMAL PROTEINS, HEAT SHOCK, AND DIAMIDE INDUCE ENZYMES OF THE γ-GLUTAMYL CYCLE IN EUCARYOTES. Lawrence E. Hightower, University of Connecticut, Storrs, Connecticut 06268



A small number of proteins accumulate dramatically in chicken embryo cells exposed to the arginine analog canavanine (CAN), or the phenylalanine analog fluorophenylalanine. Three of these proteins, P_{88}, P_{71} and P_{23} are shown in the accompanying electropherogram (SDS-PAGE). To elicit the response, the analog must be incorporated into protein. Actinomycin D or cordycepin added with the analogs blocks the enhancement. Thus, abnormal proteins and peptide fragments generated by puromycin induce a select group of proteins which are expressed at much lower levels in untreated cells (C). Diamide (DIA), which alters the redox state of cells mainly by oxidizing glutathione, and heat shock (HS) (Kelly and Schlesinger, Cell, Dec. 1978) are also effective inducers. Preliminary experiments indicate that enzymes of the γ-glutamyl cycle, the metabolic pathway responsible for the synthesis and degradation of glutathione, are among the induced proteins. Like diamide, heat shock and abnormal proteins appear to create a demand for glutathione, triggering a common cellular response, induction of enzymes of the γ-glutamyl cycle. In cultured Drosophila cells, diamide and puromycin induce a subset of the heat shock proteins which are very similar in size to the inducible chick cell proteins. It is likely that enzymes of the γ-glutamyl cycle are among the Drosophila heat shock proteins as well.

072 THE TESTOSTERONE DOMAIN OF SOLUBLE AND MICROSOMAL PROTEINS IN RAT PROSTATE AND SEMINAL VESICLE, Donald B. Carter, Alan B. Silverberg, and Stephen E. Harris, Laboratory of Environmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

Two-dimensional gel electrophoresis has been used to map the pattern of protein synthesis in rat prostate and seminal vesicle from castrate, testosterone stimulated castrates and intact animals. Proteins are detected by autoradiography of S^{35}-methionine labeled proteins. In the prostate soluble fraction, three major proteins having molecular weights less than 25,000 daltons and isoelectric points (I.P.) less than 6.0 are under androgen control. Three 50 to 70 thousand dalton proteins appear in the castrate soluble maps but are not detectable in the testosterone stimulated castrate map. A major androgen dependent protein having a molecular weight of 12,000 daltons and I.P. of 5.4 appears in the soluble fraction as well as the microsomal fraction of prostates from stimulated animals. Using non-equilibrium pH gradient electrophoresis, proteins having isoelectric points greater than 7 were investigated. One major 30,000 dalton protein having I.P. 8 was found to be androgen dependent in prostate. Three major seminal vesicle soluble proteins under androgen control are found in the pH range from 7 to 9. All have molecular weights less than 25,000 daltons. Conversely, the castrate soluble fraction has several basic proteins not detectable in the androgen stimulated castrate. The presence or absence of androgen appears to effectuate the appearance of specific groups of proteins in both tissues. The mRNA for the major low molecular weight proteins of prostate and seminal vesicle is also androgen dependent.