REGULATORY MECHANISMS IN THE CONTROL OF PROTEIN KINASES

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I. INTRODUCTION

Over the quarter century since the first report of a protein kinase in liver by Burnett and Kennedy[1] this type of enzyme (NTP: protein phosphotransferase) has been identified and studied in a wide range of vertebrate and invertebrate cells. It has become clear as a result that the simple transfer of a phosphate moiety from a nucleoside triphosphate to a protein molecule is a powerful posttranslational mechanism which nature has frequently exploited to alter the properties of uniquely situated proteins responsible for any of a large and diverse number of regulatory events in cells. In the last several years, the regulation and function of protein kinases have become important to virologists, immunologists, plant biochemists, neurochemists, and students of cellular growth mechanisms as well as to the biochemists and metabolic endocrinologists whose interest was originally attracted[2] by the involvement of glycogen phosphorylase kinase in the regulation of glycogen metabolism in skeletal muscle. (For review see References 3 to 7).

The reactions catalyzed by protein kinases can be generally represented as indicated in Equation 1:

$$\text{NTP.Mg}^{++} + \text{protein} \rightarrow \text{protein-P} + \text{NDP.Mg}^{++} \qquad (1)$$

In all the systems which have been studied to this time this posttranslational protein modification can be reversed by the action of a phosphoprotein phosphatase[5,8] as indicated in Equation 2:

$$\text{Protein-P}_n + n\text{H}_2\text{O} \rightarrow \text{protein} + n\ \text{P} \qquad (2)$$

The biochemical basis for the wide-ranging ability of protein kinases to dramatically change the activity of key proteins lies in part in the ability of many protein substrates to preserve some of the high energy of the nucleoside triphosphate bond within their structure when phosphate is covalently bound to a specific residue. The free energy of hydrolysis ($\Delta G°$) of the serine-phosphate bond in casein has been calculated to be -6.5 kcal/mol under conditions where the same parameter is only -2.9 kcal/mol for free serine-phosphate[9]. ($\Delta G°$ for ATP = -8.4 kcal/mol). The finding of such a high free energy of hydrolysis of protein bound

phosphate has two clear consequences. First, it is clear that the considerable energy made available upon cleavage of a protein-phosphate bond might be used to bring about significant conformational changes in protein structure. Second, the high free energy of hydrolysis means that the phosphorylation reaction can be limited to circumstances when the appropriate protein kinase is activated and able to catalyze phosphorylation.

This review is concentrated upon a biochemical consideration of the protein kinases which have been purified to homogeneity up to this time. Other important aspects of the control of cellular events by protein phosphorylation-dephosphorylation reactions such as the enzymology of protein phosphatases and the study of protein substrates are not extensively addressed herein, but are the subject of several excellent reviews.[5-8,10-12] The reader is also referred to several recent reviews which deal with individual protein kinases, notably cAMP-dependent protein kinases (cAK),[7,10-13] cGMP-dependent protein kinases,[11,14] and phosphorylase kinase.[6,13] These latter enzymes will be covered insomuch as studies on them have set precedents of terminology and methodology for the study of other protein kinases and in order to highlight properties which appear to be held in common and which may, therefore, enlighten researchers through the existence of common structural features and mechanisms of effector regulation or similar protein substrate specificities. Throughout, we wish to emphasize the importance of comparison of diverse protein kinases since we believe that when nature has discovered a particularly effective biochemical means of regulation it is apt to employ it in multiple and varied systems.

A full understanding of the involvement of any particular protein kinase in the control of cellular events clearly hinges upon a knowledge of the enzymology and biochemistry of the enzyme in question. Protein kinases play a *transducer* role in nature, acting as mediators for effector molecules such as haem, calcium, cAMP, or cGMP which may be involved in the action of hormones such as EGF,[15] catecholamines,[16-19] glucagon,[16,21] vasopressin, angiotensin II,[22] or insulin[23-27] on targeted cellular events. As a result, one can expect to identify within the structure of most protein kinases an *effector binding domain* or subunit and a *catalytic domain* or subunit. The unique structural, thermodynamic, and kinetic properties of these two domains and the *mechanism of action* of one domain upon another in individual protein kinase structures lie at the heart of those regulatory events wherein these enzymes are involved. This review will focus upon these aspects of the protein kinases which have been isolated up until this time.

Accurate biochemical data on protein kinase structure have been possible since the first purification of a protein kinase, phosphorylase *b* kinase, to chemical homogeneity in 1964.[29] Twelve other protein kinases have since been purified to apparent homogeneity. The gross physical and kinetic properties of these proteins are summarized in Table 1. It is clear that protein kinases have a wide range of molecular weights and a variety of different subunit structures. However, it is equally clear that the vast majority prefer ATP rather than GTP, UTP, or ITP as the phosphoryl donor and that all have Kms for ATP which are well below the concentrations of the nucleotide (2 to 6 m*M*) thought to exist in most cells. It is immediately clear, therefore, that little regulation of protein kinase activity can be achieved by mechanisms which simply change the Km for ATP. Other means must be sought and it is towards a better understanding of these means that this review is addressed.

II. cAMP-DEPENDENT PROTEIN KINASE

The protein kinase which has been most extensively characterized is the cAMP-

Table 1
PURIFIED PROTEIN KINASES

Protein kinase	Monomer molecular weights	Subunit structure	Autophosphorylated subunits	Km for ATP
cAMP-dependent type I	49,000 (R) 39—42,000 (C)	R_2C_2	C	3—15 μM
cAMP-dependent type II	54—56,000 (R) 39—42,000 (C)	R_2C_2	R,C	3—15 μM
cGMP-dependent	74—81,000 (E)	E_2	E	10—20 μM
Phosphorylase kinase	118—145,000 (α) 108—128,000 (β) 41—42,000 (γ) 17,000 (δ)	$(\alpha\beta\gamma\delta)_4$	α,β	200—400 μM
Myosin light chain kinase	77—125,000 (M) 17,000 (C)	MC	M	50—300 μM
Hemin-dependent eIF-2α kinase	80—95,000		+	
dsRNA-dependent eIF-2α kinase	67—70,000		+	
Casein kinase I	37—42,000	Monomer	+	13—200 μM
Casein kinase II	42—44,000 (α) 38—40,000 (α') 24—26,000 (β)	$\alpha\alpha'\beta_2$	β	4—10 μM
Viral tyrosine kinase I	60,000		+	
II	120,000		+	
III	85—142,000		+	
EGF-dependent tyrosine kinase	150,000 (E)		E	
Pyruvate dehydrogenase kinase	50,000			
Insect cyclic nucleotide-dependent protein kinase	180,000 (I)	I_2		86 μM
Rhodopsin kinase	50—52,000	Monomer		8 μM

dependent protein kinase. The enzyme has two principal classes of isozymes,[30] named as type I and type II on the basis of their elution from DEAE-cellulose.

The relative distribution of these two isozymes varies from species to species and from tissue to tissue[30] within a wide range of sources from which the enzyme has been isolated. Recently, it has been reported that isozyme II has a neural subclass which is present in bovine cerebral cortex, cerebellum, medulla, mid-brain, brain stem, anterior pituitary, pineal and adrenal medulla, but not in bovine skeletal muscle, liver, kidney, heart, or adrenal cortex.[28] Erlichman et al.[28] have pointed out the great potential value of this subclass of isozyme in developmental, anatomic and pathological studies of the brain. No significant evidence is at present available to challenge the postulate[31] that all the cellular actions of cAMP in mammalian tissues are mediated by the action of these isozymes.

The purification of both isozymes to chemical homogeneity has been achieved and has recently been reviewed in detail by Carlson et al.[13] Biochemical studies on the subunit structure and physical properties of the purified kinase have been fundamental to our understanding of the regulation of cellular events by cAMP. The cAMP-dependent protein kinases which have been studied appear to be tetramers of two regulatory and two catalytic subunits (Table 1). The binding of cAMP to the regulatory subunit (R) causes a change in the affinity of the regulatory dimer for the catalytic subunits (C)[32-34] so profound that they physically dissociate when the holoenzyme is present at cellular concentrations (~0.2 to 0.7 μM). The activation by cAMP can be represented as indicated in Equation 3.[35]

$$R_2C_2 + 4\ cAMP \leftrightarrows R_2 \cdot (cAMP)_4 + 2C \quad (3)$$

The exact affinity of the regulatory dimer for the catalytic subunit has been hard to determine because of the difficulty of purifying regulatory subunit in the absence of cAMP. The nucleotide has an affinity for the regulatory subunit of the enzyme which is dependent upon concentration of protein and which increases as the protein is diluted.[36-38] At cellular kinase concentrations of 0.2 to 0.7 μM the apparent dissociation constant of cAMP from the protein is 10^{-8} M. As a result of this high affinity at dilute concentrations, the nucleotide is exceptionally difficult to remove.[35] In recent years researchers have prepared R subunit by elution with cAMP from affinity columns prepared with 8(6-aminohexyl)-cAMP,[39,40] N^6-(2-aminoethyl)-cAMP,[39,41] or N^6(6-aminohexyl)-cAMP[42]. It is also possible to elute using high (2 to 8 M) concentrations of urea in the eluting buffer.[32,33,43-45]

Although the cAMP-binding properties of the R subunit appear sensitive to this procedure,[45] the ability of R subunit to inhibit the C subunit may be preserved intact after extensive dialysis to remove urea.[32,33] It has also been possible to prepare R subunit devoid of cAMP by elution from these affinity columns with cGMP followed by extensive dialysis which does succeed in removing the nucleotide.[44] The affinity of protein prepared by these procedures for C subunit has been reported to be ~0.2 nM in the absence of cAMP for both types of isozymes in the presence of 150 mM NaCl and 1 mM magnesium.[32] The affinity is decreased 10-fold by phosphorylation and 10^4-fold by the binding of cAMP[46] to a value ($2 \times 10^{-6} M$) which exceeds the estimated intracellular concentration of the R_2C_2 holoenzyme complex (2 to $5 \times 10^{-7} M$) and therefore results in net dissociation. The structural basis for the 10^4-fold lowering in affinity caused by cAMP binding remains obscure.

Care must be taken in determinations of affinity, since the interaction of the effector-binding subunit with the catalytic moiety is affected by salt concentration[29,32] and by the presence of protein substrates,[29,47] basic polypeptides,[29,47,48] or MgATP.[49] Furthermore, the validity of these determinations rests on the assumption that the holoenzyme form is devoid of all catalytic activity and that heterologous protein substrates can only undergo phosphorylation when cAMP is bound.[29]

A. cAMP-Binding Domains

The discovery of two cAMP-binding sites per R subunit monomer[34,35,50] has led to an avid interest in the action of the effector on protein kinase activation. The existence of two sites within one polypeptide chain could allow for intrachain cooperativity for cAMP binding.[51] At present it is not known which site, or sites, is responsible for catalytic subunit activation, although studies pertaining to this question will be described below.

Both of the major isozyme classes contain the two intrachain sites referred to as Sites 1 and 2.[51-53] Although it is clear that the isozymes exhibit differences in cyclic nucleotide analog specificity[54] and MgATP effects on cAMP binding,[55] the effects of analogs on cAMP dissociation behavior suggest that intrachain Sites 1 and 2 of one isozyme are quite similar, and perhaps homologous, to the corresponding sites of the other isozyme.[52] There is compelling evidence to indicate that Sites 1 and 2 are different. When labeled cAMP is removed by exchange with an excess of the unlabeled nucleotide, the presence of certain competing cyclic nucleotide analogs before the addition of cold nucleotide and at levels which inhibit [^{3}H]cAMP binding by more than 50%, alters the relative amount but not the slope of each of the two dissociation components which are observed.[52] Two distinct [^{3}H]cAMP dissociation rates, reflecting approximately equal amounts of each component, can be observed when

purified R subunit is used.[52,53] Certain competing cyclic nucleotide analogs at low concentrations inhibit [^{3}H]cAMP binding approximately 50%, while much higher concentrations of the analogs are required for further inhibition.[43,52,56] It appears, therefore, that analogs have a relative preference for either Site 1 or Site 2.[52]

If the binding of a radiolabeled analog which prefers one of the sites is measured, one would expect that only those unlabeled analogs which prefer the same site would compete well in a binding reaction. This is found to be the case.[45,56] The biphasic quenching of the fluorescence of 1,N^6-etheno-cAMP when bound to R also suggests different intrachain binding sites.[43] The data indicating two different cAMP-binding sites are thus quite convincing. The postulate that these different sites are intrachain rather than interchain assumes that the R dimer contains two identical chains. To date, no separation procedure has resolved two distinct chains of the R dimer with different cAMP-binding properties. Intrachian binding site variability is supported by the fact that variation of regions within a single protein chain occurs much more frequently than variation between homologous chains of a protein dimer. Contiguous duplication of a gene coding for a single binding site, followed by modification and fusion, is one possible evolutionary process which could explain the existence of the two different intrachain sites.

Some preliminary insights into the nature of Sites 1 and 2 have been gained from studies of [^{3}H]cAMP dissociation and from the effects of cyclic nucleotide analogs on [^{3}H]cAMP binding and dissociation.[52] The dissociation rate constant for [^{3}H]cAMP at 25° is approximately 0.025 to 0.050/min for Site 1 and 0.23 to 0.26/min for Site 2. These rate constants are similar, but not identical, for isozymes I and II. If one assumes that both sites have similar association rates, it follows that Site 1 would have a higher affinity for cAMP.

Cyclic nucleotide analogs with C-8 modification of the purine ring have relative preference for Site 1, whereas, those analogs with C-6 modification prefer Site 2 as indicated by the model presented in Figure 1.[52] It is possible that Site 1 binding either requires the amino group at position 6 for hydrogen bonding or other interaction, or a bulky substituent at this position imposes spatial restraints. By the same reasoning, modification at C-8 could restrict binding at Site 2. The analogs could exist in different conformational states (*syn* or *ante*), or different purine ring positional electron densities, which confer site selectivity to them. The possibility that two different cyclic nucleotides or two different forms of cAMP react with Sites 1 and 2 in vivo cannot be ruled out. The existing knowledge of cyclic nucleotide binding to the two intrachain sites and the availability of diverse cyclic nucleotide analogs should allow detailed studies of function and mapping of cyclic nucleotide binding to Sites 1 and 2 to be done in the near future.

That the cAMP-binding domains are located in discrete regions of the primary sequence of R, as indicated in Figure 1, is suggested by the finding that small proteolytic fragments of 10,000 to 16,000 daltons, which retain cAMP-binding activity, can be isolated from both isozymes.[53,57] A 16,000-dalton fragment from type I isozyme resembles Site 2 in its cAMP dissociation behavior, but it cannot be ruled out that it either represents a modified Site 1 or that it contains both sites, at least one of which is modified. A 14,000-dalton fragment from type II isozyme appears, on the basis of its dissociation properties and analog effects on cAMP binding, to be a modified Site 2.[56,57] The latter fragment occurs in the middle portion of the primary sequence of R[56,58] and seems to be separate from the 8-azido-[^{32}P]cAMP affinity-labeled peptide.[56,59] The 8-azido-[^{32}P]cAMP labeling, which might be expected to occur mainly at Site 1, has been shown to be covalently linked to a tyrosine residue and very close to one of the phosphorylation sites near the carboxyl end of the R chain.[59]

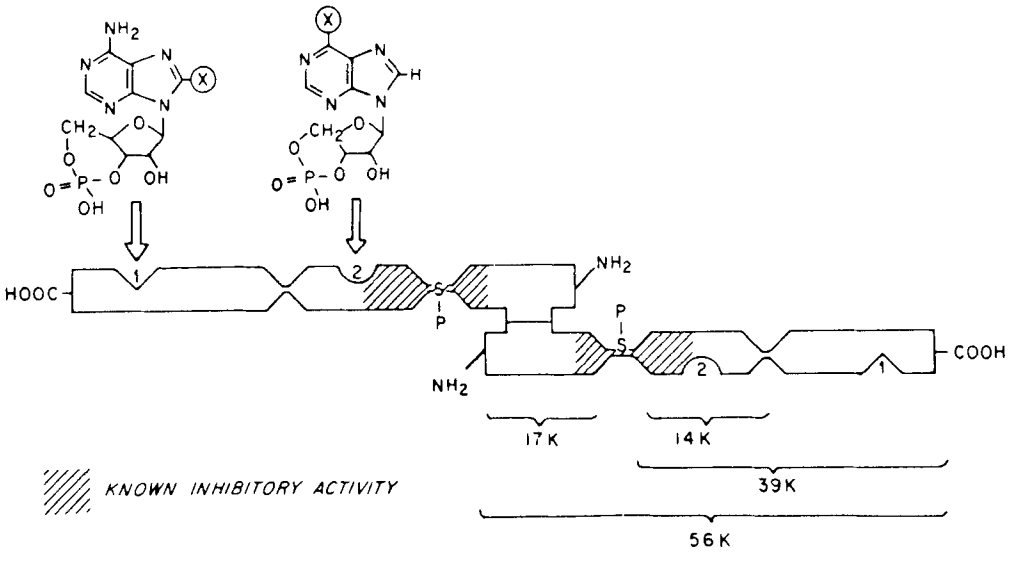

FIGURE 1. Model of domains in type II isozyme of regulatory subunit. cAMP-binding sites are designated as 1 and 2 as described in the text. The preference of analogs modified in the 8-position for Site 1 and of those modified in the 6-position for Site 2 is indicated, as is the only region known to possess inhibitory activity. Phosphorylatable serine residues are indicated by S-P.

Since probably neither the cAMP-binding fragments nor the affinity-labeled peptide represents an entire binding site, it is too early to state confidently that the readily obtainable 16,000- and 14,000-dalton fragments represent Site 2 of the respective R, whereas the principal 8-azido-[^{32}P]cAMP-labeled peptide is derived from Site 1.

Several studies are now being directed toward understanding the function(s) of the two intrachain cAMP-binding sites. Since the binding of a Site 2-preferring analog such as [^{3}H]cIMP is stimulated by the binding of Site 1-preferring analogs, at least one function of Site 1 appears to be to induce binding to Site 2 in a cooperative manner.[56] The reverse experiment, which tests whether Site 2 binding stimulates Site 1 binding, has also demonstrated significant stimulations.[45,60,61] This rather direct method of determining cooperativity of cyclic nucleotide binding differs from kinetic methods, which have occasionally shown cooperativity.[37,62] Such a method promises to be useful in studies of effector binding to other protein kinases. Although the data suggest intrachain cooperativity of Sites 1 and 2, they can also be interpreted as interchain cooperativity. Were that the case, cAMP binding to Site 1 of one chain of the dimer would presumably stimulate binding to Site 2 of the other chain. Whether or not cAMP binding to Site 1 or Site 2, or both, is responsible for the C subunit activation cannot be unequivocally stated at this time. However, a Site 2—preferring analog such as cIMP is more efficient than cAMP itself, in terms of moles bound to R per mole of active C subunit released.[45,60,61] On the other hand, it is known that low concentrations of Site 1—preferring analogs will activate protein kinase. Finally, since the cAMP-binding domains appear to interact with each other, one should consider the possibility that two molecules of bound cAMP could themselves interact. Dimerization of cAMP does occur under certain conditions,[64] albeit at low affinity, and such a process could be favored by the proximity of two interacting sites.

B. Catalytic Domains

As indicated in Table 1, the C subunit of cAMP-dependent protein kinase has been

Table 2
SUBSTRATES FOR cAMP-DEPENDENT PROTEIN KINASE

Physiological substrates	Possible physiological substrates	Nonphysiological substrates
Phosphorylase kinase	Histone	Casein
Glycogen synthase	Phospholamban	RCMM-lysozyme
Hormone-sensitive lipase	Fructose diphosphatase	RNA polymerase (*E. coli*)
Pyruvate kinase	Diglyceride lipase	eIF-2 (*E. coli*)
Troponin I	Protamine	cGMP-dependent protein kinase
Regulatory subunit	Phosphatase inhibitor I	Artificial peptides
Cholesterol esterase	Phosphofructokinase	
Acetyl CoA carboxylase	Reverse transcriptase	
Tyrosine hydroxylase	S-6 ribosomal protein	
ATP-citrate lyase	Myelin basic protein	
	HMG CoA reductase kinase	

isolated and shown to be a monomer of 39,000 to 42,000 daltons. This protein has been purified to homogeneity from bovine liver,[65] heart,[66-70,33] adrenal cortex,[71] rabbit skeletal muscle,[70,72,73] rabbit liver,[70] rat skeletal muscle,[74] porcine skeletal muscle,[75] and porcine gastric mucosa.[75] In contrast to R subunit, C subunit, isolated from either the type I isozyme or the type II isozyme or obtained from any of these sources, appears to have similar chemical,[65,70,75,77] physical,[37,65,69,73,75,77] catalytic,[65,69,70,73] and immunological[78] properties, as well as similar Km for ATP,[65,69,79,80] protein substrate specificity,[65,69,70,73] and the ability to interact with either type I or type II R subunit.[79,80] Nevertheless, a number of investigators[65,70,81] have separated up to three different forms of the protein which have similar molecular weights but different isoelectric points as revealed by isoelectric focusing. (For a summary see Reference 81). The different migration of these forms does not appear to be due to different amounts of endogenous phosphate or carbohydrate,[70] nor to any immediately obvious differences in the content of Asx, Glx, His, Lys, or Arg residues.[70]

The C subunit has a very broad protein substrate specificity in vitro as indicated in Table 2. This breadth does not appear to be due to differing specificities between forms of the protein which have different isoelectric points. However, it has been necessary to define strict experimental criteria[10] which must be satisfied in order to clearly demonstrate that any protein is a substrate for the enzyme in vivo.[10] Such criteria are all the more important in that they can legitimately be employed during the examination of any protein kinase. They have been carefully applied by Beavo and Krebs[10] to the substrates of cAMP-dependent protein kinases and it is on this basis that substrates have been designated as "physiological", "probably physiological", or "nonphysiological" in Table 2.

The molecular basis for the protein and peptide substrate specificity of cAMP-dependent protein kinase in vitro appears to lie in part in the requirement of this enzyme for a pair of basic amino acids on the amino terminal side of phosphorylated serines or threonines.[82-84] Among the known substrate proteins, arginine residues are particularly common in this position and they usually occur two or three residues from the phosphorylatable amino acid.[5,7,10,11] Studies using peptide substrates, recently reviewed by Glass and Krebs[11] and by Carlson et al.,[13] indicate that two arginyl residues provide the best combination for protein kinase action when they are one residue removed from the phosphorylated amino acid, but that a Lys.Arg sequence is preferred if there are two intervening residues.[109] At present, there are no data which

indicate that histidine, lysine, arginine, or tyrosine residues can undergo phosphorylation catalyzed by this kinase. It has been postulated, on the basis of structural predictions from known sequences of phosphorylated sites in proteins,[85] that a β-turn in the protein structure near the phosphorylatable residue may be a further determinant which is recognized by cAMP-dependent protein kinase. However, studies of peptide substrate and substrate analog binding using NMR spectroscopy indicate that, if there is any *obligatory* structural determinant other than arginine residues, then it must be a coil structure,[46] and not a β-bend.

It is interesting to consider the requirement for basic residues in protein substrates in the light of other enzymes, notably the trypsin-like serine proteases, which also recognize sites in proteins adjacent to basic amino acid residues. It has recently been shown that the C subunit from rat intestinal mucosa is retained by an affinity column of soybean trypsin inhibitor[86] which can therefore be used to separate C subunit from the holoenzyme.[86] Furthermore, it was found that soybean trypsin inhibitor was capable of inhibiting the pure C subunit obtained from skeletal muscle, albeit with low affinity[86,95] (Ki $\sim 10^{-4}$ *M*). Lastly, it has been shown that α-N-tosyl-L-lysine chloromethyl ketone (TLCK), an affinity labeling reagent originally designed for labeling the active site of trypsin, specifically attacks a sulfhydryl group at the active site of the C subunit of cAMP-dependent protein kinase and inactivates the enzyme.[87] It therefore seems possible that the retention of the C subunit on soybean trypsin inhibitor affinity columns is due to an affinity between the inhibitor and the recognition subsite at the active site of the C subunit. Experiments in this laboratory have not revealed any ability of the protein inhibitors of C subunit, i.e., the regulatory subunits or the Walsh inhibitor, to inhibit trypsin[88] despite the fact that both trypsin inhibitor[96] and the protein kinase inhibitor[97,98] contain essential basic amino acids. An examination of the published sequences of trypsin[87] and of catalytic subunit[90] reveals little clear sequence homology. Nevertheless, there do seem to be similarities in the recognition properties of this particular kinase and trypsin and in the ability of both enzymes to be inhibited by heat-stable protein inhibitors in a manner competitive with substrate and with K_is in the nanomolar range. An awareness of the chemistry and enzymology of well-characterized proteases such as trypsin promises to be useful during the future study of protein kinases which have similar substrate recognition characteristics.

In order to probe the chemistry of the active site, chemical reagents have been used to modify the C subunit. These include sulfhydryl modifying reagents: iodoacetamide,[65] *p*-hydroxymercuribenzoate,[65] N-ethylmaleimide,[65] *p*-chloromercuribenzoic acid,[73] and 5,5′-dithiobis (2-nitrobenzoic acid),[70,73,110] all of which appear to destroy activity. The enzyme can be protected, at least partially, against these agents by the presence of Mg.ATP but not of protein substrate.[65,70,73] Interestingly, thiocyanylation of the -SH groups, which represents a relatively small modification, does not appear to affect either the catalytic properties of the molecule or the ability to be inhibited by the homogeneous kinase inhibitor.[70] The magnesium nucleotide has also been reported to protect the kinase activity from modification by ethoxyformic anhydride,[91] or the affinity label 5′-*p*-fluorosulfonyl benzoyl-5′-adenosine[90,93] which modifies a lysine residue,[92,93] as does the 2′,3′-dialdehyde derivative of ATP.[94] Cibacron Blue F3GA inhibits the C subunit in a reaction which can be prevented by the presence of either Mg.ATP or protein substrate.[99] 3-(3-Dimethylaminopropyl)-1-ethyl carbodiimide in the presence of glycine ethyl ester modifies what appears to be an essential glutamate residue at the active site.[48] This modification can be prevented by the inclusion of protein substrate but not of Mg.ATP in the modification reaction.[48] These data taken together indicate the presence of sulfhydryl groups and glutamic residues around the active site, but do not prove their direct involvement in the catalytic mechanism.

The data obtained from experiments using affinity labels based on the structure of ATP indicate the presence of a lysine residue in the proximity of the nucleotide binding site, but do not prove involvement of this residue in the binding of ATP. The use of a large number of ATP analogs modified in numerous parts of the molecule has nevertheless provided considerable information about the ATP-binding site and the relationship of this site to equivalent sites in other kinases or proteins which bind ATP.[100,101] In this way researchers have been able to study which parts of the nucleotide molecule are important determinants for binding to the protein kinase in question. This approach to the study of the nucleotide binding sites of protein kinases has particular attractions since it has the potential to identify nucleotides which are useful, specific inhibitors of a particular enzyme in the presence of several others.

It is clear from the studies of Freist et al.[100] (confirmed in this laboratory[101]) that the C subunit has a high specificity for the purine moiety of ATP and that modifications to the triphosphate moiety affect binding to the protein less, whereas analogs modified at the ribose moiety have binding properties very similar to that of ATP. This is in contrast to some t-RNA synthetases[102] and potassium ion-stimulated adenosine triphosphatase[103] which appear to possess strong binding determinants for the ribose moiety, but is similar to data on DNA polymerase[46] which indicate very tight binding to the adenine moiety.

These data have been used to argue that the high affinity ATP-binding site on the holoenzyme of the type I isozyme is related to the ATP-binding site of the C subunit rather than the cAMP-binding site of the R subunit.[100] This has been confirmed by the use of the photoaffinity label [γ^{32}P]8-N_3-ATP which appears in the C subunit after photolysis in the presence of holoenzyme followed by dissociation with cAMP.[104] Furthermore, these studies demonstrate that cAMP can bind competitively to the ATP site of the C subunit (K_i = 210 μM).[100] It is therefore clear that concentrations of cAMP in this range are able to inhibit the C subunit when concentrations of ATP below the Km (5 to 10 μM) are used in assays. This has led to the false identification of protein kinase which is inhibited, rather than stimulated, by cAMP.

ATP analogs have also been used in attempts to probe the Mg.ATP (*syn* or *ante*) conformation preferred by enzymes. Should there exist a metal ion-ATP complex in which the metal is coordinated to the N-7 atom of the adenine moiety and to oxygen atoms of the phosphate moiety while ATP is in the *ante* conformation (Figure 2), then substituents at the N-7 position should inhibit the formation of the *ante* conformation.[104] In the same way it has been argued that Mg-ATP in the *syn* conformation may have metal coordinated to the N-1 or N-3 atoms of the adenine moiety and to phosphate oxygens. Hence substituents at these positions would block formation of the Mg.ATP *syn* conformation. Data obtained by the use of NMR[46] and Raman spectroscopy,[105] however, do not indicate any direct chelation of the adenine moiety by magnesium. At neutral pH the Raman data indicate that the binding to the phosphate moiety is the only interaction between ATP and Mg^{++} since noticeable spectral changes in adenine ring vibrations are not found.[105] Nevertheless, it is clear from model building of ATP analogs (see Figure 2) that bulky substituents at the C-8 position of ATP severely restrict adoption of the *ante* conformation. Credence is given to this idea by the known conformation of 8-substituted adenosines in both crystals[106] and solution.[107] As a result, protein kinases which can bind such analogs well must be able to use the *syn* conformation of the magnesium nucleotide. In the case of cAMP-dependent protein kinase, increasing the size of the substituent at the C-8 position (-SH>-Br>-NH_2) progressively decreases ability to bind the kinase.[100,101] This could indicate either a strong steric requirement for binding at this site or a preference for *ante* conformations.

Model building also makes clear that only the largest and most charged substituents

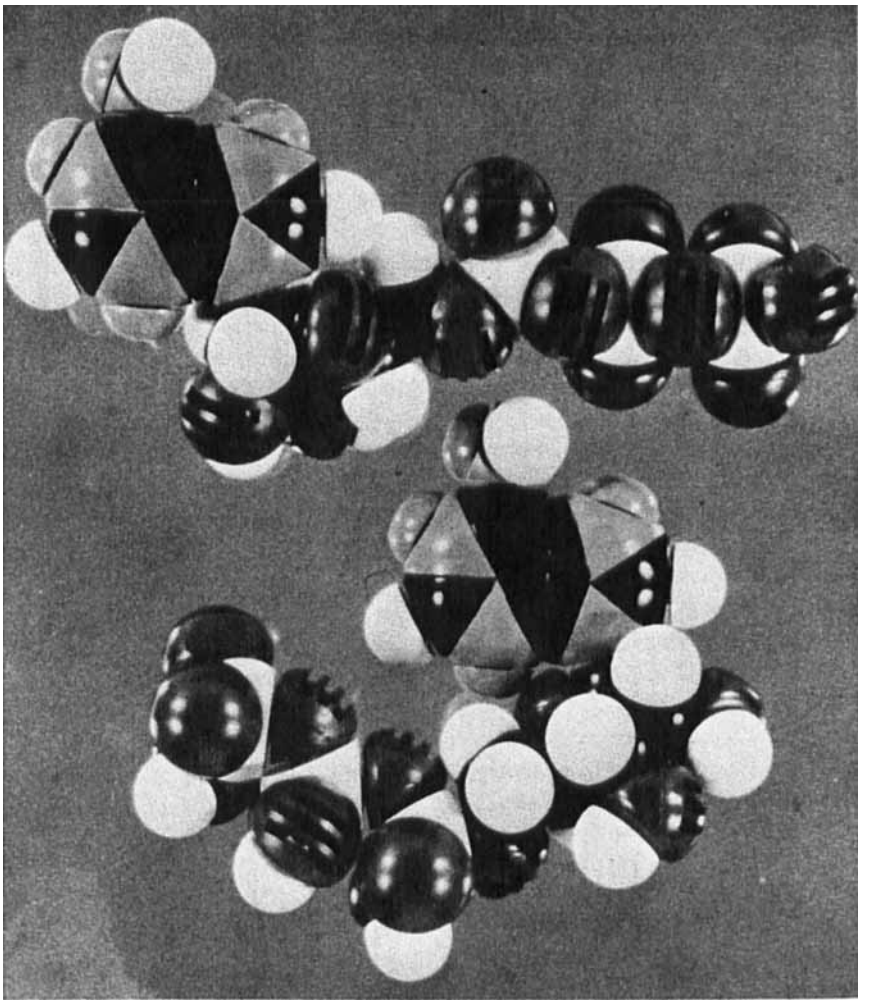

FIGURE 2. Space-filling models of the *syn* (lower) and *ante* (upper) forms of ATP.

at the C-6 position can affect the conformation of the nucleotide. Therefore, the large changes in binding affinity caused by modification at this position (ITP has a K_i at least 100-fold above the binding constant for ATP) cannot be due to conformational change (*syn* or *ante*) and must be due to either a need for the hydrogen-bonding amino group, a need for a defined electron density in the ring structure, or strict steric requirements for the molecular organization at this position.

The molecular organization of the triphosphate moiety at the binding site has also been the subject of study and is of particular relevance to the reaction mechanism which removes the γ-phosphate in order to transfer it to protein, to peptide,[82,84,108,109] or to water.[65,76,110,111] Indeed the various possible chelated structures of the triphosphate moiety have been suggested[112] to be the structural basis for the catalytic versatility of Mg.ATP in biochemical reactions. Clearly, it is possible for enzymes to determine the phosphate which is to undergo nucleophilic attack by arranging the relative positions and the conformations of the bound substrates such that the phosphorus atom that is to undergo substitution is proximal to the appropriate nucleophilic atom of the substrate.[112] Such is the case for the C subunit for which the relationship of peptide substrate

conformation to that of the triphosphate moiety has been described in detail.[67,68] This has been possible because catalysis requires a divalent cation such as Mg^{++} which can be replaced by $Mn.^{++}$ The paramagnetic properties of Mn^{++} allow the use of electron paramagnetic resonance (EPR) to determine stoichiometries and binding constants for the cation.[46] The paramagnetic effect of bound Mn^{++} on the longitudinal nuclear relaxation rates ($1/T_1p$) of nearby atoms such as phosphorus, substrate protons, or water protons allows precise measurements of the metal to substrate distances.[46] Careful studies of the triphosphate conformation have depended upon the preparation of stable complexes of ATP with Mn(II),[67] Cr(III),[46] and tetraamine Cobalt III,[68] which have two important properties:

1. They bind to ATP-utilizing enzymes but are relatively inert to substitution and have low activity (about 1/1000th of that with Mg.ATP)
2. They have known absolute stereochemistry around phosphorus and known chelate structures.

M^{3+}.ATP complexes chelated at the β- and γ-phosphates have two stereoisomers denoted Δ and Λ.[46] It is clear that the bovine heart C subunit uses the Δ isomer as a substrate in preference to the Λ isomer. The stoichiometry of metal binding to enzyme-nucleotide complex is 2:1.[67] Since numerous studies indicate that ATP binding to C subunit has a stoichiometry of 1,[92-94,100] it follows that there are two metal binding sites. The NMR data indicate that the higher affinity metal binding site is on the enzyme-bound nucleotide and the lower affinity site is induced on the enzyme by the bound nucleotide. This latter metal binding forms a bridge between enzyme and metal nucleotide substrate and inhibits activity.[68] These data account for the strong inhibition ($\simeq$80%) of C subunit activity which occurs at higher concentrations ($>$20 m*M*) of magnesium.

A determination of the secondary structure of peptide bound to protein kinase has also been possible through the use of ATP chelates of heavy metals. Manganese, cobalt, or chromium ions can be used to cause shifts in the relaxation times of substrate or water protons and this has allowed the stereochemistry of heptapeptide binding to C subunit to be described by the calculation of precise distances between metal and proteins and by model building.[46] It was thus determined that the α-helix and β-pleated sheet structures are not preferred as substrates, that various β-turns could conceivably be preferred, but that a coil structure for peptide substrates could well be obligatory.[46] It is of interest that the conformation of that part of the phosphorylase *a* molecule which contains the serine phosphorylated by phosphorylase *b* kinase is a coil as determined by X-ray analysis.[113]

Knowledge of the precise stereochemistry of peptide and metal nucleotide substrates at the active site contributes greatly to an understanding of the reaction mechanism employed by the kinase to catalyze the transfer of phosphate from nucleotide to peptide. Kinetic studies on the beef heart enzyme, which indicate the absence of ping-pong kinetics,[67,68,114] can be combined with studies of active site stereochemistry to argue against a phosphoryl-enzyme intermediate and for a mechanism wherein the γ-phosphate dissociates and forms a metaphosphate intermediate before nucleophilic attack by the seryl hydroxyl.[46] This is in contrast to reports which have indicated that the brain enzyme does employ a phosphoryl-enzyme intermediate linked to a histidyl residue in the reaction mechanism.[94]

The studies discussed above have been the first which have yielded stereochemical data on any protein kinase active site. It will be of great interest in the future to determine whether other protein kinases use similar reaction mechanisms or whether different methods of catalysis are employed. Such studies will inevitably contribute greatly to our

understanding of the means which nature has designed for the regulation of active site catalysis by these enzymes and for the recognition of physiological substrate enzymes and proteins.

From the point of view of practical considerations, observations on the cAMP-dependent protein kinase catalytic domain indicate several areas of potentially fruitful study on other protein kinases which are less well understood. These include studies of active site stereochemistry (as described above), ATPase reactions, ATP analog specificity, and the reversal of protein kinase reactions. The ability of C subunit to transfer phosphate from ATP to water appears genuine and not due to a contaminating ATPase activity.[111] This property allows the study of kinetics and reaction mechanism in the absence of protein or peptide substrate. High concentrations of Mg.ADP can be used to force the C subunit to "reverse" catalytic activity, remove phosphate from a protein substrate, and transfer it to ATP.[9,115] The Km of the reverse reaction for ADP is 40 μM.[9,100,101,115] This is a useful method for removing phosphate from protein substrates in the absence of an appropriate or pure protein phosphatase if the reverse reaction has a relatively low Km for Mg.ADP and particularly if the protein substrate specificity of the reverse reaction is similar to that of the forward reaction and the kinase responsible for catalyzing phosphorylation of a substrate is unknown or uncertain. This method has been used to carefully study the effect of the phosphorylation state on the activity of rat liver pyruvate kinase,[115] and is an absolute requirement for the determination of the free energy of hydrolysis of covalently bound phosphate.[9] It is doubtful, however, that the reverse reaction has any physiological meaning.

C. Autophosphorylation

The holoenzyme of cAMP-dependent protein kinase has been known since 1974 to undergo "autophosphorylation",[117] i.e., to catalyze phosphorylation of a residue within its own structure in an intramolecular reaction.[118] As is indicated in Table 1 there are now many protein kinases which have been shown to undergo autophosphorylation and, while this phenomenon may not be a general property of all protein kinases, it may well be of fundamental importance to the regulation of the activity of a large number of these enzymes and the cellular events which they control. The effect of autophosphorylation on the catalytic and regulatory properties of any kinase promises to be important to an understanding of why protein kinases should undergo this reaction. This is indeed the case for cAMP-dependent protein kinase.

The C subunit is able to catalyze the incorporation of 2 mol of phosphate per mole of R monomer,[119,120] and the C monomer itself can incorporate 2 mol of phosphate upon incubation with Mg.ATP in the absence of protein substrate.[70,121] Although the sequence of the autophosphorylation sites in the C subunit has been determined,[122] no clear functional role has as yet been assigned to them. In the case of the R subunit, the two phosphorylation sites have different kinetics of phosphorylation since one site (Site 2) is phosphorylated extremely quickly even at 0° whereas the other (Site 1) requires incubation with large concentrations of C subunit at 30°.[119] Autophosphorylation under conditions where the faster site (Site 2) is phosphorylated results in R subunit which has a lower reassociation rate with C subunit.[123-125] The recent observation that the two autophosphorylation sites in the R subunit are proximal to the two cAMP-binding sites[56] at least in terms of the primary structure is interesting and supports our original contention[35] that the two cAMP-binding sites may have evolved as the result of genetic duplication of a domain. No effect of phosphorylation on the binding of cAMP by either domain has been demonstrated to date, although the juxtaposition of a cAMP-binding site with each phosphorylation site in the primary structure may indicate that appropriate assay conditions have not yet been designed.

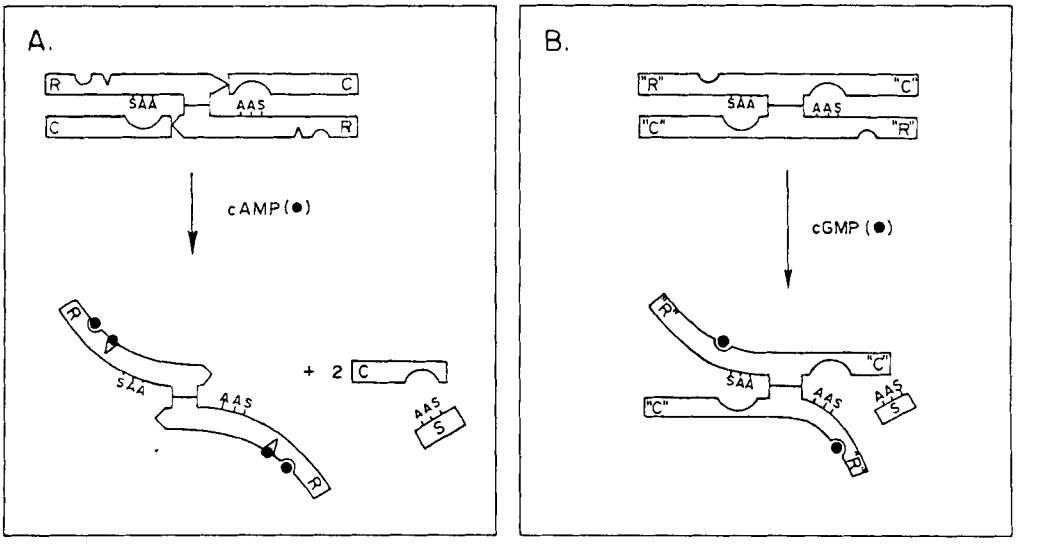

FIGURE 3. Models of modes of action of cyclic nucleotide-dependent protein kinases. (A) cAMP-dependent protein kinase. (B) cGMP-dependent protein kinase.

The Site 2 phosphorylation site has been shown to be within three residues of the most sensitive part of the R subunit to tryptic cleavage.[119,120] This site has been determined to have the primary sequence Asp-Arg-Arg-Val-Ser-Val-Cys-Ala and trypsin cleaves between the two arginyl residues as one might expect. The high tryptic sensitivity of this site suggests that it may exist on a connecting peptide between domains.[119,120] A model depicting the structure of the isozyme II of cAMP-dependent protein kinase is shown in Figure 3. While this model is designed as a working hypothesis and is by no means complete or unequivocally accurate it does demonstrate several testable features which we consider important. Each monomer has three major domains: two-cAMP-binding domains[35,119,120] and a dimerization domain as first described by Taylor et al.[126,127] One of the two phosphorylated residues is located either near or within a cAMP-binding domain.[56,119,128] No such detailed model of the domain structure of the regulatory moiety of any other kinase can be presented at this time. Data on the mechanism of action of the R moiety upon the C subunit of this kinase are of interest in the study of other protein kinases which may or may not employ similar mechanisms to regulate active site catalysis.

D. Mechanism of Action

The molecular basis for the regulation of cAMP-dependent protein kinase by cAMP lies in the means by which active site catalysis in the C subunit is inhibited by the R subunit and the means by which cAMP can reverse this inhibition by binding to the R moiety. As discussed above, the two subunits have an exceptionally high affinity for each other in the absence of cAMP[32,37] and this affinity is decreased 10^4-fold by binding of the nucleotide. It is generally considered[10,13,32,37] that the holoenzyme complex has no significant activity towards peptide and protein substrates although the isozyme II is clearly capable of catalyzing autophosphorylation in the absence of cAMP.[117]

Several pieces of evidence have now been gathered which suggest that cAMP activates the holoenzyme by a mechanism which involves formation of an intermediate ternary complex of nucleotide and holoenzyme before dissociation of the dissimilar subunits. In this postulated mechanism cAMP would bind to the R subunit while it was part of the

holoenzyme complex rather than to that small amount of free R subunit which was dissociated simply as a result of the equilibrium between holoenzyme and free R. As a result of cAMP binding the R and C subunits are presumed to dissociate from one another so that the whole process can be represented as:

$$R_2C_2 + cAMP \rightarrow cAMP.\ R_2C_2 \rightarrow cAMP.\ R_2 + 2C \qquad (4)$$

The evidence for this sequence of events is mainly kinetic, although two groups have reported data[117,129] which may indicate physical isolation of a cAMP-holoenzyme complex. Huang et al.[129] have used CL-Sepharose 6B gel filtration to study the interaction of [^{3}H]cAMP (10^{-8} *M*) and holoenzyme, and report that although [^{3}H]cAMP binds to the holoenzyme, it does not dissociate it until higher concentrations (10^{-7} *M*) of the nucleotide are used. Erlichman et al.[117] have shown that preincubation of "dephosphorylated" isozyme II holoenzyme with 20 μM cAMP for 10 min at 0° followed by chromatography on a column of Bio-Gel A-1.5m in the presence of 2 μM cAMP resulted in binding activity which emerged in the same elution volume as untreated holoenzyme and which co-eluted with cAMP-dependent catalytic activity. However, similar experiments carried out using S-200 chromatography and in the presence of 50 m*M* KCl, 50 m*M* KH_2PO_4 and after preincubation with 0.5 m*M* cAMP do result in complete dissociation.[37] The interpretation of experiments which purport to demonstrate physical isolation of such a ternary complex of isozyme II must clearly be qualified because of the ability of the subunits to quickly reassociate, particularly in the absence of high salt concentrations,[30] and because of the difficulty in separating free R from holoenzyme using conventional procedures.

Despite the inherent difficulty in the isolation of a ternary complex it is clear that the R subunit and C subunit can interact in the presence of cAMP. In the case of the type II isozyme the C subunit is clearly capable of catalyzing phosphorylation of the R subunit in the presence of saturating concentrations of cAMP. Modification of C subunit by 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) causes rapid inactivation of C subunit, yet "phospho-"R subunit decreases the rate of inactivation and "dephospho-"R subunit increases the rate even in the presence of 0.5 m*M* cAMP.[110] These results may clearly be explained by a ternary complex [$cAMP_n$. R_2C_2] in which the -SH group(s) on the C subunit is (are) activated toward reaction with DTNB. The slower rate of inactivation of the phosphoholoenzyme compared to the dephosphoenzyme may be the result of slower recombination of the subunits of the former. The authors of these studies correctly point out the possibility that inactivation of C subunit in the ternary complex is not caused by direct reaction of C subunit with DTNB. It remains possible that a stepwise mechanism is involved wherein DTNB first reacts with cAMP.R and then subunit recombination allows intracomplex transfer of TNB (trinitrobenzoate anion of DTNB) from R to C.[110] Nevertheless, it is clear that changes in sensitivity to DTNB are due to interaction of the dissimilar subunits in the presence of cAMP.

Such interactions are also indicated by experiments which show that the presence of C subunit stimulates the rate of dissociation of labeled cAMP or of (1,N^6-etheno-cAMP) from R subunit.[33,39,49] Data on the binding of cAMP to *Drosophila* embryo protein kinase at 0° indicate a ternary complex as the most likely of three postulated mechanisms to fit the data.[130] The data on the binding of cAMP in all these experiments are compromised quantitatively by the use of the simple Millipore filtration method which is known to underestimate the cAMP bound to R subunit.[33,35,50] Nevertheless, the data do support the ternary complex mechanism qualitatively. Perhaps the most convincing evidence for this mechanism comes, however, from studies with the type I isozyme from bovine skeletal muscle which have demonstrated that in the absence of cAMP the dissociation of R from

C is exceptionally slow ($t_{1/2}$ = 1 hr) as compared to the almost immediate activation of holoenzyme by saturating concentrations of cAMP.[33] As discussed above (Section II.A), it is unclear at the moment whether both moles of cAMP which bind to each R monomer are responsible for activation, nor has it been possible to determine whether or not one or the other site is primarily responsible. Furthermore it is unclear whether either or both sites are involved in the formation of the ternary complex before dissociation and activation.

In the absence of bound cAMP the R subunit exerts a very effective inhibitory action on the C subunit. It has been postulated[91] that R subunit inhibition of the C subunit from the type II isozyme of bovine protein kinase occurs through shielding of the Mg.ATP binding site. However, the data which show intramolecular autophosphorylation of this isozyme in the absence of cAMP make it clear that the Mg.ATP site is indeed available.[46,117] There could, of course, exist a distinct Mg.ATP binding site for the phosophorylation of exogenous substrates by the C subunit, but the kinetic,[65,66,70,73] NMR,[67,68] and analog modification data[100,101] indicate there to be only one type of site.

It has been suggested[35,119] that R subunit inhibits the catalytic activity by acting as a "substrate analog" of high affinity which shields the protein or peptide substrate binding site on the C subunit. Modifications to the R subunit such as tryptic proteolysis or treatment with an arginine-modifying reagent[35] remove inhibitory activity and the ability of R subunit to act as a phosphoryl-acceptor substrate. In addition NMR studies of substrate proton relaxation rates make it clear that it is the binding of peptide substrate which is blocked by the type II isozyme of the R subunit, rather than that of Mg. ATP.[34] Since R subunit clearly blocks the ATPase activity[110] which has been demonstrated to be intimately associated with the C subunit,[65,76,110] it follows that the interaction of the two subunits is even able to prevent water (at a concentration of 50 *M*) from acting as a substrate for the kinase activity. It is therefore clear that R subunit may exert at least two actions to inhibit catalytic activity. First, it may act as a high affinity substrate analog and competitive inhibitor and second, it may block the ability of catalytic subunit to transfer phosphate from ATP to substrate.

It remains possible that there is more than one interaction site for the protein substrate on the C subunit and that R subunit blocks interaction with that site also. Whether or not an additional inhibitory interaction site is the Site 1 phosphorylation site is uncertain. The possibility of a second interaction site seems particularly attractive in light of data which indicate heat lability of inhibitory activity but not of phosphoryl-acceptor or substrate activity for the isozyme II of the R subunit.[119]

The heat-stable protein kinase inhibitor may also work by acting as a "substrate analog" since inhibitory activity of the homogeneous protein is blocked by treatment with the arginine-specific reagent, 2,3-butanedione and the kinetics of interaction are consistent with those of a high affinity, competitive inhibitor.[97] However, the mechanism of inhibition by R subunit is clearly different in some respects in that the inhibitory activity of the heat-stable inhibitor is obviously insensitive to heat treatment, whereas that of the type II isozyme of the R subunit is destroyed by mild heat treatment[119] and the inhibitor contains no phosphorylation sites.[100,101]

Experiments on the interaction of fragments of R subunit which retain inhibitory activity with the C subunit will better elucidate the biochemical mechanisms which are involved in inhibition. Few such fragments have been isolated up to this time. Careful kinetic experiments to determine whether or not R subunit inhibits C subunit in a manner which is competitive with substrate have not been reported. However, the type I isozyme is indeed capable of inhibiting cGMP-dependent protein kinase in a competitive manner. It is clear that R subunit possesses superior inhibitory properties ($K_i \simeq 1$ n*M*) to those of synthetic peptide analogs ($K_i \simeq 1$ m*M*) of protein substrates.[132] The demonstration of

high affinity inhibitory kinetics for the heat-stable protein kinase inhibitor[97] raises the possibility that it contains a uniquely effective inhibitory sequence within its structure or that this protein also possesses properties other than the ability to act as a substrate analog which confer great inhibitory activity upon it.

E. Activation in Intact Tissues

The state of protein kinase activation, referred to as protein kinase activity ratio (activity minus cAMP divided by activity plus cAMP), can be measured in crude extracts of intact tissues after treatment of the tissues or whole animal with hormones or other agents.[21,133-139] In most cases the activity ratio has been shown to reflect the level of cAMP in the tissue. The activity ratio has been useful in various studies up to this time:

1. It provides an approach to the understanding of qualitative and quantitative in vivo cAMP-dependent protein kinase regulation.
2. It has been a better indicator of cAMP changes than measurement of cAMP itself. One might even argue that in metabolic studies measurement of the activity ratio is more informative than measurement of cAMP levels. Many investigators have had difficulties measuring significant changes in cAMP after treatment with hormones which cause physiological effects on phosphorylase, heart function, lipolysis, etc. There have been claims that cAMP did not change and could not be the mediator of such actions.
3. Noninvolvement of cAMP can be studied in this manner. For example, it has been used to demonstrate that cAMP is *not* the mediator of alpha agonist effects in liver.[21] Since the protein kinase is a crucial step in the overall control of metabolism in tissues, it is an important step to analyze for possible blocks under experimental conditions (e.g., adrenalectomy).
4. Using the activity ratio one can explore the presence of non-cAMP mechanism(s) for kinase regulation.
5. One can attempt to determine the existence of "sequestered" cAMP in vivo. The activity ratio has been a useful tool for many workers and has cleared up several misinterpretations in the literature.

Since many investigators have used the original method for determining the protein kinase activity ratio, it is important to recognize the pitfalls one has to contend with. These pitfalls, and methods to circumvent them, have been discussed individually in several papers over the years,[21,133-136,140,141] and are summarized in Table 3. Obviously, some of the potential pitfalls in the list would apply to several other enzymes which are assayed in crude extracts. Many of the items of Table 3 have also been emphasized by Palmer et al. who have suggested the inclusion of exogenous protein kinase in homogenizing media in order to monitor and correct for dissociation-association artifacts.[139] Another improvement in the method is the suggestion of using artificial peptides, instead of histones, as substrate in the kinase assay.[138] Other workers have investigated homornal effects on the protein kinase by determining the endogenous cAMP-R complex either by measuring [^{3}H]cAMP binding to empty sites on R[142] or by measuring protein-bound cAMP directly.[143-145]

The protein kinase activity ratio probably compares in nature and value to other assays involving activatable enzymes, even those involving covalent modification, e.g., phosphorylase, phosphorylase kinase, pyruvate kinase, and lipase. There are many effectors which change in concentration and form after cell breakage. One can only approach the real value while ruling out as many artifacts as possible. It is anticipated, as with all enzyme assays, that improvements in the activity ratio determination will be

Table 3
POTENTIAL PITFALLS IN DETERMINATION OF THE PROTEIN KINASE ACTIVITY RATIO

ATPases
Phosphodiesterases
Phosphoprotein phosphatases
Release of sequestered cAMP
Artifactual RC dissociation (histone, salt)
C binds to particles
Salt inhibits kinase
Artifactual R and C reassociation
ATP effects on reassociation
Endogenous or added cAMP too high
Isozyme distribution (tissue and species)
cAMP-independent protein kinase
Presence of endogenous substrates
Heterogeneity of cells in a tissue
Proteases

made with time. As the regulation of other protein kinases becomes understood in more detail at an enzymological level, one would expect it to be possible to develop similar activity ratio assays for these enzymes in order to test their involvement in regulatory events mediated by hormones or viruses, or in cellular growth mechanisms.

III. cGMP-DEPENDENT PROTEIN KINASE

The purification of cyclic GMP-dependent protein kinase to homogeneity[146-148] from bovine lung and heart has made it clear that this enzyme is regulated in a manner different from the cAMP-dependent protein kinase, although the enzymes are similar in that each is specifically activated by a cyclic nucleotide. cGMP binding and catalytic activities of the enzyme are clearly on one polypeptide chain since the subunit obtained by limited proteolysis contains both cGMP binding and phosphotransferase activities.[146] The activities exist on separate domains, however, since further proteolysis of the enzyme can separate the two activities.[149] The enzyme is a dimer[146-149] (M_r = 175,000 to 180,000) consisting of similar subunits (M_r = 74,000 to 82,000) which are linked in part by interchain disulfide bridges.[149-151] The domain responsible for dimerization can be isolated as a 16,000-dalton dimer composed of two 7000-dalton monomers which represent the blocked amino-terminal ends of the two polypeptide chains.[151] Activation which occurs by the binding of 1 mol of cGMP per monomer[146,149,148] can therefore be represented as:

$$E_2 + 2\ cGMP \rightleftharpoons E_2.cGMP_2 \qquad (5)$$

Purification of cGMP-dependent protein kinase to homogeneity has also allowed study of the detailed biochemistry of the enzyme and has cast some light upon the role of this kinase and the transduction of cGMP effects to cellular events.

The low levels of cGMP and cGMP-dependent protein kinase in cells have made the study of cGMP effects a much more difficult task than study of cAMP or the cAMP-dependent enzyme. Furthermore, reports of proteins which bind cGMP specifically and with high affinity, but which have no phosphotransferase activity.[152-154] raise the possibility that the biological effects of cGMP are mediated by the action of more than

one protein transducer. At the present time the relationship of phosphorylation of any specific substrate protein to cellular events mediated by cGMP-dependent protein kinase has not been unequivocally established. These considerations have led Lincoln and Corbin[155] to postulate that the versatility of the cGK may be limited to the control of only one or very few processes. It follows that cGMP itself, as compared with cAMP, might be more specific in its biological function.

Crucial to any discussion of the role of cGMP is a consideration of the protein substrate specificity of enzymes that mediate the action of the nucleotide. cGMP-dependent protein kinase [cGK] was initially thought to possess highly specific protein recognition properties, distinct from those of cAMP-dependent protein kinase since opposite physiological effects appeared to be associated with rises in the cellular content of either cAMP or cGMP.[156,157] Furthermore, cGK has been reported to catalyze phosphorylation of distinct tryptic peptides on mixed histones and to be unable to activate phosphorylase kinase.[149,158,159] Subsequently, however, more careful examination of the protein[14,160,161] and peptide substrate[163] specificity of cGK in vitro has revealed remarkable similarities between the two enzymes. Both protein kinases can catalyze phosphorylation of several protein substrates.[13,160-162] The rates of phosphorylation of substrates are usually 10- to 15-fold slower when cGK is used than when the cAMP-dependent enzyme is the catalyst.[11,160-162]

All the known in vitro substrates for cGK are described in Table 4. The columns have been arranged according to the criteria which have been outlined as requirements for physiological substrates for a protein kinase.[10] As is clear, the application of criteria that must be fulfilled by a physiological protein substrate[10] leaves no substrates which one can be certain are phosphorylated in vivo.

Detailed studies using synthetic peptides recently reviewed by Glass and Krebs[11] show that the two cyclic nucleotide-dependent protein kinases have overlapping but not entirely identical specificity determinants.[163] Certain differences in protein, as opposed to peptide specificity, are indicated by studies using histone H2B,[163,164] H_1[166] the skeletal muscle phosphorylase kinase,[162] "G-substrate" from cerebellum,[167,168] or the type I regulatory subunit.[169,170] All of these proteins possess two phosphorylation sites, both of which can undergo phosphorylation catalyzed by either of the cyclic nucleotide-dependent protein kinases. In each protein one site is preferred by cGK and the other preferred by the C subunit. G-substrate,[167,168] proteins G-1 and G-2 from smooth muscle,[168a] and type I regulatory subunit[169,170] represent the few native proteins which are phosphorylated faster by cGK than by C subunit.[167,168] Since certain proteins which act as good substrates for cGK in vitro probably are not physiological substrates, it will be of great interest to determine whether or not these proteins act as substrates for cGK in vivo.

The most dramatic difference in substrate specificity between the two enzymes is for the type I isozyme of the R subunit[169,170] which can incorporate 2 mol of phosphate per polypeptide chain when the reaction is catalyzed by cGK, but not when catalyzed by C subunit.[170] C subunit does not catalyze the incorporation of phosphate even at concentrations (10^{-5} *M*) which are 4 times the concentration of cGK used[170] and are at least 20 times higher than those estimated to be present in cells.[69] There are data which indicate that the type I isozyme of R subunit contains phosphate in vivo when isolated from bovine skeletal muscle,[169] rat soleus muscle,[169] or cultured S-49 mouse lymphoma cells.[171] However, the site phosphorylated by cGK in vitro is clearly different from any site phosphorylated in vivo in rat soleus muscle.[169] Since the level of cGK in rat sekletal muscle is exceptionally low,[152] and since the phosphorylation of the type I isozyme of R subunit is relatively slow [using high concentrations of R subunit (7×10^{-6} *M*) and cGK (6×10^{-6} *M*) the phosphorylation reaction required 2 to 3 hr to plateau at 30°][170] it remains possible that in vivo phosphorylation is due to some kinase other than cGK. This

Table 4
SUBSTRATES FOR cGMP-DEPENDENT PROTEIN KINASE

Physiological substrates	Possible physiological substrates	Nonphysiological substrates
?	Troponin I	Casein
	Phospholamban	RCMM-lysozyme
	cAMP-kinase (type I R subunit)	Artificial peptides
	Phosphorylase kinase	
	Glycogen synthase	
	Phosphatase inhibitor I	
	Pyruvate kinase	
	Fructose diphosphatase	
	Cholesterol esterase	
	Diglyceride lipase	
	Hormone-sensitive lipase	
	S-2 ribosomal protein	
	S-10 ribosomal protein	
	L-5 ribosomal protein	
	Protamine	
	Histone	
	cGMP-kinase (self phosphorylation)	
	Unidentified brain protein	
	Myelin basic protein	

possibility has not been tested to date. Alternatively, appropriate conditions for the optimum phosphorylation of the type I isozyme R subunit by cGK in vitro may not have been designed, or cellular factors needed for the optimum rate of reaction may be missing. Specific substrates for cGK do appear to exist in vivo since the phosphorylation of a number of unidentified proteins in broken cell preparations has been reported to be selectively stimulated by low concentrations of cGMP but not by cAMP.[11,172-178] The actual in vivo phosphorylation of any of these proteins has not been demonstrated.

The reported differences in protein substrate specificity are therefore seen against the background of large similarities between the two cyclic nucleotide-dependent protein kinases in this respect. This similarity originally led Lincoln and Corbin[155] to propose that the two types of enzyme were homologous proteins. While it is clear that there are differences in the mode of action and cyclic nucleotide specificity of these two kinases, they do have very similar physical properties,[149,155] Kms for ATP,[149,155] affinity for cyclic nucleotide,[155] mode of action,[35,150] and kinetics of modification by 5′-*p*-fluorosulfonylbenzoyladenosine.[92,93,179] A comparison of the amino acid compositions of cGK and the isozyme II of cAMP-dependent protein kinase has been used to predict a 70 to 90% sequence homology.[155] While knowledge of the actual extent of homology must await the sequence determinations, the concept of homology has been valuable in that it has allowed predictions about one enzyme to be drawn from data on the other and vice versa. Models have been proposed for the structure of the two enzymes[35,150,180] as indicated in Figure 3. As is the case for the cAMP-dependent enzyme, it is clear that the structure of cGK contains functional domains which can be separated by limited proteolytic digestion.[149,151]

A. Effector-Binding Domains

1. cGMP

It is believed that one molecule of cGMP binds to each monomer of cGK kinase. This is supported by the stoichiometry of [^{3}H]cGMP binding[146,149,148] to homogeneous enzyme eluted with the radioactive nucleotide from either of two types of affinity column: 8-(2-aminoethyl)-amino-cAMP-Sepharose[182] or 8-(2-aminoethyl)-thio-cGMP-Sepharose.[41] The binding of cGMP to cGK is of similarly high affinity as is the binding of cAMP to the regulatory subunit ($\sim 10^{-8}$ *M*).[146-148,155] The homogeneous enzyme can be 50% activated by 0.02 to 0.05 μM cGMP in a highly specific manner.[146,183] The only nucleotide tested with a comparable effectiveness is 8-Br-cGMP.[146] Cyclic GMP is 100 times as effective as cAMP in the decrease of [^{3}H]cGMP binding to the enzyme.[11,184] Interestingly, the guanine nucleotide is only 25 times as effective as cAMP in the activation of histone phosphorylation,[11] but 1000-fold better when hormone-sensitive lipase is the substrate.[14,161] The cause of these discrepancies probably lies in the effect of histone itself on the cGMP binding and catalytic properties of the enzyme as discussed below.

Data have been presented by Gill et al.[184] which indicate that the binding of cGMP exhibits positive cooperativity. The addition of cold cGMP to cGK fractionally saturated with [^{3}H]cGMP retarded the release of [^{3}H]cGMP from the complex brought about by dilution. This is consistent with cooperativity as are Scatchard plots of [^{3}H]cGMP binding. As might be expected, binding of cGMP is sensitive to conditions of temperature, and pH.[184]

ATP is able to decrease the affinity of cGK for cGMP by noncompetitively inhibiting the rate of association of the cyclic nucleotide.[184] Although this effect appears to be due to interaction with the catalytic site of the enzyme since it is half-maximal at a concentration close to the Km of cGK for ATP (10 μM),[184] this has not been rigorously proven to be the case by the use of photoaffinity labels or analog specificity studies. The ATP effect cannot be due to an autophosphorylation of the cGK since no magnesium is required for it, and it is generally evident after very short incubation times, which is inconsistent with the relatively slow autophosphorylation reaction known to occur in the presence of Mg.ATP.[150,185] A model can be outlined to explain the effects of ATP on cGMP binding which draws strong analogies with the type I isozyme of the cAMP-dependent enzyme. The latter has a high affinity (10 n*M*) binding site for ATP[37] which is known to stimulate reassociation of the two dissimilar subunits[30] and a consequent increase in the rate of dissociation of cAMP.[49] However, in the case of the cGK, no binding of ATP has been observed in the absence of cGMP, and it is clear that phosphorylation is unlikely to be the mechanism involved. It remains likely nevertheless that ATP in some way induces a conformational change in the catalytic domain which stimulates it to interact with the cGMP-binding domain in such a way as to inhibit the rate of association of the free cyclic nucleotide.

The cGMP-binding domain can be separated from the catalytic domain by proteolysis,[149,186-188] however, it is also possible to copurify the cGMP binding and catalytic domains on a monomeric, globular fragment of Mr = 65,000 by the use of trypsin[146] or chymotrypsin.[157] The precise relative locations of these latter two functional domains have not been further determined. Changes in catalytic and binding properties which might be caused by proteolysis and which might contribute to our understanding of the protein structure have not been studied.

2. Polyarginine

In the same way that cAMP-dependent protein kinase can interact with the heat-stable protein inhibitor,[97,98] cGK also has been reported to possess within its structure binding

sites for regulatory proteins. Calmodulin has been reported to stimulate cGK in a calcium-independent manner by increasing the maximum velocity without altering the Km for ATP,[189] in a manner analogous to heat-stable modulators.[192-194] The lack of calcium dependence makes this report suspect, however, as does the impurity of the enzyme preparations used. Of considerably greater interest are the reported effects of arginine-rich histones and synthetic poly-L-arginine. As is the case for cAMP-dependent protein kinase,[30] these proteins stimulate cGK activity when used at low concentrations, but inhibit the enzyme at higher concentrations.[190] At low concentrations these proteins also cause a decrease in the binding of cGMP.[190] No studies have been made of the enzymological mechanism involved in these effects. For example, it is not yet known whether the Km or Vmax for ATP or substrate is changed or whether cGMP binding affinity is altered. It is perplexing that the consequences of interaction with arginine-containing peptides and proteins do not appear to be reversible.[190] Although the possibility of contamination of histone by proteases has been considered carefully and excluded by the use of synthetic poly-L-arginine and histone heated at 90° C,[190] it remains possible that a polyarginine-stimulated protease contaminates the cGK preparation. In this regard it is particularly disturbing that soybean and lima bean trypsin inhibitors were able to exert small inhibitory effects on the action of histones. Nevertheless, independent data do indicate another site of interaction with histone. Preliminary evidence has been presented by Glass and Miller[191] which indicates that a synthetic peptide analog of the phosphorylation site in histone H_2B is capable of inhibiting phosphorylation of peptides but not of whole histone H_2B. These data suggest that the interaction of histone with cGK at a site distinct from the catalytic site prevents the peptide from access to the active site. It remains possible therefore that low concentrations of arginine-rich proteins can interact with cGK in such a way that they prize apart the two isologous chains indicated in Figure 3 through interaction with a site very close to the cGMP-binding sites. The proximity of these two sites is suggested by the decrease in [^{3}H]cGMP binding caused by poly-L-arginine and the prevention of polyarginine effects by high cGMP concentrations.[190] At high concentrations one might expect the basic histones to inhibit cGK through competition for those sites on the enzyme which bind the arginine residues proximal to the phosphorylation sites of cGK protein substrates. This is indeed found.[190]

The effects of arginine-rich histones and poly-L-arginine can be blocked by a protein[192-194] which "stimulates" cGK through the prevention of inhibition by high concentrations of histone.[190] This latter protein, the stimulatory modulator, has been the subject of extensive study and has been reported to have a similar tissue distribution to cGK.[195] It will not be discussed in detail here since its effects are clear only when some histones are used as substrates for cGK, and Shoji et al.[194] have clearly demonstrated by sucrose density gradient centrifugation that the modulator does not interact with the kinase, but exerts its effects by binding to histone.

B. Catalytic Domain

Although the search for a cellular role for cGK has generated extensive studies on the protein and peptide specificity of cGK relatively little is known about the ATP-binding properties of the enzyme. The Km for ATP is 10 to 20 μM,[149,159] and GTP and ITP will not serve as substrates. No studies of the stereochemistry of metal-ATP complexes at the active site have been reported, although NMR studies on the subject could provide invaluable data with which to compare cGK with the cAMP-dependent enzyme and other protein kinases. In particular, it is impossible at this time to discuss the catalytic mechanism which is involved in the transfer of phosphate from ATP to protein or peptide substrate.

Preliminary data have been reported which suggest that there are considerable

similarities in the ATP-binding properties of the C subunit and of cGK. Both proteins can be modified with the affinity label 5′-*p*-fluorosulfonylbenzoyladenosine.[92,93,179] The modification can be prevented in each case by MgATP, MgADP, or MgAppNp, but not by magnesium alone, or by peptide substrate. Interestingly, the rate of affinity label modification of cGK can be stimulated up to 10-fold by the presence of basic polypeptides such as histone II A or protamine.[179] While it is conceivable that this effect is due to binding to the polyarginine-binding site described above, it seems unlikely since synthetic polylysine can stimulate the rate of inactivation[179] whereas this polypeptide is unable to stimulate cGK activity in a manner analogous to polyargine.[190] Polyarginine does not affect either the activity of the holoenzyme of isozyme II of cAMP-dependent protein kinase[190] or the rate of affinity label inactivation of the C subunit.[92] The pseudo first-order rate constant for inactivation with 1 m*M* affinity label was 0.048 ± 0.0003/min^{-1} for C subunit and 0.067 ± 0.005/min^{-1} for cGK. The K_i for affinity label was 0.24 m*M* for C subunit and 0.82 m*M* for cGK. These data are consistent with preliminary studies of the binding of ATP analogs to the active site.[101] These studies indicate that cGK has an affinity for nearly all of 27 analogues which is 2- to 3-fold lower than that for C subunit. Furthermore, it is clear that whereas the affinities of the two enzymes for nucleoside triphosphates are consistently different, the *relative* affinities of a range of analogs for either protein kinase are very similar and both enzymes have a very high specificity for the purine moiety, relative to the ribose or triphosphate moieties. This indicates substantial similarity between the two ATP binding sites.

C. Autophosphorylation

Incubation of Mg. [γ-^{32}P]-ATP with homogeneous cGK results in the incorporation of ^{32}P into the enzyme itself.[150,185] The reaction is intramolecular and independent of enzyme concentration,[150] as is the case for the autophosphorylation of the isozyme II of cAMP-dependent protein kinase in the absence of cAMP.[118] It has recently become clear that both cGK[151] and the isozyme II of the R subunit[169,170] can incorporate 4 mol of phosphate per mole of enzyme dimer. In the presence of cGMP only 1 mol of phosphate is incorporated[151] whereas in the presence of cAMP it has been reported that 2 mol are incorporated.[151] Autophosphorylation can be prevented by limited tryptic cleavage of cGK or by treatment with the arginine-specific protein modifying reagent 2,3-butanedione.[150] Cyclic AMP and histone H2B[185,185a] stimulate the autophosphorylation whereas it is inhibited by cGMP.[150,185] It has been postulated that cGMP decreases autophosphorylation by the same conformational change which allows the catalytic site to become available to substrate (See Figure 3).[150]

Stimulation of autophosphorylation by histone H2B appears inconsistent with the effects of polyarginine on histone kinase activity.[190] However, the same stimulation is consistent with the effect of histone IIA to increase the rate of modification by affinity label.[179] It appears therefore that polycationic proteins like histone may have a dual effect: first, to make the catalytic site available to protein substrate and second, to increase the rate of reaction of MgATP with the enzyme.

The effect of cAMP on the rate of reaction with affinity label, and the effects of histone on distribution of phosphate in cGK have not been reported on, so it is impossible to tell at present whether stimulation of autophosphorylation by cAMP occurs through a similar mechanism to that employed by histone H2B.

D. Mechanism of Action

Detailed quantitative studies upon the relationship between cGMP bound to cGK and the activity of the enzyme upon protein substrates have not been performed, however, qualitative studies have provided considerable insight into how the cylic nucleotide

brings about activation of the enzyme. A model for the mode of action of cGMP involving such a conformational change is depicted in Figure 3. The model is analogous to that for cAMP-dependent protein kinase wherein the activity is inhibited in the absence of cyclic nucleotide by high affinity binding of the autophosphorylation site to the substrate binding determinants of the catalytic site. The model is consistent with the following evidence:

1. cGMP stimulates activity upon substrates, but inhibits autophosphorylation.[150]
2. Low concentrations of trypsin activate cGK and destroy the ability of the enzyme to undergo autophosphorylation.[150]
3. The arginine-specific protein modifying reagent 2,3-butanedione blocks autophosphorylation of the enzyme and also stimulates the activity in the absence of cGMP.[150]
4. The effects of 2,3-butanedione are evident only when the enzyme is modified in the presence of cGMP.[150]
5. Cyclic GMP brings about no detectable change in the Stokes radius of the enzyme.
6. The rate of modification of cGK by 5'-*p*-fluorosulfonylbenzoyladenosine is not affected by cGMP.[179]
7. The dimer cannot be dissociated into monomers by treatment with either 2-mercaptoethanol or 2,3-butanedione, however, the combination of the two reagents succeeds in causing monomerization.[150]

Although cGMP binding to the protein kinase has been reported to be cooperative,[184] no reports of cooperativity in activation of the enzyme have appeared. As a result one can only speculate as to the consequence of interactions between the two cGMP-binding sites. There appear to be interactions of the enzyme with basic polypeptides through binding to a "polyarginine-binding site" on cGK. Binding of peptide to this site appears to decrease cGMP binding, suggesting that the two sites may be close to one another. Recent data which show one of the cAMP-binding sites in the isozyme II of the R subunit to be close to a highly acidic region containing four adjacent glutamate residues are interesting in this regard.[120] The presence of an endogenous protein, which might bind to the polyarginine-binding site has not been tested for, although this is an attractive possibility.

In summary, less detailed and quantitative biochemistry has been performed upon cGK than upon the cAMP-dependent protein kinases. Studies performed to date have cast some light upon the role of the enzyme and upon the regulation by cGMP. Further studies of the active site stereochemistry and catalytic mechanism are needed in order to compare cGK with other protein kinases and to more fully understand the regulatory mechanisms involved. For example, no reports of ATPase activity associated with cGK have appeared and so it is not known whether cGMP might stimulate it, or whether the inhibited enzyme prevents the transfer of phosphate from ATP to water. Studies of the primary sequence of the enzyme also promise to be important to our understanding of cGK, since the sequence of the autophosphorylation sites will help elucidate both the in vivo substrate specificity and the regulation of cGK.

Lastly, data which show changes in the activity of the enzyme in vivo are critical to the demonstration of the involvement of cGK in the action of cGMP upon any cellular process. A method to study the state of activation of the enzyme in heart when cGMP is raised by nitroprusside or by acetylcholine has been developed in the laboratory of Lincoln and Keely.[196] This method has been useful in distinguishing the effects of these two agents on contractile force. The data imply cGMP produced by acetylcholine, but not by nitroprusside, is coupled to protein kinase activation in this tissue. These authors have emphasized the difficulties in making sensitive determinations of the cGK activity ratio.

A greater knowledge of the binding determinants for both polypeptide and nucleoside triphosphate substrates will help immeasurably in the design of conditions under which it will be easier to study the state of activation of the enzyme in vivo.

IV. PHOSPHORYLASE KINASE

The enzyme which converts phosphorylase *b* to its active form, phosphorylase *a*, was the first protein kinase to be characterized[197] and the first to be purified to homogeneity.[197,199] The role of phosphorylase kinase in catalyzing the phosphorylation of phosphorylase has been thoroughly documented in muscle,[2-5,198a] but the enzyme is also clearly important in the control of glycogenolysis in heart[2-5] and liver.[17,200] The skeletal muscle protein is composed of four types of subunit (α, β, γ, δ) organized in a tetrameric structure ($\alpha_4 \beta_4 \gamma_4 \delta_4$) with an overall molecular weight of 1.3×10^6.[198,201] The enzyme has different isozymic forms in red and white skeletal muscle,[202] the α subunit in red muscle having about 3% lower molecular weight,[198,202] and being named α'.[198,203] In liver, phosphorylase kinase has a less clearly delineated structure, since it has proven difficult to purify the enzyme intact. The data obtained in relation to the liver enzyme have been reviewed by Chrisman et al.[200]

An activity present in sarcoplasmic membranes has also been reported to catalyze phosphorylation of phosphorylase.[204,205] In addition, fluorescent antibodies to soluble muscle phosphorylase kinase are found to stain cardiac or diaphragm sarcoplasmic reticular membranes and sarcolemma.[204,205] This activity has been reported to catalyze phosphorylation of phospholamban,[206,207] the proposed activator protein of the cardiac sarcoplasmic reticulum calcium pump,[208,209] as well as exogenous phosphorylase *b*. However, it does not appear to be identical to soluble phosphorylase kinase. The enzyme appears to have no γ subunit[205] and no tightly bound calmodulin as a δ subunit.[207] In contrast, the δ subunit from the soluble skeletal muscle enzyme has been shown to be tightly bound, to bind calcium,[210] and, by determination of part of the amino acid sequence, to be closely related to calmodulin.[211]

A vast body of work has been directed toward clarifying the roles of the other subunits in this complicated structure. Recently methods have been developed for the dissociation of the γ subunit from the complex,[212] and this work has at last laid the groundwork for more detailed studies of the domains within the structure of each polypeptide chain responsible for effector binding, regulation, and catalytic activity. The identification of two different strains of animal which lack phosphorylase kinase in either muscle[213,214] or liver[215] tissue promises to be important in the elucidation of the roles of the enzyme and of the individual subunits in these tissues. For an excellent and detailed discussion of the chemical and physical properties of phosphorylase kinase and of currently available purification methods the reader is referred to the recent review by Carlson et al.[13] The following discussion will therefore be limited to a consideration of the mechanisms involved in regulation of phosphorylase kinase activity.

A. Regulatory Domains

Two distinct mechanisms for the regulation of phosphorylase kinase activity are thought to be of physiological significance. First, the enzyme activity has been shown to have an absolute requirement for calcium.[216,217] Second, the enzyme has very little activity when isolated from resting muscle and assayed at physiological pH.[13] The activity exhibits a pH optimum at pH 8.2, however, and the ratio of activity at pH 6.8 to that at pH 8.2 can be raised from less than 0.05 to 0.35 to 0.40 when phosphorylation of the enzyme is catalyzed by cAMP-dependent protein kinases.[197,198,201,218] The pH 6.8/8.2 ratio can also be increased to 0.5 by autophosphorylation[197] or to 0.7 by tryptic

proteolysis[197,198] or to a similar extent by dissociation with LiBr.[212] The enzyme also exhibits sensitivity in vitro to many other effectors. These include an activating effect of free magnesium ion, described in elegant experiments performed by Clerch and Huijing[219] in which free magnesium concentration was varied under conditions of constant saturating Mg.ATP and constant free ATP.[219] Several phosphorylated carbohydrates influence the activity. These include glycerophosphate, which inhibits the nonactivated kinase, but stimulates the activated enzyme,[220] and glucose-6-phosphate (G6P), which inhibits enzyme activity[197] both through binding to phosphorylase *b*[221] and through a direct effect on the enzyme.[220] The G6P effect can be mimicked by UDPG, glucose-1-phosphate, and fructose-1-phosphate.[220] Heparin stimulates the unactivated enzyme[197] by an unknown mechanism. Polyaspartic acid decreases the activity at pH 8.2, as does protamine.[197]

All the effects described above are on the holoenzyme form of phosphorylase kinase. The inability of researchers to separate or clearly designate the functions of any of the subunits until recently means that it is only possible at the moment to discuss a few of the above effectors for which binding to a particular subunit has been shown. This section therefore concentrates upon the action of the regulatory domains for calcium, calmodulin, troponin C, and cyclic nucleotide-dependent protein kinase upon the catalytic site(s) of phosphorylase kinase.

It is clear that the α and β subunits play a regulatory role, although this may not be their exclusive function. Limited proteolysis of these subunits in the absence of any apparent degradation of the γ subunit causes activation of the enzyme by decreasing the Km for phosphorylase *b*.[197,198,201] A very similar effect is produced upon phosphorylation of the α and β subunits by purified cAMP-dependent protein kinase,[197,198,201,218] through autophosphorylation of these two subunits[197,220] or, interestingly, by the presence of high concentrations of organic solvents, particularly acetone or 2-propanol.[222]

There appears to be a good correlation between the phosphorylation of the β subunit and the increase in activity which results.[201,218,220] However, a discrete role for the α subunit in regulation cannot be ruled out since a continuing increase in activity can be observed after a plateau in the phosphorylation of the β subunit has been reached.[201] Experiments wherein cGK has been used to catalyze phosphorylation of the α and β subunits have recently been reported.[223] This kinase is a particularly useful tool since it catalyzes phosphorylation of the α subunit much faster than the β subunit in the conditions under study. Activation was closely correlated with phosphorylation of the β subunit and poorly correlated with that of the α subunit.[223] Both the α and β subunits also appear to be involved in the loose binding of calmodulin in vitro since cross-linking reagents are found to link them to ^{14}C-labeled calmodulin.[224]

1. Calmodulin

It has been suggested that extrinsic calmodulin may be a "δ' subunit" of the enzyme. However, it has also been made clear[201,225] that troponin C, which is known to possess extensive sequence homology with calmodulin,[220,227] can substitute for the calcium-dependent regulator in this regard. Subsequently, the determination of the half-maximal effects of calcium on the troponin C or δ' complexes with phosphorylase kinase[210] have indicated that troponin C must be the dominant form of interaction at these sites at the concentrations of calcium one expects in vivo.[228,229] The author[210] points out that this concurs with what one might expect from knowledge of the concentrations of calmodulin and troponin C in fast twitch muscle fibers. If a δ' subunit is bound to phosphorylase kinase in vivo $(\alpha\beta\gamma\delta\delta')_4$, then it is clear that a molar excess of calmodulin over phosphorylase kinase does not exist, but calmodulin is thought to interact with many other proteins in muscle, notably the myosin light chain kinase.[230,231] On the other hand,

troponin C is present at very high concentrations (100 μM)[228,210] and is therefore available to interact with phosphorylase kinase as well as with the troponin-tropomyosin complex.

The precise means by which troponin C or loosely bound calmodulin mediate the effects of calcium on activity through binding to the α or to the β subunit remain unclear. However, neither protein appears to be capable of increasing the activity of phosphorylase kinase which has been previously activated by phosphorylation or by proteolysis. This suggests a mechanism which employs common structural features at some point as is suggested by the binding of δ' and troponin C to the same subunits which undergo phosphorylation or proteolysis during activation. Both phosphorylation and proteolysis cause a large decrease in Km for phosphorylase *b* (25-fold in the case of phosphorylation; 100-fold in the case of proteolysis) in the absence of large changes in Vmax or Km for ATP. This is in contrast to the activation of the cyclic nucleotide-dependent protein kinases by cAMP ro cGMP, each of which causes changes in the Vmax of enzyme activity, with no change in Km for substrate or ATP.

As indicated above, the δ subunit of skeletal muscle phosphorylase kinase is essentially identical in amino acid sequence and all determined properties to calmodulin isolated from bovine uterus.[211] It is tightly bound, as evidenced by the fact that it is not removed during preparations of the enzyme in the presence of 20 mM EGTA and the absence of calcium, and in that it is not dissociated from the γ subunit even by 8 M urea.[224] The rate of exchange of ^{14}C-calmodulin with the δ subunit has been reported to be 15%/week at 0°.[224] Data obtained from experiments using cross-linking reagents also show that the δ subunit is in the close proximity of the γ subunit in the native holoenzyme complex. It follows that the activating effects of calcium which are mediated by the δ subunit are probably, although not certainly or exclusively, a result of interaction between it and the γ subunit. Determination of the sensitivity of the various forms of phosphorylase kinase to calcium indicates that the most important role of this interaction is in the phosphorylated, activated enzyme which can be half-maximally activated by 1.6 μM $CaCl_2$ at pH 6.8 and 0.6 μM $CaCl_2$ at pH 8.2.[210] The unphosphorylated phosphorylase kinase *b* appears less sensitive to calcium,[232,210] and takes 20 μM $CaCl_2$ to cause half-maximal activation in the presence of extrinsic calmodulin at pH 6.8, but 4 μM $CaCl_2$ in the presence of troponin C at the same pH.[20] It therefore seems that the phosphorylation of the α and β subunits is able in some way to increase the affinity of the δ subunit for calcium as well as lower the Km for phosphorylase at the active site. This implies either interaction of the α and β subunits directly with tightly bound calmodulin, or indirectly through association with the γ subunit, which appears so tightly bound to it.[224]

2. Autophosphorylation

Both the α and β subunits of phosphorylase kinase incorporate phosphate upon incubation with Mg.ATP. This reaction is particularly significant in the presence of high concentrations of magnesium (> 10 mM) or of ATP (~ 1 mM) and at pH 8.2 as opposed to pH 6.8.[220,234] That autophosphorylation might activate the enzyme is suggested by the lag in the time course of activity at pH 6.8,[201,233,235] when progressive autophosphorylation appears to coincide with the increase in activity.[220,234] In addition, glycogen has been noted to stimulate both autophosphorylation and activity,[237] and many other effectors of autophosphorylation, including phosphate,[220] glycerophosphate,[220] and glucose-6-phosphate,[220] all affect activity in parallel. The mechanism through which autophosphorylation stimulates activity is unclear, although it may well cause the removal of an inhibitory action upon the catalytic subunit(s). It is impossible to tell whether a "substrate analog" autophosphorylation site is involved in inhibition, in analogy with the model of regulation postulated for the cyclic nucleotide-dependent protein kinases

(Figure 3). This seems inadequate for phosphorylase kinase since such a model would predict an increase in the Vmax through an increased number of available binding sites for substrates, whereas a decrease in the Km is the most significant kinetic change observed.

B. Catalytic Domain

Although it is clear that the major substrate for phosphorylase kinase in vivo is phosphorylase *b*, the purified enzyme in vitro is also capable of catalyzing phosphorylation of itself,[237,220,234] troponin I,[238-240] troponin T,[241-245] rabbit sarcoplasmic reticulum,[204,205,244] cardiac sarcolemma,[245] casein,[237] glycogen synthase,[246-249] phospholamban,[206-209] or synthetic peptides.[13]

At the present time it is unclear whether the phosphorylation of these substrates is catalyzed by one single catalytic subunit or whether the β subunit as well as the γ subunit are involved in catalysis. A role for the β subunit in catalysis cannot be excluded. An affinity label based on the structure of ATP is reported to be incorporated equally into the β and γ subunits, about 1 mol of label being incorporated per monomer.[250] The decrease in activity caused by affinity label appeared to correlate best with modification of the β subunit. Proteolysis of dogfish phosphorylase kinase phosphorylated mainly in the β subunit allows a low molecular weight phosphoprotein to be isolated which appears to retain activity.[251]

The γ subunit has recently been purified to approximate homogeneity after slow dissociation of the complex by either 1 *M* LiBr or 100 m*M* ATP at 0° C.[212] It appears to exist as a dimer of Mr = 42,000 subunits upon isolation. This subunit clearly has catalytic activity which is insensitive to calcium concentration but which can be stimulated 60% by calmodulin.[212] Detailed studies on the protein or peptide specificity of the purified subunit have not been performed. It will be of great interest to compare these with the known specificity of the holoenzyme since it remains possible that the β subunit contributes to the catalytic activity. The possibility that two sites might exist is suggested by the inability of troponin T to compete with phosphorylase *b* as a substrate for phosphorylase kinase,[252] and by the observation that antibodies prepared to the apparently homogeneous kinase inhibit activity on both substrates but with different patterns.[252] Although contamination is hard to exclude as a possible artifactual cause of these data, it could indicate the existence of two catalytic sites or of two protein binding or recognition sites associated with a single active site.

The specific activity of the peak fractions of the purified γ subunit preparation are very close (650,000 units/mg) to what one would predict based on the activity of the fully activated holoenzyme (95,000 units/mg). The purified γ subunit is highly active at pH 6.8, whereas the holoenzyme is virtually inactive.[13,212] The increase in activity is a result of a decrease in the Km for phosphorylase *b*. The authors conclude that "the pH 6.8 activity of nonactivated phosphorylase kinase is strongly inhibited by regulatory subunit(s) that modulates the pH 6.8 activity by affecting the Km for phosphorylase *b*."[212]

Peptide specificity studies have made clear the important binding determinants for the phosphorylation of phosphorylase *b*.[253,254] Substitution in the synthetic peptide which is based on the primary sequence immediately around the phosphorylatable serine of phosphorylase *b*—Lys-Gln-Leu-Ser-Val-Arg—has more deleterious effects than substitution in areas on either side of this sequence.[253,254] This is consistent with determinations of the primary sequence in human, rat, and dogfish phosphorylase, in which this sequence is highly conserved.[13] The arginine residue on the carboxyl side of the phosphorylated serine seems to be particularly important. Substitution of this residue with a glycine or lysine residue causes a dramatic decrease in the Vmax and increase in the Km for the peptide.[253] The importance of arginine for substrate activity is emphasized by

the observed competitive inhibition by arginine ethyl ester.[255] On the other hand, the sequence in glycogen synthase which undergoes phosphorylation has no such arginyl residue on the carboxyl side. No explanation for this apparent anomaly is currently available. The apparent importance for an arginyl residue is reminiscent of the cyclic nucleotide-dependent protein kinases and is emphasized by the similarity in size of the γ subunit to the cAMP-dependent protein kinase catalytic subunit (Mr = 39,000 to 42,000). Few other obvious similarities exist, however, and it has been shown that it is possible to synthesize a peptide containing two seryl residues, one of which is phosphorylated by phosphorylase kinase and the other by cAMP-dependent protein kinase.[256] This indicates that there are different exact specificity determinants for the two protein kinases and this has indeed been confirmed by careful comparison of the specificity determinants for the two enzymes.[82,257]

No extensive study of the ATP-binding site of phosphorylase kinase has been performed by the use of either analogs of ATP or of ATP chelated with heavy metals. Nevertheless, it is clear that the enzyme activity can be reversed[258] by the use of high concentrations of Mg.ADP in the presence of glucose, which dissociates tetrameric muscle phosphorylase *a* to the dimer.[258] Current knowledge of the enzyme suggests that there is no change in either Km for ATP or Vmax of the enzyme caused by either calcium-calmodulin, calcium-troponin C, organic solvent, proteolytic or phosphorylation-related interactions with phosphorylase kinase. It follows that control of enzyme activity is unlikely to be expressed through the ATP-binding site or through modulating catalytic action upon the nucleotide. These activators appear to derive their effectiveness from an ability to release the catalytic subunit(s) from the inhibitory activity of other subunits, which keeps the Km for phosphorylase high by a mechanism which remains tantalizingly obscure.

V. MYOSIN LIGHT CHAIN KINASE

The majority of studies on the structure and function of myosin light chain kinase has been done on the enzyme from muscle cells.[231,259] There is much evidence which suggests that myosin light chain kinase may play a particularly important role in the control of contraction in smooth muscle.[259,260] It is assumed that the basic mechanism of contraction in smooth muscle is similar to that in skeletal muscle and that length changes occur as the result of interactions between the thick and thin filaments.

Two forms of regulation have been proposed for the regulation of the interaction of smooth muscle myosin and actin. These are the leiotonin systems of Ebashi et al.[261,262] and the phosphorylation of myosin.[231,259,260] Of these, the phosphorylation of myosin has been reported in most detail and has, at the moment, the most evidence to support it. The myosin phosphorylation is mainly that of the light chains by a myosin light chain kinase. Although myosin light chains prepared from skeletal, cardiac, or smooth muscle can all be phosphorylated by a myosin light chain kinase from their respective tissues,[259,263] skeletal and cardiac muscle myosin MgATPase activity can be activated by actin without prior myosin phosphorylation.[231,259,260,264] In contrast, the smooth muscle and nonmuscle myosins require phosphorylation of the 20,000-dalton light chain as a prerequisite for activation of their ATPase activity by actin.[231,264] In smooth muscle,[268] platelets,[265,265a] and BHK-21 cells[266] the extent of phosphorylation has been quantitatively correlated with stimulation of the actin-activated ATPase. Consequently, myosin light chain kinase has been proposed as a major regulator of calcium-dependent contraction in smooth muscle and may also have an important function in nonmuscle tissues such as brain, platelets, and BHK-21 cells. Having said this, it must be added that the myosin light chain kinase which is present in skeletal muscle may well play a modulatory role, although not

an obligatory one, in the control of contraction in that tissue. The phosphorylated L_2 light chain of skeletal myosin has been reported to increase the ATPase activity of actomyosin at low concentrations (0.05 to 5 μM) of calcium.[284] Recent in vivo studies confirm this possibility. It has been shown in frog[285,280] and rabbit muscle[287] that an increase in the phosphorylated form of the light chain occurs during tetanic contraction. Phosphorylation of myosin light chains has also been demonstrated in perfused rat heart under the influence of both negative and positive inotropic agents.[285a]

The myosin light chain kinase has been purified to chemical homogeneity from skeletal,[267,269] heart,[270,271] smooth muscle,[265,272] platelets,[273,274] and BHK-21 cells.[266] There are also preliminary reports of purification of myosin light chain kinase from brain.[275,276] The enzyme is a dimer when isolated from skeletal,[267,269] cardiac,[270,271] smooth muscle,[265,272] platelets,[273,274] or BHK-21 cells.[266] It has been reported to consist of one subunit of molecular weight 77,000 to 125,000 daltons[231] and another subunit which binds calcium and has been identified as calmodulin.[265,269,277-279] Neither component alone appears to possess myosin light chain kinase activity.

A. Effector-Binding Domains

1. Calmodulin and Calcium

The chemical and physical properties of calmodulin and the characteristics of calcium binding to it have been extensively reviewed[230,280-282] and will not be covered here. A quantitative model for the activation of skeletal muscle myosin light chain kinase through interaction with the calcium-calmodulin complex has been presented.[279] The data indicate that activation by calcium and calmodulin is a fully reversible process which absolutely requires both calcium ion and calmodulin. The first step of the activation appears to require binding of 4 mol of calcium to each mole of calmodulin.[279] This complex subsequently combines with the inactive catalytic subunit to form the active calcium-calmodulin-myosin light chain kinase holoenzyme wherein there is a 1:1 ratio of calmodulin to catalytic subunit.[279,283] The intrinsic activation constants, K_{Ca++} and K_{CaM} have been calculated to be 10 μM and 0.86 μM, respectively, and the rate equation obtained using these constants is able to predict the state of enzyme activation at known concentrations of calcium and calmodulin. No method exists at present to determine the state of activation of this protein kinase in vivo.

Light tryptic proteolysis of myosin light chain kinase has been shown to activate the enzyme and to remove the absolute dependence on the presence of calcium-calmodulin. This may reflect either a loss in activity or the assumption of an inhibitory domain of a particularly active conformation at the catalytic site. While interference with the calmodulin-binding domain may also occur, this need not follow from the data, and it is possible that calmodulin does bind to the proteolyzed enzyme but has no activating effect since the inhibiting domain has already been removed by the protease.

The consequences of light proteolysis are reminiscent of similar effects to activate and remove the calcium-calmodulin dependence of phosphorylase kinase[201,210] or to remove cyclic nucleotide-independence from cAMP- or cGMP-dependent protein kinase by the proteolysis of inhibitory domains.[35,150]

2. Phosphorylation by Cyclic Nucleotide-Dependent Protein Kinase

Neither cAMP or cGMP appears to directly affect the activity of purified myosin light chain kinase.[231] However, the enzyme isolated from turkey gizzard smooth muscle,[272] human platelets,[273] or bovine cardiac muscle[271] has been shown to be phosphorylated by the catalytic (C) subunit of cAMP-dependent protein kinase. The C subunit can catalyze the incorporation of one mol of ^{32}P from [$\gamma - {}^{32}P$]ATP into light chain kinase.[271-273,300] The total amount of chemical phosphate in the enzyme before phosphorylation has not

been reported, and it therefore remains possible that there is more than one phosphorylatable site.

The effect of phosphorylation is a decrease in activity. For the smooth muscle kinase the activity dropped from 4.23 to 0.42 μmol/mg/min[260] after phosphorylation catalyzed by the cAMP-dependent enzyme. It has been suggested[260] that the loss in activity is due to a decrease in the binding affinity of calmodulin to the phosphorylated enzyme and to a small decrease in Vmax. From the data presented, it is also possible that the effect is due to a decrease in the ability of bound calmodulin to activate since no direct calmodulin binding studies have been done.

3. Autophosphorylation

Myosin light chain kinase has been reported to undergo calcium and calmodulin-dependent autophosphorylation[271,288] in the apparent absence of any other protein kinase. The autophosphorylation may be at the same site as is phosphorylated in the presence of pure catalytic subunit of cAMP-dependent protein kinase since incubation of myosin light chain kinase with calcium, calmodulin, and catalytic subunit results in no greater incorporation than with calcium and calmodulin alone.[271] Since a proteolyzed form of the cardiac enzyme cannot undergo either cAMP-dependent phosphorylation or autophosphorylation[270] it appears the phosphorylated site can be removed by mild proteolysis during preparation of the enzyme. No effects of autophosphorylation on activity have been reported.

B. Catalytic Domain

The principal substrates for active myosin light chain kinase in vivo are almost certainly Mg.ATP and the myosin light chain.[231,289,299] Although the enzyme is able to catalyze phosphorylation of several other proteins, none has as fast a rate of phosphorylation as the 18,000- to 22,000-dalton light chain. The sequence of the phosphorylation site has been determined in myosin light chain isolated from four different sources:

Chicken skeletal muscle[290]
Lys-Arg-Arg-Ala-Ala-Glu-Gly-Ser-Ser[P]-Asn-Val-Phe-Ser
Rabbit skeletal muscle[291-293]
Lys-Arg-Arg-Ala-Ala-Ala-Glu-Gly-Ser-Ser[P]-Asn-Val-Phe-Ser-Met
Chicken gizzard[294]
Arg-Ala-Thr-Ser[P]-Asn-Val-Phe-Ala-Met
Scallop[294]
Ala-Asp-Lys-Ala-Ala-Ser[P]-Gly-Val-Leu-Thr

Although the scallop sequence exhibits significant differences, it is clear that there is conservation of at least the Ser[P]-Asn-Val-Phe primary sequence among two species and two tissues, and one can infer that this cluster of amino acids may be an important specificity determinant. Of further note are the conserved Lys-Arg-Arg sequences five residues to the amino-terminal side of the phosphorylated serine. In keeping with the requirement of cAMP-dependent protein kinase for arginyl residues adjacent to the active site, the 20,000-dalton light chain of chicken gizzard myosin has been shown to undergo phosphorylation catalyzed by a homogenous preparation of this kinase at the same site as is phosphorylated in the presence of myosin light chain kinase in vitro.[295]

Proteins which have been reported not to undergo significant phosphorylation catalyzed by myosin light chain kinase include casein,[267,270,273] skeletal muscle phosphorylase *b*,[267,270,273] histone IIIS,[267] histone IIA,[270,273] histone VS,[273] troponin,[267] and phosvitin.[270,273]

No studies using synthetic peptides as substrates have been reported on this enzyme.

We must await such studies before our faintly perceived impressions of the specificity determinants for a protein substrate of myosin light chain kinase can become firm convictions.

Initial studies performed using the skeletal muscle enzyme have shown that ATP is preferred as the phosphoryl donor over GTP, UTP, or ITP.[267] Free ATP^{4-} appears to inhibit the enzyme,[267] and there is also strong inhibition by MgADP.[267] No successful attempts to reverse the enzyme reaction have been reported.

The Km for ATP varies according to the source of the enzyme. The Km of the smooth muscle kinase for ATP is about 65 μM,[87] a value lower than that reported for the platelet enzyme (121 μM),[273] the cardiac enzyme (175 μM, 220 μM),[270,271] or the skeletal myosin light chain kinase (200 to 400 μM).[231,267] Detailed studies on the nucleoside triphosphate specificity of myosin light chain kinase have not been reported. The Km for the myosin light chain appears to be in the range of 15 to 30 μM.[267,289,297] The Vmax was reported to be about 10 μmol/min/mg kinase for the chicken gizzard[296] or turkey gizzard[272] enzymes, and values reported for the skeletal muscle enzyme vary (4.3 μmol/min/mg;[297] 17 μmol/min/mg;[277] 26 μmol/min/mg).[267] The Vmax for the cardiac enzyme has been determined as 20 to 30 μmol/min/mg[271] or 30 nmol/min/mg for a proteolytically degraded form of the enzyme.[271] Kinetic studies of the active site have not progressed to a stage where any catalytic mechanism can be delineated.

C. Mechanism of Action

The biochemical basis for regulation of myosin light chain kinase by calcium and calmodulin, by cAMP-dependent protein kinase, or by autophosphorylation cannot be described at the moment; however, it is possible to emphasize some points of particular interest. First, light proteolysis activates the intact enzyme,[231,276,298] but has no effect on the enzyme which has undergone some considerable breakdown already.[270] Such proteolysis appears to remove the phosphorylatable sites.[270] Second, phosphorylation by cAMP-dependent protein kinase in vitro decreases enzyme activity dramatically,[260] either by decreasing the affinity for calmodulin, or by decreasing the efficacy of the bound regulator. One cannot tell at the moment whether the Vmax of the enzyme in the presence of saturating calmodulin is affected.[260]

These data suggest a regulatory mechanism in which an inhibitory domain is removed by calmodulin, but is endowed with greater efficacy by phosphorylation. Since the action of calmodulin is principally to increase the Vmax of the enzyme it seems possible that such an inhibitory domain has high affinity for the active site in the absence of calmodulin. The interesting observation that calmodulin does not activate the ATPase activity of the enzyme in conditions where activation of myosin light chain phosphorylation is observed suggests that the inhibitory domain blocks the binding of myosin light chain, rather than that of Mg.ATP. Although alternative explanations, such as contamination with an ATPase activity or the existence of two catalytic sites are clearly possible, such a model is analogous to the ability of the isozyme II regulatory subunit of cAMP-dependent protein kinase to prevent peptide substrate, rather than MgATP, binding to the catalytic subunit.[34]

VI. HEMIN-CONTROLLED PROTEIN KINASE (HRI) (HCR)

A protein kinase has been isolated from reticulocyte lysates which is involved in the inhibition of protein synthesis through the phosphorylation of the smallest subunit (eIF-2 α) of the initiation factor eIF-2 which binds Met-$tRNA_f$ and GTP.[301-305] This subunit has a molecular weight of 38,000 daltons and the other two subunits of the eIF-2

complex (Mr = 50,000 and 52,000 daltons) do not undergo phosphorylation by this kinase.[303-305] The kinase has been referred to as HCR (heme-controlled repressor)[306] or HRI (heme-regulated inhibitor).[306,304,305] The striking biphasic inhibition of peptide elongation in the absence of hemin appears to be due, at least in part, to the biphasic activation of this protein kinase. Both the inhibition of protein synthesis and the activity of the kinase are blocked by the addition of hemin.[304] The hemin-controlled eIF-2α kinase has been highly purified as a polypeptide of Mr = 95,000 or 80,000 daltons on SDS-polyacrylamide gel electrophoresis,[304,305] however, glycerol density gradient certrifugation indicates a molecular weight of 120,000 for the native enzyme.[304] It is thus impossible to assign either a monomeric or dimeric structure to the enzyme at this time.

The activity of this eIF-2 kinase is strongly inhibited by hemin (20 μM),[324,325] and this inhibition can be studied with the purified form of the "reversible HRI".[304,307] This latter protein kinase activity can still be inhibited by hemin in contrast to the other purified form of the enzyme, the "irreversible HRI", which cannot be inhibited by hemin.[308] The irreversible form of the enzyme appears to be a modified form of the reversible HRI which has been altered either by brief treatment with sulfhydryl reagents[309] or by prolonged incubation in the absence of hemin.[310] Both the reversible and irreversible forms have similar monomer molecular weights[307,308] and have similar biphasic kinetics when used to inhibit protein synthesis.[307,308] After addition of either protein the initial control rate is followed by an abrupt inhibition in the rate of protein synthesis. Both forms of the kinase appear to undergo autophosphorylation. The data suggest, but do not prove, that autophosphorylation results in activation of the enzyme. Preincubation of the HRI with reticulocyte lysates enriched in Mg.ATP results in an increased activity of the protein kinase.[304,305] Hemin inhibits both autophosphorylation and activity toward eIF-2α as a substrate.[304,308] However, the possibility that an intermediary protein kinase might be responsible for the phosphorylation and subsequent activation of the enzyme has not been ruled out.[305,311] Since dependence of the autophosphorylation reaction on enzyme concentration has not been determined we can only speculate as to whether we are observing an intermolecular or intramolecular phosphorylation. Should an intermediary kinase exist it would follow that the effects of hemin might be exerted upon it rather than directly on the eIF-2α kinase itself.

Other possible regulators of the hemin-dependent eIF-2 kinase include G6P[312] and reduced glutathione (GSSG),[313] however, the purity of the systems in which these effectors have been tested makes it difficult at present to define their site of action or discuss direct regulation of the kinase. The postulate that cAMP may affect this system through binding to the regulatory subunit of the cAMP-dependent protein kinase and modulating the effect of this kinase on eIF-2α kinase has been firmly rejected,[311,314] and extensively reviewed.[305] The ability of high concentrations of cAMP (10 mM) to inhibit kinase activity is likely to be due to competition of the cyclic nucleotide with ATP (0.1 mM)[304] as is found to be the case for the catalytic subunit of cAMP-dependent protein kinase.[100,101]

VII. dsRNA-DEPENDENT PROTEIN KINASE

The site of phosphorylation in the eIF-2α (Mr = 38,000) subunit which is acted upon by the hemin-regulated protein kinase appears to be very close to, if not the same as,[315,316] the site which undergoes phosphorylation catalyzed by a double-stranded RNA (dsRNA)-dependent activity[317-321] which can be distinguished immunologically from the HRI[322] and which is ribosome associated. The dsRNA-dependent protein kinase is of particular interest because of the role it has been suggested to play in the antiviral action of interferon upon cells.[305,323-326] The activated enzyme has been purified to approximate

Table 5
CASEIN KINASES

Source	Type	Monomer molecular weights	Subunit structure	$S_{20,w}$	ATP Km	GTP Km	Ref.
Yeast	I	42,000	Monomeric	—	200 μM	ND	330
Novikoff ascites tumor cells	I	37,000	Monomeric	—	20 μM	4.1 mM	331
Rabbit reticulocytes	I	37,000[a]	Monomeric	3.25	13 μM	900 μM	332
Novikoff ascites tumor cells	II	44,000 (α) 40,000 (α') 26,000[a] (β)	$\alpha\alpha'\beta_2$	7.65	4 μM	7 μM	333
Rat liver	II	42,000 (α) 39,000 (α') 26,000 (β)	$\alpha\alpha'\beta_2$	7.0	ND	ND	334
Rabbit reticulocytes	II	42,000 (α) 38,000 (α') 24,000[a] (β)	$\alpha\alpha'\beta_2$	7.55	10 μM	40 μM	335

Note: ND: not determined.

[a] Polypeptides that have been shown to contain endogenous phosphate or to be labeled with ^{32}Pi.

homogeneity from rabbit reticulocyte lysates.[318-321] It appears to have a subunit size of 67,000 to 70,000 daltons. The enzyme is capable of catalyzing phosphorylation of histone[318] as well as eIF-2α and can undergo phosphorylation itself when the enzyme is activated by dsRNA.

Very little is known about the mechanism by which dsRNA activates the eIF-2α kinase.[305] It is not possible at the moment to tell whether autophosphorylation is part of that mechanism. No apparent change in molecular weight occurs, however,[317-320] and this probably rules out a dissociative mechanism. The binding of dsRNA has been suggested[318,320] to be the first step in increasing the activity of the enzyme, and a scheme wherein autophosphorylation is required as an intermediate step during the process of activation has been presented.[318] The requirement for ATP in the activation of eIF-2α kinase by dsRNA[320,321] supports such a scheme as does the fast rate of phosphorylation of the polypeptide doublet that migrates as bands of 67,000 and 68,500 daltons on SDS-polyacrylamide gel electrophoresis[320,321] and is thought to possess the protein kinase activity.[312-321] Questions as to how the phosphorylation of dsRNA-dependent protein kinase occurs, or how it stimulates enzyme activity are subjects which deserve much further research, but which can draw only conjecture at the present.

VIII. CASEIN KINASES

While a large number of protein kinases, including phosphorylase kinase and both the cyclic nucleotide-dependent enzymes have the ability to catalyze the phosphorylation of casein, a number have been named as casein kinases since they prefer casein over histone as a substrate. Those casein kinases which have been purified to apparent homogeneity are summarized in Table 5. As is clear, these protein kinases can be divided into two groups which have been termed casein kinase I and casein kinase II in order of their elution from DEAE-cellulose.[329] The role of these kinases in the regulation of cell function and their in vivo substrate specificity remains obscure.

Some progress towards such an understanding has been made by Traugh et al.,[332-334]

who have shown that both types of casein kinases are capable of catalyzing phosphorylation of eucaryotic initiation factors. Casein kinase I can stimulate the incorporation of phosphate into eIF-4B and eIF-5, whereas casein kinase II acts upon eIF-2, eIF-3, eIF-4B, and eIF-5. Neither casein kinase, however, acts upon the eIF-2α subunit which undergoes phosphorylation under the influence of the heme-controlled repressor[304,335-337] or of dsRNA-dependent protein kinase.[315,316] In contrast, it is clear that casein kinase II can catalyze the incorporation of 2 mol of phosphate into the eIF-2β (Mr = 53,000) subunit.[336]

Determination of the in vitro protein substrate specificity has been approached by using casein variants.[332,333] Chymotryptic peptides have been made of the phosphorylated variants and these peptides have been subjected to amino acid analysis in order to determine their origin in the known primary sequence of the complete polypeptide. This method has made clear the primary structure in the region of the phosphorylation sites in casein. A preference of casein kinase I for a glutamate residue two frames towards the amino terminal of the phosphorylated serine has thus been revealed.[332,334] In contrast, casein kinase II catalyzes the phosphorylation of threonine residues in both α_{s1}-casein A and β-casein A^2. In both cases a Glu-Asp sequence was predicted to be immediately adjacent to the carboxyl end of the phosphorylated threonine.[333,334]

The two types of casein kinase are also clearly different in their subunit structure and specificity for nucleoside triphosphate (Table 5). Casein kinase I appears considerably more capable of distinguishing between GTP and ATP. It follows that GTP could be used as a relatively specific inhibitor of casein kinase II during in vitro assays.

Casein kinase II, but not casein kinase I, can be inhibited by heparin[338] and possibly other naturally occurring glycosaminoglycans[339] through a mechanism which is competitive with substrate (Ki = 20 ng/mℓ). Competitive kinetics indicate the binding of heparin to the catalytic subunit, but it has not been determined which of the subunits of casein kinase II (α,α' or β) is involved directly in catalysis. No regulatory mechanism for casein kinase I has been described.

Both casein kinases can undergo phosphorylation themselves, casein kinase II in the β subunit[329-330] and casein kinase I in the single Mr = 37,000 polypeptide chain.[329] Autophosphorylation has not been reported to change casein kinase activity under the conditions employed.[329,334]

In summary, the data presented prove beyond doubt that the two casein kinases are different enzymes which probably have different cellular functions and means of regulation. Further studies on the precise biochemical means by which these enzymes might be regulated are clearly necessary. Studies of the peptide and nucleoside triphosphate analog specificity to determine the mechanism of catalysis will be important to the understanding of the catalytic mechanism and the means by which catalysis might be stimulated, inhibited, changed, or even prevented.

IX. VIRAL TYROSINE KINASES

Recently, a great amount of research activity has been stimulated by observations which indicate that the viral gene product responsible for transformation by a number of viruses is a protein kinase which catalyzes the phosphorylation of a low level of tyrosine residues in cellular proteins, a phenomenon not previously observed. This has been shown to be the case for the *src* gene product of avian sarcoma virus[340,341] and Rous sarcoma virus[342] as well as feline sarcoma virus[343] and Abelson murine leukemia virus.[344] These findings indicate a role for tyrosine protein kinase in some types of viral transformation and possibly also in other methods of neoplastic transformation of cells.

Of particular interest, however, is the discovery of a number of normal cell proteins which appear to be similar in structure and function to the transformed gene product[345-347] and which also catalyze tyrosine phosphorylation. It follows that this activity may have a role in normal cellular growth control mechanisms.

Crucial to further research must be data on the primary substrate specificity of tyrosine protein kinases. The normal assay for these kinases involves the phosphorylation of the immunoglobulin heavy chain of antibody directed against the pp60^{src}.[356,357] Cellular substrates for the avian sarcoma virus kinases have been proposed. It has been shown that a 34,000- to 36,000-dalton polypeptide which serves as a substrate for the avian sarcoma virus-transforming gene product (pp60^{src}) in vitro is also phosphorylated in vivo at a site located in the same tryptic peptide.[349] Rous sarcoma virus has also been shown, by the use of antibody precipitation of ^{32}P-labeled substrate, to induce in vivo phosphorylation of vinculin, a protein of 130,000 daltons which may be involved in the attachment of normal cells to anchor actin-containing filaments and substratum.[350] Vinculin and vimentin also appear to be in vitro substrates.[357] Other cytoskeletal proteins, fibronectin, myosin heavy chain, α-actinin and actin have been examined using the same approach but have either been shown to contain no phosphotyrosine in vivo, or to undergo insignificant changes in their state of tyrosine phosphorylation.[350,357] Although they do not appear to undergo phosphorylation in the cell, tubulin, actin, and vimentin have been reported by one laboratory to serve as in vitro substrates for the pp60^{src} kinase activity from avian sarcoma virus-treated cells.[351] The physiological significance of these data is unclear. Lastly, of particular interest during any discussion of tyrosine kinase substrate specificity is the reported sequence of the site phosphorylated by the pp60^{src} in transformed cells: Leu-1Le-Glu-Asp-Asn-Glu-Tyr(P)-Thr-Ala-Arg.[352] As yet no detailed peptide specificity studies or specific inhibitors have been reported.

The definitive substrate specificity and therefore the role of the individual tyrosine kinases remains obscure. Methods which rely on the observation of changes in ^{32}P content of polypeptides suffer from the disadvantages of artifacts due to changes in specific activity of the cellular phosphoryl donor molecule, and of changes which are missed because of high endogenous protein phosphate or lack of resolution on the gel systems employed. On the other hand, experiments which use antibodies to determine which specific proteins become phosphorylated are limited in their scope and cannot identify the kinase involved in vivo.

Evidence has been presented which locates at least a fraction of both the pp60^{src} kinase and normal cell *src* protein kinase activity within the plasma membrane of cells.[353-356] Investigation of the functional domains of the Rous sarcoma virus tyrosine kinase indicates that the amino terminal of the Mr = 60,000 polypeptide is responsible for attachment to the plasma membrane whereas the catalytic domain is located towards the carboxyl end of the molecule.[356a] This constrained locus for the enzyme would clearly narrow the in vivo substrate specificity to proteins which could approach the plasma membrane.

Both the endogenous cellular *sarc* gene, from which the *src* gene of Rous sarcoma virus is derived and the *src* gene itself encode proteins of Mr = 60,000 daltons which undergo phosphorylation in at least two positions of the polypeptide chain.[357,359] Insufficient enzymological studies have been published which show unequivocally the auto-phosphorylation of pp60^{src} for any effect of autophosphorylation on activity or regulation to be seriously discussed.

Viral tyrosine kinases have recently been classified according to their monomer molecular weight, nucleoside triphosphate specificity, and preference for Mn^{++} or Mg^{++} as nucleotide chelators.[357] The Rous sarcoma gene product (pp60^{src}) has been designated as a type I tyrosine protein kinase.[357] It has a molecular weight of 60,000 and can use

either ATP or GTP as phosphoryl donor. On the other hand, the Abelson murine leukemia viral gene product (p120) has Mr = 120,000 daltons and prefers ATP to GTP and has been designated as a type II tyrosine kinase. The type III tyrosine kinases, which are of varied molecular weight, use ATP and like the type II tyrosine kinase, have a preference for Mn^{++} over Mg^{++}.[357]

Detailed enzymological studies on the peptide or nucleoside triphosphate specificities have not been done as yet. Such studies will undoubtedly help determine the similarities and differences between the three types of viral tyrosine kinases as well as help determine the extent of structural and functional similarity of each viral gene product to the naturally occurring homologue. Furthermore, these kind of data could enlighten scientists as to the cellular role of these kinases through the development of specific nucleotide, protein or peptide inhibitors, and the determination of the specificity determinants for protein and peptide substrates. Although it is not clear at present how the phosphorylation of tyrosine leads to transformation of cells, it is clear that greater knowledge of the enzymes involved will provide insight into a new type of protein kinase and its regulation and allow new models of cellular growth control mechanisms to be put forward and tested.

X. EGF-DEPENDENT PROTEIN KINASE

A protein kinase activity which is dependent upon the presence of epidermal growth factor (EGF) has recently been identified[360,361] and purified[362] from A-431 human epidermoid carcinoma cell membranes using affinity chromatography over resin coupled to EGF. These membranes are unusual in that they have a very high receptor density (2 to 3×10^6/cell).[363] Protein kinase activity appears to reside in the same protein complex as EGF-binding activity, a unique observation.[362] Study of the solubilization of these activities from the membrane indicate the EGF-binding polypeptide to be an integral membrane protein.[362] Activation of kinase activity towards exogenous histone[360,361] or towards membrane proteins including the receptor polypeptide itself is brought about by the binding of EGF[360-362] and is reversible.[362] It has been pointed out that reversibility rules out the possibility that EGF releases a sequestered kinase activity from the membrane.

Since EGF is clearly involved in the control of biochemical events which lead to cell growth and multiplication, it has been exciting to realize that EGF-dependent protein kinase has many characteristics which are reminiscent of the viral tyrosine kinases, described in the previous section, which appear to be involved in malignant transformation.[361] Not only does the EGF-dependent enzyme catalyze tyrosine phosphorylation,[364] but it also has a rapid rate of reaction at 0°[362] and a preference for Mn^{++}.[360,361]

It is clear that this protein kinase probably contains at least three functional domains: an effector-binding domain for EGF, a catalytic domain, and phosphorylatable site. Trypsin appears to decrease all three activities, however, and may therefore not be a useful tool for separation of the domains or studies of how they might interact with one another.[362] The EGF-binding activity has high affinity for the hormone in either intact ($K_{0.5} = 10^{-8}\ M$) or solubilized ($K_{0.5} = 2 \times 10^{-8}\ M$) preparations.[362] Very little is known about the catalytic domain other than its ability to catalyze phosphorylation of histone, membrane proteins, or the Mr = 150,000 polypeptide itself, and its ability to catalyze the fast reaction at 0° and its preference for Mn^{++}.[360-362] No effect of autophosphorylation on this tyrosine kinase activity has been reported, and at present the existence of a separate kinase responsible for phosphorylation of the Mr = 150,000 polypeptide cannot be ruled out. It does not appear that trypsin can activate the kinase by damaging an endogenous,

inhibitory domain although it remains possible that this would be observed if low amounts of protease were used.

The nucleoside triphosphate analog specificity of EGF-dependent protein kinase has not yet been determined, and this combined with the early stage of the study of protein and peptide substrate specificity makes careful comparison with the viral tyrosine kinases and other protein kinases difficult at present. It will be of great interest to further probe the biochemical mechanisms involved in the stimulation of tyrosine kinase activity by EGF and the role which autophosphorylation might play in the control of enzyme activity by regulators.

XI. PYRUVATE DEHYDROGENASE (PDH) KINASE

PDH kinase[366,367] is particularly important because of the role phosphorylation catalyzed by it plays in the control of PDH activity by insulin and other hormones.[27,368,369] The enzyme is exclusively mitochondrial in mammalian cells and it is clear that it very specifically catalyzes phosphorylation of three sites in the α-subunit of pyruvate decarboxylase. The amino acid sequences of these sites have been determined[370,371] and the kinase has been shown to bring about phosphorylation of synthetic peptides constructed on the basis of these known sequences.[372] These data make clear that the enzyme has a substrate specificity different from other protein kinases that have been examined. Although serine residues are the receptors for phosphate, few arginine residues are evident in the primary sequences immediately proximal to the phosphorylation sites.[370,371] The effects of phosphorylation to inhibit activity of the decarboxylase[373] appear to be most pronounced when Site 1 is phosphorylated,[370,374] although there is some evidence that Site 2 may also be involved.[369,375] The real, physiological function of these second two phosphorylation sites is a matter of controversy. On the one hand data have been obtained by Sugden et al.[376] which indicate that phosphorylation at these sites inhibits the reactivation of PDH by the phosphatase. On the other hand Teague et al.[375] found that the presence of thiophosphoryl groups, which are relatively resistant to the action of phosphatase, at Sites 2 and 3 had no effect on the rate of dephosphorylation of Site 1.

PDH kinase appears to be stimulated by the products of the reaction which catalyzes conversion of pyruvate to acetyl CoA, notably acetyl CoA itself and NADH, and is inhibited by the substrates for pyruvate dehydrogenase, pyruvate, coenzyme A, and NAD^+.[27,369,377-380] Tryptic peptide substrates for the kinase have been used to distinguish between substrate- and kinase-directed actions of these effectors. Using a highly purified preparation of kinase and a tryptic digest of PDH it has been shown that Acetyl CoA and NADH stimulate kinase activity, whereas pyruvate, dichloroacetate, and ADP inhibit it.[369] These data imply the direct interaction of regulators with domains in the protein kinase structure. Firm conclusions as to the specific protein target of these regulators will have to await rigorous purification of the kinase and the use of synthetic peptides. The transacetylase subunit of PDH also appears to play a role in the regulation of protein kinase activity in that the apparent Km of the kinase for its protein substrate is decreased about 30-fold in the presence of this subunit.[369]

No data are available which suggest autophosphorylation of PDH kinase. The fact that there appear to be only 2 to 5 kinase polypeptides which are tightly bound to the overall PDH complex which has close to 150 polypeptide chains of varying molecular weight and an overall mass of 7 to 10×10^6 daltons[372] makes it difficult to purify large quantities of the kinase upon which to do detailed effector-binding studies or protein chemistry. Discussion of the interaction of functional domains within the structure of PDH kinase must attend greater knowledge of the mechanism of effector action on the substrate, on domains within the kinase structure, and on activity.

XII. OTHER PROTEIN KINASES

There have been preliminary reports of the purification of a number of protein kinases not discussed heretofore. These include a calmodulin-dependent protein kinase from rabbit liver which appears to be very specific for glycogen synthase,[381] a light-dependent rhodopsin kinase from bovine rod outer segments,[382] a calcium and phospholipid-dependent protein kinase which is present in many tissues,[383,384] and an interesting protein kinase from insects which has high affinity for both cAMP (Ka = 43 n*M*) and cGMP (Ka = 25 n*M*),[385] and may be related in evolution to the well-described protein kinases regulated by the individual nucleotides. Insufficient data on the purity or regulation of these activities have been presented to date to allow these reports to be evaluated, or considered in relation to the regulation of activity through the interaction of domains.

XIII. PERSPECTIVES ON REGULATION OF PROTEIN KINASES

Whereas a great deal is known about the regulation of a few protein kinases, notably cAMP-dependent protein kinase, cGMP-dependent protein kinase, and phosphorylase kinase, it is clear that there is very little information about the biochemical basis for regulation of the majority of protein kinases which have been isolated to sufficient purity to allow biochemical studies to be performed. The diversity of protein kinases in protein substrate specificity and regulation is striking. On the other hand pronounced similarities exist between the enzymes which have been studied up until now. Nearly all prefer ATP as a phosphoryl donor and all the reported Km for ATP appear well below the estimated concentration of the nucleotide in cells. Furthermore, it appears that all the protein kinases which have been tested can undergo phosphorylation themselves. This reflects both the ability of some protein kinases to catalyze phosphorylation of themselves and the ability of some to undergo phosphorylation by other enzymes.

All the protein kinases tested are also capable of catalyzing the phosphorylation of small peptides, an important fact which indicates that the recognition determinants for phosphorylation of a protein by a particular kinase are largely contained in the primary structure of the substrate. The secondary, tertiary, and quaternary levels of protein organization appear less important. A powerful tool is thus provided for distinguishing between protein kinases in the form of synthetic peptides which can be used as specific substrates or inhibitors. Similarities have been observed in the substrate specificity of a number of groups of protein kinases, e.g., the cyclic nucleotide-dependent protein kinases, the eIF-2 kinases, the casein kinases, and the tyrosine protein kinases. We propose that these groups may represent families of homologous proteins by analogy with the cytochromes, globins, dehydrogenases, and other protein families. If this is the case, then more confident extrapolations could be made from one enzyme to another within a given protein kinase family.

The relationship between protein kinases and other enzymes which bind ATP and transfer the terminal phosphate to small molecules or to water is an unexplored area, but one of considerable interest. The tertiary structures of arginine kinase, pyruvate kinase, hexokinase,[386] and phosphoglycerate kinase,[387] which involve two domains separated by a cleft which is closed upon the binding of substrate, have been suggested to be common to several other kinases.[386] Although no protein kinase has been studied at this level of resolution, it will be useful and fascinating in the future to determine whether relationships exist with other types of kinases as well as among families of protein kinases. Regulatory mechanisms which are found to be held in common by families of kinases will be particularly powerful precedents for the study of novel protein kinases involved in the control of cellular events.

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REFERENCES

1. **Burnett, G. and Kennedy, E. P.**, The enzymatic phosphorylation of proteins, *J. Biol. Chem.*, 211, 969, 1954.
2. **Fisher, E. H. and Krebs, E. G.**, Conversion of phosphorylase *b* to phosphorylase *a* in muscle extracts, *J. Biol. Chem.*, 216, 121, 1955.
3. **Krebs, E. G.**, Protein kinases, *Curr. Top. Cell. Reg.*, 5, 99, 1972.
4. **Walsh, D. A. and Ashby, C. D.**, Protein kinases: aspects of their regulation and diversity, *Recent Prog. Horm. Res.*, 29, 329, 1973.
5. **Nimmo, H. G. and Cohen, P.**, Hormonal control of protein phosphorylation, *Adv. Cyc. Nuc. Res.*, 8, 145, 1977.
6. **Walsh, D. A. and Cooper, R. H.**, *Biochem. Actions Horm.*, 6, 1, 1979.
7. **Cohen, P.**, The role of cAMP-dependent protein kinases in regulation of glycogen metabolism in mammalian skeletal muscle, *Curr. Top. Cell. Reg.*, 14, 118, 1978.
8. **Lee, E. Y. C., Silberman, S. R., Ganapathi, M. K., Petrovic, S., and Paris, H.**, The Phosphoprotein phosphatases: properties of the enzymes involves in the regulation of glycogen metabolism, *Adv. Cyc. Nuc. Res.*, 13, 95, 1980.
9. **Shizuta, Y., Beavo, J. A., Bechtel, P. J., and Krebs, E. G.**, Reversibility of cAMP-dependent protein kinase reactions, *J. Biol. Chem.*, 250, 6891, 1975.
10. **Beavo, J. A. and Krebs, E. G.**, Phosphorylation-dephosphorylation of enzymes, *Ann. Rev. Biochem.*, 48, 923, 1979.
11. **Glass, D. B. and Krebs, E. G.**, Protein phosphorylation catalyzed by cyclic AMP-dependent and cyclic GMP-dependent protein kinases, *Ann. Rev. Pharmacol. Toxicol.*, 20, 363, 1980.
12. **Rubin, C. S. and Rosen, O. M.**, Protein phosphorylation, *Ann. Rev. Biochem.*, 44, 831, 1975.
13. **Carlson, G. M., Bechtel, P. J., and Graves, D. J.**, Chemical and regulatory properties of phosphorylase kinase and cAMP-dependent protein kinase, *Adv. Enzym.*, 50, 41, 1979.
14. **Gill, G. N. and McCune, R. W.**, Guanosine 3′, 5′-monophosphate-dependent protein kinase, *Curr. Top. Cell. Reg.*, 15, 1, 1979.
15. **Cohen, S., Carpenter, G., and King, L., Jr.**, Epidermal growth factor-receptor-protein kinase interactions, *J. Biol. Chem.*, 255, 4834, 1980.
16. **Garrison, J. C.**, The effects of glucagon, catecholamines, and the calcium ionopore A23187 on the phosphorylation of rat hepatocyte cytosolic proteins, *J. Biol. Chem.*, 253, 7091, 1978.
17. **Exton, J. H.**, Mechanisms involved in α-adrenergic phenomena: role of calcium ions in actions of catecholamines in liver and other tissues, *Am. J. Physiol.*, 238, E3, 1980.
18. **Keely, S. L. and Corbin, J. D.**, Involvement of cAMP-dependent protein kinase in the regulation of heart contractile force, *Am. J. Physiol.*, 233, H269, 1977.
19. **Stull, J. T. and Mayer, S. E.**, in *Handbook of Physiology: The Cardiovascular System*, Vol. I, Berne, R. M. and Sperelakis, N., Eds., American Physiological Society, Washington, D.C., 1979, 741.
20. **Brownsey, R. W., Hughes, W. A., and Denton, R. M.**, Adrenaline and the regulation of acetyl-coenzyme a carboxylase in rat epididymal adipose tissue, *Biochem. J.*, 184, 23, 1979.
21. **Cherrington, A. D., Assimacopoulos, F. D., Harper, S. C., Corbin, J. D., Park, C. R., and Exton, J. H.**, Studies on the α-adrenergic activation of glucose output. II. Investigation of the roles of adenosine 3′:5′-monophosphate and adenosine 3′:5′-monophosphate-dependent protein kinase in the actions of phenylephrine in isolated hepatocytes, *J. Biol. Chem.*, 251, 5209, 1976.
22. **Garrison, J. C., Borland, M. K., Florio, V. A., and Trible, D. A.**, The role of calcium ion as a mediator of the effects of Angiotensin II, catecholamines, and vasopressin on the phosphorylation and activity of enzymes in isolated hepatocytes, *J. Biol. Chem.*, 254, 7147, 1979.
23. **Benjamin, W. D. and Singer, L.**, Actions of insulin, epinephrine and dibutyryl cAMP on fat cell protein phosphorylation, *Biochemistry*, 14, 3301, 1975.

24. **Avruch, J. A., Leone, G. R., and Martin, D. B.,** Effects of epinephrine and insulin on phosphopeptide metabolism in adipocytes, *J. Biol. Chem.*, 252, 1511, 1976.
25. **Hughes, W. A., Brownsey, R. W., and Denton, R. M.,** Studies on the incorporation of [^{32}P]-phosphate into pyruvate dehydrogenase in intact rat fat cells, effects of insulin, *Biochem. J.*, in press.
26. **Brownsey, R. W. and Denton, R. M.,** Role of phosphorylation in the short-term regulation of acetyl CoA carboxylase by insulin and adrenaline in fat cells, *INSERM*, 87, 195, 1979.
27. **Randle, P. J., Hutson, N. J., Kerbey, A. L., and Sugden, P. H.,** Regulation of pyruvate dehydrogenase by phosphorylation/dephosphorylation, in *From Gene in Protein: Information Transfer in Normal and Abnormal Cells*, Academic Press, New York, 1979, 501.
28. **Erlichman, J., Sarkar, D., Fleischer, N., and Rubin, C. S.,** Identification of two subclasses of type II cAMP-dependent protein kinases, *J. Biol. Chem.*, 225, 8179, 1980.
29. **Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H.,** Purification and properties of rabbit skeletal muscle phosphorylase *b* kinase, *Biochemistry*, 3, 1027, 1964.
30. **Corbin, J. D., Keely, S. L., and Park, C. R.,** The distribution and dissociation of cAMP-dependent protein kinase in adipose, cardiac and other tissues, *J. Biol. Chem.*, 250, 218, 1975.
31. **Kuo, J. F. and Greengard, P.,** Cyclic nucleotide-dependent protein kinases. IV. Widespread occurrence of cAMP-dependent protein kinase in various tissues and phyla of the animal kingdom, *Proc. Natl. Acad. Sci. U.S.A.*, 64, 1349, 1969.
32. **Hofmann, F.,** Apparent constants for the interaction of regulatory and catalytic subunit of cAMP-dependent protein kinase. I and II, *J. Biol. Chem.*, 255, 1559, 1980.
33. **Builder, S. E., Beavo, J. A., and Krebs, E. G.,** The mechansim of activation of bovine skeletal muscle protein kinase by adenosine 3′:5′ monophosphate, *J. Biol. Chem.*, 255, 3514, 1980.
34. **Granot, J., Mildvan, A. S., Hiyama, K., Kondo, H., and Kaiser, E. T.,** Magnetic resonance studies of the effect of the regulatory subunit on metal and substrate binding to the catalytic subunit of bovine heart protein kinase, *J. Biol. Chem.*, 255, 4569, 1980.
35. **Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M., and McCarthy, D.,** Studies on the properties and mode of action of the purified regulatory subunit of bovine heart cAMP-dependent protein kinase, *J. Biol. Chem.*, 253, 3997, 1978.
36. **Beavo, J. A., Bechtel, P. J., and Krebs, E. G.,** Activation of protein kinase by physiological concentrations of cAMP, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 3580, 1974.
37. **Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E. G.,** Comparison of cAMP-dependent protein kinase from rabbit skeletal and bovine heart muscle, *J. Biol. Chem.*, 250, 7795, 1975.
38. **Schwechheimer, K. and Hofmann, F.,** Properties of regulatory subunit of cAMP-dependent protein kinase (Peak I) from rabbit skeletal muscle prepared by urea treatment of the holoenzyme, *J. Biol. Chem.*, 252, 7690, 1977.
39. **Dills, W. L., Goodwin, C. D., Lincoln, T. M., Beavo, J. A., Bechtel, P. J., Corbin, J. D., and Krebs, E. G.,** Purification of cyclic nucleotide receptor proteins by cyclic nucleotide affinity chromatography, *Adv. Cyc. Nuc. Res.*, 10, 199, 1979.
40. **Ramseyer, J., Kaslow, H. R., and Gill, G. N.,** Purification of the cAMP receptor protein subunit of protein kinase by affinity chromatography using 8(6-aminohexyl)-amino-adenosine 3′,5′ monophosphate coupled to agarose, *Biochem. Biophys. Res. Commun.*, 59, 813, 1974.
41. **Dills, W. L., Beavo, J. A., Bechtel, P. J., Meyer, K. R., Sakai, L. J., and Krebs, E. G.,** Binding of cAMP-dependent protein kinase to immobilized cyclic nucleotide derivatives, *Biochemistry*, 15, 3724, 1976.
42. **Rieke, E., Panitz, N., Eigel, A., and Wagner, K. G.,** On the detachment of the regulatory subunit of brain protein kinase from a cAMP-polyacrylamide gel, *Hoppe-Seyler's Z. Physiol. Chem.*, 356, 1177, 1975.
43. **LaPorte, D. C., Builder, S. E., and Storme, D. R.,** Spectroscopic studies of the cAMP binding sites of the regulatory subunits of type I and II protein kinase, *J. Biol. Chem.*, 255, 2343, 1980.
44. **Buss, J. E., McCune, R. W., and Gill, G. N.,** Comparison of cyclic nucleotide binding to adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate-dependent protein kinases, *J. Cyc. Nuc. Res.*, 5, 225, 1979.
45. **Rannels, S. R. and Corbin, J. D.,** Studies on the function of the two intrachain cAMP binding sites of protein kinase, *J. Biol. Chem.*, 256, 7871, 1981.
46. **Granot, J., Mildvan, A. S., and Kaiser, E. T.,** Studies of the mechanism of action and regulation of cAMP-dependent protein kinase, *Arch. Biochem. Biophys.*, 205, 1, 1980.
47. **Miyamoto, E., Petzold, G. L., Harris, S. J., and Greengard, P.,** Dissociation and concomitant activation of adenosine 3′,5′-monophosphate-dependent protein kinase by histone, *Biochem. Biophys. Res. Commun.*, 44, 305, 1971.
48. **Matsuo, M., Huang, C-H., and Huang, L. C.,** Evidence for an essential arginine recognition site on adenosine 3′:5′-cyclic monophosphate dependent protein kinase of rabbit skeletal muscle, *Biochem. J.*, 173, 441, 1978.

49. **Brostrom, C. O., Corbin, J. D., King, C. A., and Krebs, E. G.,** Interaction of the subunits of cAMP-dependent protein kinase of skeletal muscle, *Proc. Natl. Acad. Sci. U.S.A.*, 68, 2444, 1971.
50. **Weber, W. and Hilz, H.,** Stoichiometry of cAMP binding and limited proteolysis of protein kinase regulatory subunits R_I and R_{II}, *Biochem. Biophys. Res. Commun.*, 90, 1073, 1979.
51. **Corbin, J. D., Rannels, S. R., Flockhart, D. A., Robinson, A. M., and Atkins, P. D.,** Cyclic nucleotide binding sites of protein kinases, *Cold Spring Harbour Conf. Cell Prolif.*, 8, 45, 1981.
52. **Rannels, S. R. and Corbin, J. D.,** Two different intrachain cAMP binding sites of cAMP-dependent protein kinases, *J. Biol. Chem.*, 255, 7085, 1980.
53. **Rannels, S. R. and Corbin, J. D.,** Studies of functional domains of the regulatory subunit from cAMP-dependent protein kinase isozyme I, *J. Cycl. Nuc. Res.*, 6, 201, 1980.
54. **Yagura, T. S., Sigman, C. C., Storm, P. A., Reist, E. J., Johnson, H. L., and Miller, J. P.,** Mapping cAMP binding sites on type I and type II cAMP-dependent protein kinase using 2-substituted derivatives of cyclic AMP, *Biochem. Biophys. Res. Commun.*, 92, 463, 1980.
55. **Hoppe, J., Marutzky, R., Freist, W., and Wagner, K. G.,** Activation of the protein kinase I from rabbit skeletal muscle. (The high-affinity ATP site of the holoenzyme), *Eur. J. Biochem.*, 80, 369, 1977.
56. **Corbin, J. D. and Rannels, S. R.,** Perturbation and structural organization of the two intrachain binding sites of cAMP-dependent protein kinase II, *J. Biol. Chem.*, 256, 11671, 1981.
57. **Rannels, S. R. and Corbin, J. D.,** Characterization of small cAMP-binding fragments of cAMP-dependent protein kinase, *J. Biol. Chem.*, 254, 8605, 1979.
58. **Takio, K., Walsh, K. A., Neurath, H., Smith, S. B., Krebs, E. G., and Titani, K.,** The amino acid sequence of a hinge region in the regulatory subunit bovine cardiac of muscle cAMP-dependent protein kinase II, *FEBS Lett.*, 114, 83, 1980.
59. **Kerlavage, A. R. and Taylor, S. S.,** Covalent modification of an adenosine 3′:5′-monophosphate binding site of the regulatory subunit of cAMP-dependent protein kinase II with 8′-azidoadenosine 3′:5-monophophate identification of a single modified tyrosine residue, *J. Biol. Chem.*, 255, 8433, 1980.
60. **Rannels, S. R. and Corbin, J. D.,** Studies on the intrachain cAMP binding sites of cAMP-dependent protein kinase, *Adv. in Myocardiol.*, in press.
61. **Rannels, S. R. and Corbin, J. D.,** Binding to and activation of cAMP-dependent protein kinase by cyclic nucleotides, *Fed. Proc.*, 40(402Abst.), 1981.
62. **Hoppe, J., Lawaczeck, R., Rieke, E., and Wagner, K. G.,** Mechanism of activation of protein kinase I from rabbit skeletal muscle, *Eur. J. Biochem.*, 90, 585, 1978.
63. **Soderling, T. R.,** Regulatory functions of protein multisite phosphorylation, *Mol. Cell. Endocrinol.*, 16, 157, 1979.
64. **Hayashi, F., Akasaka, K., and Hatano, H.,** Conformation of adenosine 3′,5′-monophosphate in solution as studied by the NMR-desert method. II. Self-association and temperature-dependent glycosidic isomerication at pH 7, *Biochim. Biophys. Acta*, 588, 181, 1979.
65. **Sugden, P. H., Holladay, L. A., Reimann, E. M., and Corbin, J. D.,** Purification and characterization of the catalytic subunit of adenosine 3′-5′-cyclic monophosphate-dependent protein kinase from bovine liver, *Biochem. J.*, 159, 409, 1976.
66. **Erlichman, J., Rubin, C. S., and Rosen, O. M.,** Physical properties of a purified cAMP-dependent protein kinase from bovine heart muscle, *J. Biol. Chem.*, 248, 7607, 1973.
67. **Armstrong, R. N., Kondo, H., Granot, J., Kaiser, E. T., and Mildvan, A. S.,** Magnetic resonance and kinetic studies of the manganese II ion and substrate complexes of the catalytic subunit of adenosine 3′,5′-monophosphate-dependent protein kinase from bovine heart, *Biochemistry*, 18, 1230, 1979.
68. **Granot, J., Kondo, H., Armstrong, R. N., Mildvan, A. S., and Kaiser, E. T.,** Nuclear magnetic resonance studies of the conformation of tetraaminecobalt (III)-ATP bound at the active site of bovine heart protein kinase, *Biochemistry*, 18, 2337, 1979.
69. **Hofmann, F., Bechtel, P. J., and Krebs, E. G.,** Concentrations of cAMP-dependent protein kinase in various tissues, *J. Biol. Chem.*, 252, 1441, 1977.
70. **Peters, K. A., Demaille, J. G., and Fischer, E. H.,** Adenosine 3′:5′-monophosphate-dependent protein kinase from bovine heart, characterization of the catalytic subunit, *Biochemistry*, 16, 5691, 1977.
71. **Steiss, R. G. and Finn, F. M.,** Bovine adrenal cortical protein kinase: isolation of the type II catalytic subunit, *Biochem. Biophys. Res. Commun.*, 89, 1245, 1979.
72. **Beavo, J. A., Bechtel, P. J., and Krebs, E. G.,** Preparation of homogeneous cAMP-dependent protein kinase from rabbit skeletal muscle, *Methods Enzymol.*, 38(C), 299, 1974.
73. **Bechtel, P. J., Beavo, J. A., and Krebs, E. G.,** Purification and characterization of catalytic subunit of skeletal muscle adenosine 3′:5′-monophosphate-dependent protein kinase, *J. Biol. Chem.*, 252, 2691, 1977.

74. **Kinzel, V. and Kubler, D.,** Single step purification of the catalytic subunit(s) of cyclic 3',5'-adenosine monophosphate-dependent protein kinase(s) from rat muscle, *Biochem. Biophys. Res. Commun.*, 71, 257, 1976.
75. **Taylor, S. S. and Stafford, P. H.,** Characterization of adenosine 3':5'-monophosphate-dependent protein kinase and its dissociated subunits from porcine skeletal muscle, *J. Biol. Chem.*, 253, 2284, 1978.
76. **Reimann, E. M. and Umfleet, R. A.,** Selective precipitation of ^{32}P; onto filter papers application to ATPase and cyclic AMP phosphodiesterase determination, *Biochim. Biophys. Acta*, 523, 516, 1978.
77. **Zoller, M. J., Kerlavage, A. R., and Taylor, S. S.,** Structural comparisons of cAMP-dependent protein kinases I and II from porcine skeletal muscle, *J. Biol. Chem.*, 254, 2408, 1979.
78. **Schwoch, G., Hamaan, A., and Hilz, H.,** Antiserum against the catalytic subunit of adenosine 3':5'-cyclic monophosphate-dependent protein kinase, *Biochem. J.*, 192, 223, 1980.
79. **Yamamura, H., Kumon, A., Nishiyama, K., Takada, M., and Nishizuka, Y.,** Characterization of two cAMP-dependent protein kinases in rat liver, *Biochem. Biophys. Res. Commun.*, 45, 1560, 1971.
80. **Yamamura, H., Nishiyama, K., Shimomura, R., and Nishizuka, Y.,** Comparison of catalytic units of muscle and liver adenosine 3',5'-monophosphate-dependent protein kinases, *Biochemistry*, 12, 856, 1973.
81. **Kubler, D., Gagelman, M., Pyerin, W., and Kinzel, V.,** Isolation of the catalytic subunits of cAMP-dependent protein kinases from different mammalian tissues on the basis of charge differences of their subunits, *Hoppe-Zeyler's Z. Physiol. Chem.*, 360, 1421, 1979.
82. **Kemp, B. E., Bylund, D. B., Huang, T. S., and Krebs, E. G.,** Substrate specificity of cAMP-dependent protein kinase, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 3448, 1975.
83. **Daile, P., Carnegie, P. R., and Young, J. D.,** Synthetic substrate for cAMP-dependent protein kinase, *Nature*, 257, 416, 1975.
84. **Kemp, B. E., Benjamini, E., and Krebs, E. G.,** Synthetic hexapeptide substrates and inhibitors of cAMP-dependent protein kinase, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1038, 1976.
85. **Small, D., Chou, P. Y., and Fasman, G. D.,** Occurrence of phosphorylated residues in predicted B-turns: implications for B-turn participation in control mechanisms, *Biochem. Biophys. Res. Commun.*, 79, 341, 1977.
86. **Alhanaty, E., Bashan, N., Moser, S., and Shaltiel, S.,** Immobilized soybean trypsin inhibitor in the stabilization, resolution, and purification of adenosine 3':5'-monophosphate-dependent protein kinases, *Eur. J. Biochem.*, 101, 283, 1979.
87. **Kupfer, A., Gani, V., Jimenez, J. S., and Shaltiel, S.,** Affinity labeling of the catalytic subunit of cyclic AMP-dependent protein kinase by N^2-tosyl-L-lysine chloromethyl ketone, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 3073, 1979.
88. **Flockhart, D. A. and Corbin, J. D.,** unpublished observations.
89. **Hartley, B. S.,** Homologies in serine proteinases, *Phil. Trans. R. Soc. (London)*, B257, 77, 1970.
90. **Shoji, S., Ericcson, L. H., Demaille, J. G., Walsh, K. A., Neurath, H., Fischer, E. H., and Titani, K.,** Primary structure of catalytic subunit of bovine heart cAMP-dependent protein kinase type II, *Fed. Proc.*, 39(Abst.), 2589, 1980.
91. **Witt, J. J. and Roskoski, R., Jr.,** Bovine brain cAMP-dependent protein kinase: mechanism of action, *Biochemistry*, 14, 4503, 1975.
92. **Hixson, C. S. and Krebs, E. G.,** Affinity labeling of catalytic subunit of bovine heart muscle cAMP-dependent protein kinase, *J. Biol. Chem.*, 254, 7509, 1979.
93. **Zoller, M. J., and Taylor, S. S.,** Affinity labeling of the nucleotide binding site of the catalytic subunit of cAMP-dependent protein kinase using *p*-fluorosulfonyl-[^{14}C]-benzoyl 5'-adenosine, *J. Biol. Chem.*, 254, 8363, 1979.
94. **Severin, E. S., Sushchenko, L. P., Kochetkov, S. N., and Karochkin, S. N.,** Structure and function of protein kinase from pig brain, *Adv. Cyc. Nuc. Res.*, 9, 171, 1978.
95. **Kinzel, W. and Koenig, N.,** Interaction of protease inhibitors with the catalytic subunit of cAMP-dependent protein kinase, *Biochem. Biophys. Res. Commun.*, 93, 349, 1980.
96. **Stroud, R. M., Kay, L. M., and Dickerson, R. E.,** The crystal and molecular structure of DIP-inhibited bovine trypsin at 2.7 A resolution, *Cold Spring Harbour Symp. Quant. Biol.*, 36, 125, 1971.
97. **Demaille, J. G., Peters, K. A., and Fischer, E. H.,** Isolation and properties of the rabbit skeletal muscle protein inhibitor of cAMP-dependent protein kinase, *Biochemistry*, 16, 3080, 1977.
98. **Demaille, J. G., Ferraz, C., and Fischer, E. H.,** The protein inhibitor of cAMP-dependent protein kinase, *Biochim. Biophys. Acta*, 586, 374, 1979.
99. **Witt, J. J. and Roskoski, R, Jr.,** Cyclic AMP-dependent protein kinase: active site directed inhibition by cibacron blue F3GA, *Biochemistry*, 19, 143, 1979.

100. **Hoppe, J., Freist, W., Marutzky, R., and Shaltiel, S.,** Mapping the ATP binding site in the catalytic subunit of cAMP-dependent protein kinase: Spatial relationships with the ATP binding site of the undissociated enzyme, *Eur. J. Biochem.*, 90, 427, 1978.
101. **Flockhart, D. A., Freist, W., Hoppe, J., and Corbin, J. D.,** Use of ATP analogues to map the ATP binding sites of the cyclic nucleotide-dependent protein kinases, *Fed. Proc.*, 39(Abst.), 3165, 1980.
102. **Freist, W. and Cramer, F.,** Phenylalanyl-tRNA, lysyl t-RNA, isoleucyl-tRNA and arginyl-tRNA synthetases. Substrate specificity in the ATP/PP_i exchange with regard to ATP analogs, *Eur. J. Biochem.*, 107, 47, 1980.
103. **Hegyvary, C. and Post, R. L.,** Binding of adenosine triphosphate to sodium and potassium ion-stimulated adenosine triphosphatase, *J. Biol. Chem.*, 246, 5234, 1971.
104. **Hoppe, J. and Freist, W.,** Localization of the high affinity ATP site in adenosine 3′:5′-monophosphate-dependent protein kinase, type I. Photoaffinity labeling studies with 8-azidoadenosine S′-triphosphate, *Eur. J. Biochem.*, 93, 141, 1979.
105. **Lanir, A. and Yu, N. T.,** A raman spectroscopic study of the interaction of divalent metal ions with the adenine moiety of ATP, *J. Biol. Chem.*, 254, 5882, 1979.
106. **Tavale, S. S. and Sobell, H. M.,** Crystal and molecular structure of 8-bromoguanosine and 8-bromoadenosine, two purine nucleosides in the *syn* conformation, *J. Mol. Biol.*, 48, 109, 1970.
107. **Ikehara, M., Uesugi, S., and Yoshida, K.,** Studies on the conformation of purine nucleosides and their 5′-phosphates, *Biochemistry*, 11, 830, 1972.
108. **Pomerantz, A. H., Allfrey, V. G., Merrifield, R. B., and Johnson, E. M.,** Studies on the mechanism of phosphorylation of synthetic polypeptides by a calf thymus cyclic AMP-dependent protein kinase, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 4261, 1977.
109. **Feramisco, J. R., Glass, D. B., and Krebs, E. G.,** Optimal spatial requirements for the location of basic residues in peptide substrates for the cAMP-dependent protein kinase, *J. Biol. Chem.*, 255, 4240, 1980.
110. **Armstrong, R. N. and Kaiser, E. T.,** Sulfhydryl group reactivity of adenosine 3′:5′-monophosphate-dependent protein kinase from bovine heart, *Biochemistry*, 17, 2840, 1978.
111. **Armstrong, R. N., Kondo, H., and Kaiser, E. T.,** Cyclic AMP-dependent ATPase activity of bovine heart protein kinase, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 722, 1979.
112. **Mildvan, A. S.,** The role of metals in enzyme-catalyzed substitutions at each of the phosphorus atoms of ATP, *Adv. Enzymol.*, 49, 103, 1979.
113. **Fletterick, R. J., Sprang, S., and Madsen, N.,** Analysis of the surface topography of glycogen phosphorylase *a*: implications for metabolic interconversion and regulatory mechanisms, *Can. J. Biochem.*, 57, 789, 1979.
114. **Bolen, D. W., Stingelin, J., Bramson, H. N., and Kaiser, E. T.,** Stereochemical and kinetic studies on the action of the catalytic subunit of bovine cardiac muscle adenosine 3′,5′-monophosphate-dependent protein kinase using metal ion complexes of ATPβS, *Biochemistry*, 19, 1176, 1980.
115. **El-Maghrabi, M., Haston, W. S., Flockhart, D. A., Claus, T. H., and Pilkis, S. J.,** Studies on the phosphorylation and dephosphorylation of L-type pyruvate kinase by the catalytic subunit of cAMP-dependent protein kinase, *J. Biol. Chem.*, 255, 668, 1980.
116. **Shizuta, Y., Khandelwal, R. L., Maller, J. L., Vandenheede, J. R., and Krebs, E. G.,** Reversibility of the reaction of phosphorylase *b* kinase, *J. Biol. Chem.*, 252, 3408, 1977.
117. **Erlichman, J., Rosenfield, R., and Rosen, O. M.,** Phosphorylation of a cAMP-dependent protein kinase from bovine cardiac muscle, *J. Biol. Chem.*, 249, 5000, 1974.
118. **Rangel-Aldao, R. and Rosen, O. M.,** Mechanism of self-phosphorylation of cAMP-dependent protein kinase from bovine cardiac muscle, *J. Biol. Chem.*, 251, 7526, 1976.
119. **Flockhart, D. A., Watterson, D. M., and Corbin, J. D.,** Studies on functional domains of the regulatory subunit of bovine heart cAMP-dependent protein kinase, *J. Biol. Chem.*, 255, 4435, 1980.
120. **Takio, K., Walsh, K. A., Neurath, H., Smith, S. B., Krebs, E. G., and Titani, K.,** Amino acid sequence of a hinge region in the regulatory subunit of cAMP-dependent protein kinase, *FEBS Lett.*, 114, 83, 1980.
121. **Chiu, Y. S. and Tao, M.,** Autophosphorylation of rabbit skeletal muscle cAMP-dependent protein kinase catalytic subunit, *J. Biol. Chem.*, 253, 7145, 1978.
122. **Shoji, S., Titani, K., Demaille, J. G., and Fischer, E. H.,** Sequence of two phosphorylated sites in the catalytic subunit of bovine cardiac muscle adenosine 3′:5′-monophosphate-dependent protein kinase, *J. Biol. Chem.*, 254, 6211, 1979.
123. **Rosen, O. M., Erlichman, J., and Rubin, C. S.,** Molecular structure and characterization of bovine heart protein kinase, *Adv. Cyc. Nuc. Res.*, 5, 253, 1975.

124. **Rangel-Aldao, R. and Rosen, O. M.,** Dissociation and reassociation of phosphorylated and nonphosphorylated forms of cAMP-dependent protein kinase from bovine cardiac muscle, *J. Biol. Chem.*, 251, 3375, 1976.
125. **Rangel-Aldao, R. and Rosen, O. M.,** Effect of cAMP and ATP on reassociation of phosphorylated and nonphosphorylated subunits of cAMP-dependent protein kinase from bovine cardiac muscle, *J. Biol. Chem.*, 252, 7140, 1977.
126. **Potter, R. L., Stafford, P. H., and Taylor, S. S.,** Regulatory subunit of cAMP-dependent protein kinase I from porcine skeletal muscle: purification and proteolysis, *Arch. Biochem. Biophys.*, 190, 174, 1978.
127. **Potter, R. L. and Taylor, S. S.,** Relationships between structural domains and function in the regulatory subunit of cAMP-dependent protein kinase I and II from porcine skeletal muscle, *J. Biol. Chem.*, 254, 2413, 1979.
128. **Titani, K., Shoji, S., Ericsson, L. H., Walsh, K. A., Neurath, H., Fischer, E. H., Takio, K., Smith, S. B., Krebs, E. G., and Demaille, J. G.,** Primary structure of cAMP-dependent protein kinase type II from bovine cardiac muscle, *Cold Spring Harb. Conf. Cell Prolif.*, 8, 19, 1981.
129. **Huang, L. C., Froehlich, H. C., Charlton, J. P., and Huang, C.,** Dissociation of adenosine 3′,5′-monophosphate-dependent protein kinase, *Fed Proc.*, 36(Abst.), 2203, 1977.
130. **Tsuzuki, J. and Kieger, J. A., Jr.,** A kinetic study of cAMP binding and mode of activation of protein kinase from *drosophila melanogaster* embryos, *Biochemistry*, 17, 2961, 1978.
131. **Ogreid, D. and Doskeland, S. O.,** Protein kinase II has two distinct binding sites for cAMP, only one of which is detectable by the conventional membrane filtration method, *FEBS Lett.*, 121, 340, 1980.
132. **Feramisco, J. R. and Krebs, E. G.,** Inhibition of cAMP-dependent protein kinase by analogs of a synthetic peptide substrate, *J. Biol. Chem.*, 253, 8968, 1978.
133. **Corbin, J. D., Soderling, T. R., and Park, C. R.,** Regulation of adenosine 3′,5′-monophosphate-dependent protein kinase (I) characterization of the adipose tissue enzyme in crude extracts, *J. Biol. Chem.*, 248, 1813, 1973.
134. **Soderling, J. D., Corbin, J. D., and Park, C. R.,** Regulation of adenosine 3′,5′-monophosphate-dependent protein kinase (II) hormonal regulation of the adipose tissue enzyme, *J. Biol. Chem.*, 248, 1822, 1973.
135. **Keely, S. L., Corbin, J. D., and Park, C. R.,** Regulation of adenosine 3′,5′-monophosphate-dependent protein kinase: regulation of the heart enzyme by epinephrine, glucagon, insulin, and 1-methyl-3-isobutylxanthine, *J. Biol. Chem.*, 250, 4832, 1975.
136. **Means, A. R., MacDougall, E., Soderling, T. R., and Corbin, J. D.,** Testicular adenosine 3′:5′-monophosphate-dependent protein kinase, *J. Biol. Chem.*, 249, 1231, 1974.
137. **Costa, M., Manen, C. A., and Russell, D. H.,** *In vivo* activation of cAMP-dependent protein kinase by aminophylline and 1-methyl-3-isobutylxanthine, *Biochem. Biophys. Res. Commun.*, 65, 75, 1975.
138. **Kemp, B. E. and Clark, M. G.,** Adrenergic control of the cyclic AMP-dependent protein kinase and pyruvate kinase in isolated hepatocytes, *J. Biol. Chem.*, 253, 5147, 1978.
139. **Palmer, W. K., McPherson, J. M., and Walsh, D. A.,** Critical controls in the evaluation of cAMP-dependent protein kinase activity ratios as indices of hormonal action, *J. Biol. Chem.*, 255, 2663, 1980.
140. **Corbin, J. D., Keely, S. L., Soderling, T. R., and Park, C. R.,** Hormonal regulation of adenosine 3′,5′-monophosphate-dependent protein kinase, *Advances in Cyclic Nucleotide Research*, 5th ed., Drummond, G. I., Greengard, P., and Robison, G. A., Eds., Raven Press, New York, 1975, 265.
141. **Keely, S. L., Corbin, J. D., and Park, C. R.,** On the question of translocation of heart cAMP-dependent protein kinase, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 1501, 1975.
142. **Khac, L. D., Harbon, S., and Clauser, H. J.,** Intracellular titration of cAMP bound to receptor proteins and correlation with cyclic-AMP levels in the surviving rat diaphragm, *Eur. J. Biochem.*, 40, 177, 1973.
143. **Sala, G. B., Hagashi, K., Catt, K. J., and Dufau, M. L.,** Adrenocoricotropin action in isolated adrenal cells. The intermediate role of cAMP in stimulation of corticostèrone synthesis, *J. Biol. Chem.*, 254, 3861, 1979.
144. **Hayashi, K., Sala, C., Catt, K., and Dufau, M. L.,** Regulation of sterodogenesis in isolated adrenal cells. The intermediate role of cyclic nucleotides, *J. Biol. Chem.*, 254, 6678, 1979.
145. **Fujita, K., Aguilera, G., and Catt, K. J.,** The role of cAMP in aldosterone production by isolated zona glomerulora cells, *J. Biol. Chem.*, 254, 8567, 1979.
146. **Lincoln, T. M., Dills, W. L., Jr., and Corbin, J. D.,** Purification and subunit composition of cGMP-dependent protein kinase from bovine lung, *J. Biol. Chem.*, 252, 4269, 1977.
147. **Gill, G. N., Holdy, K. E., Walton, G. M., and Kanstein, C. B.,** Purification and characterization of cGMP-dependent protein kinase, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 3918, 1976.

148. **Flockerzi, V., Speichermann, N., and Hofmann, F.,** A cGMP-dependent protein kinase from bovine heart muscle: purification and phosphorylation of histone I and IIB, *J. Biol. Chem.*, 253, 3395, 1978.
149. **Inoue, M., Kishimoto, A., Takai, Y., and Nishizaka, Y.,** Guanosine 3′:5′ monophosphate-dependent protein kinase from silkworm: properties of a catalytic fragment obtained by limited proteolysis, *J. Biol. Chem.*, 251, 4476, 1976.
149a. **Gill, G. N., Walton, G. M., and Sperry, P.,** Guanosine 3′:5′-monophosphate-dependent protein kinase from bovine lung: subunit structure and characterization of the purified enzyme, *J. Biol. Chem.*, 252, 6443, 1977.
150. **Lincoln, T. M., Flockhart, D. A., and Corbin, J. D.,** Studies on the structure and mechanism of activation of the cGMP-dependent protein kinase, *J. Biol. Chem.*, 253, 6002, 1978.
151. **Monken, C. E. and Gill, G. N.,** Structural analysis of cGMP-dependent protein kinase using limited proteolysis, *J. Biol. Chem.*, 255, 7067, 1980.
152. **Lincoln, T. M., Hall, C. L., Park, C. R., and Corbin, J. D.,** cGMP binding proteins in rat tissues, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2559, 1976.
152a. **Yamazaki, A., Sen, I., Bitensky, M. W., Casnellie, J. E., and Greengard, P.,** Cyclic GMP-specific, high affinity, noncatalytic binding sites on light-activated phosphodiesterase, *J. Biol. Chem.*, 255, 11619, 1980.
153. **Francis, S. H., Lincoln, T. M., and Corbin, J. D.,** Characterization of a novel cGMP binding protein from rat lung, *J. Biol. Chem.*, 255, 620, 1980.
154. **Coquil, J. F., Franks, D. J., Wells, J. N., Dupuis, M., and Hamet, P.,** Characteristics of a new binding protein distinct from the kinase for guanosine 3′:5′-monophosphate in rat platelets, *Biochem. Biophys. Acta,* 631, 148, 1980.
155. **Lincoln, T. M. and Corbin, J. D.,** Hypothesis: on the role of the cAMP and cGMP-dependent protein kinase in cell function, *J. Cyc. Nuc. Res.*, 4, 3, 1978.
156. **Goldberg, N. D. and Haddox, M. K.,** Cyclic GMP metabolism and involvement in biological regulation, *Ann. Rev. Biochem.*, 46, 823, 1977.
157. **Goldberg, N. D., O'Dea, R. F., and Haddox, M. K.,** Cyclic GMP, *Adv. Cyc. Nuc. Res.*, 3, 155, 1973.
158. **Kuo, J. F., Kuo, W. N., Shoji, M., Davis, C. W., Seery, V. L., and Donelly, T. E.,** Purification and general properties of cGMP-dependent protein kinase from guinea pig fetal lung, *J. Biol. Chem.*, 251, 1759, 1976.
159. **Takai, Y., Nishiyuma, K., Yamamuru, H., and Nishizuka, Y.,** cGMP-dependent protein kinase from bovine cerebellum: purification and characterization, *J. Biol. Chem.*, 250, 4690, 1975.
160. **Lincoln, T. M. and Corbin, J. D.,** Adenosine 3′:5′-cyclic monophosphate- and guanosine 3′:5′-cyclic monophosphate-dependent protein kinases: possible homologous proteins, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 3239, 1977.
161. **Khoo, J. C., Sperry, P. J., Gill, G. N., and Steinberg, D.,** Activation of hormone-sensitive lipase and phosphorylase kinase by purified cyclic GMP-dependent protein kinase, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 4843, 1977.
162. **Cohen, P.,** Phosphorylation of rabbit skeletal muscle phosphorylase kinase by cGMP-dependent protein kinase, *FEBS Lett.*, 119, 301, 1980.
163. **Glass, D. B., and Krebs, E. G.,** Comparison of the substrate specificity of adenosine 3′:5′-monophosphate- and guanosine 3:5′-monophosphate-dependent protein kinase, *J. Biol. Chem.*, 254, 9728, 1979.
164. **Kuroda, Y., Hashimoto, E., Ku, Y., and Nishizuka, Y.,** A comment on the functional specificities of cAMP-dependent and cGMP-dependent protein kinases, *J. Biol. Chem.*, 85, 1099, 1979.
165. **Zeilig, C. E., Langan, T. A., and Glass, D. B.,** Phosphorylation of histone, H_1 by cyclic GMP and cyclic AMP-dependent protein kinases, *J. Cell. Biol.*, 83(Abstr.), 1239, 1979.
166. **Zeilig, C. E., Langon, T. A., and Glass, D. B.,** Sites in histone H_1 selectively phosphorylated by guanosine 3′:5′-monophosphate-dependent protein kinase, *J. Biol. Chem.*, 256, 994, 1981.
167. **Schlichter, D. J., Detre, J. A., Aswad, D. A., Chehrazi, B., and Greengard, P.,** Localization of cyclic GMP-dependent protein kinase and substrate in mammalian cerebellum, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 5537, 1980.
168. **Schlichter, D. J.,** An endogenous substrate for cGMP-dependent protein kinase in mammalian cerebellum, *Nature (London),* 273, 61, 1978.
168a. **Casnellie, J. E., Ives, H. E., Jamieson, J. D., and Greengard, P.,** Cyclic GMP-dependent protein phosphorylation in intact medial tissue and isolated cells from vascular smooth muscle, *J. Biol. Chem.*, 255, 3770, 1980.
169. **Geahlen, R. L. and Krebs, E. G.,** Studies on the phosphorylation of the type I cAMP-dependent protein kinase, *J. Biol. Chem.*, 255, 9375, 1980.
170. **Geahlen, R. L. and Krebs, E. G.,** Regulatory subunit of the type I cAMP-dependent protein kinase as an inhibitor and substrate of the cGMP-dependent protein kinase, *J. Biol. Chem.*, 255, 1169, 1980.

171. **Steinberg, R. A., O'Farrel, P. H., Friedrich, U., and Coffino, P.,** Mutations causing charge alterations in regulatory subunits of the cAMP-dependent protein kinase of cultured S49 lymphoma cells, *Cell,* 10, 381, 1977.
172. **Casnellie, J. E. and Greengard, P.,** Guanosine 3':5'-cyclic monophosphate-dependent phosphorylation of endogenous substrate proteins in membranes of mammalian smooth muscle, *Proc. Natl. Acad. Sci. U.S.A.,* 71, 1891, 1974.
173. **de Jonge, H. R.,** Cyclic nucleotide-dependent phosphorylation of intestinal epithelium proteins, *Nature,* 262, 590, 1976.
174. **Shlatz, L. J., Klimberg, D. V., and Cattieu, K. A.,** Cyclic nucleotide-dependent phosphorylation of rat intestinal microvillus and basal-lateral membrane proteins by an endogenous protein kinase, *Gastroenterology,* 75, 838, 1978.
175. **Schlichter, D. J., Casnellie, J. E., and Greengard, P.,** An endogenous substrate for cGMP-dependent protein kinase in mammalian cerebellum, *Nature,* 273, 61, 1978.
176. **Boehme, D. H., Kosecki, R., and Marks, N.,** Protein phosphorylation in human synaptosomal membranes. Evidence for the presence of substrates for cyclic nucleotide guanosine 3':5'-monophosphate-dependent protein kinases, *Brain Res. Bull.,* 3, 697, 1978.
177. **Ram, J. L. and Erlich, Y. H.,** Cyclic GMP-stimulated phosphorylation of membrane-bound proteins from nerve roots of *anlysia california, J. Neurochem.,* 30, 487, 1978.
178. **Johnson, E. M. and Haddon, J. W.,** Phosphorylation of lymphocyte nuclear acidic proteins: regulation by cyclic nucleotides, *Science,* 187, 1198, 1975.
179. **Hixson, C. S. and Krebs, E. G.,** Affinity labeling of the ATP binding site of bovine lung cyclic GMP-dependent protein kinase with 5'-*p*-fluorsulfonylbenzoyladenosine, *J. Biol. Chem.,* 256, 1122, 1981.
180. **Gill, G. N.,** A hypothesis concerning the structure of cAMP and cGMP-dependent protein kinases, *J. Cyc. Nuc. Res.,* 3, 153, 1977.
181. **Takai, Y., Nakaya, S., Inoue, M., Kishimoto, A., Nishiyamo, K., Yamamura, H., and Nishizuka, Y.,** Comparison of mode of action of cGMP- and cAMP-dependent protein kinase, *J. Biol. Chem.,* 251, 1481, 1976.
182. **Ramseyer, J., Kanstein, C. B., Walton, G. M., and Gill, G. N.,** The use of affinity chromatography in purification of cyclic nucleotide receptor proteins, *Biochem. Biophys. Acta,* 446, 358, 1976.
183. **Khoo, J. C. and Gill, G. N.,** Comparison of cyclic nucleotide specificity of cAMP- and cGMP-dependent protein kinase, *Biochem. Biophys. Acta,* 584, 21, 1979.
183a. **Buss, J. E., McCune, R. W., and Gill, G. N.,** Comparison of cyclic nucleotide binding to cAMP- and cGMP-dependent protein kinase, *J. Cyc. Nuc. Res.,* 5, 225, 1979.
184. **McCune, R. W., and Gill, G. N.,** Positive cooperativity guanosine 3':5'-monophosphate binding to guanosine 3'-5'-monophosphate-dependent protein kinase, *J. Biol. Chem.,* 254, 5083, 1979.
185. **de Jonge, H. R. and Rosen, O. M.,** Self-phosphorylation of cyclic guanosine 3':5'-monophosphate-dependent protein kinase from bovine lung: effect of cAMP, cGMP, and histone, *J. Biol. Chem.,* 252, 2780, 1977.
186. **Kuo, J. F., Patrick, J. G., and Seery, V. L.,** Subunit structure of cGMP-dependent protein kinase from guinea pig fetal lung: dissociation of holoenzyme by cGMP and histone, *Biochem. Biophys. Res. Commun.,* 72, 996, 1976.
187. **Shoji, M., Patrick, J. G., Tse, J., and Kuo, J. F.,** Studies on the cGMP-dependent protein kinase from bovine aorta. Possible existence of a catalytic subunit, *J. Biol. Chem.,* 252, 4347, 1977.
188. **Kuo, J. F., Shoji, M., and Kuo, W. N.,** Molecular and physiopathologic aspects of mammalian cGMP-dependent protein kinase, *Ann. Rev. Pharm. Toxicol.,* 18, 341, 1978.
189. **Yamaki, T. and Hidaka, H.,** Ca^{++}-independent stimulation of cGMP-dependent protein kinase by calmodulin, *Biochem., Biophys. Res. Commun.,* 94, 727, 1980.
190. **Walton, G. M. and Gill, G. N.,** Protein effects on the activity of cGMP-dependent protein kinase, *J. Biol. Chem.,* 255, 1603, 1980.
191. **Glass, D. B. and Miller, M. D.,** Interaction of cGMP-dependent protein kinase with a synthetic peptide inhibitor and histone H2B, *Fed. Proc.,* 39(Abstr.), 3163, 1980.
192. **Kuo, W. N., Shoji, M., and Kuo, J. F.,** Isolation of stimulating modulator from rat brain, *Biochem, Biophys. Res. Commun.,* 70, 280, 1976.
193. **Kuo, W. N. and Kuo, J. F.,** Isolation of stimulatory modulator of guanosine 3':5'-monophosphate-dependent protein kinase mammalian heart devoid of inhibitory modulator of adenosine 3':5'-monophosphate-dependent protein kinase, *J. Biol. Chem.,* 251, 4283, 1976.
194. **Shoji, M., Brackett, N. L., Tse, L., Tse, J., Shapira, R., and Kuo, J. F.,** Molecular properties and mode of action of homogenous preparation of stimulatory modulator of cyclic GMP-dependent protein kinase from the heart, *J. Biol. Chem.,* 253, 3427, 1978.

195. **Kuo, J. F., Malveaux, E. J., Patrick, J. G., Davis, C. W., Kuo, W. N., and Pruitt, A. W.,** cAMP and cGMP-dependent protein kinases, modulators and phosphodiesterases in arteries and veins of dogs. Distribution and effects of arteriovenous fistula and arterial occulsion, *Biochem. Biophys. Acta,* 497, 785, 1977.

196. **Lincoln, T. M. and Keely, S. L.,** Effects of acetylcholine and nitroprusside on cGMP-dependent protein kinase in the perfused rat heart, *J. Cyc. Nuc. Res.,* 6, 83, 1980.

197. **Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H.,** Purification and properties of rabbit skeletal muscle phosphorylase *b* kinase, *Biochemistry,* 3, 1022, 1964.

198. **Cohen, P.,** The subunit structure of rabbit skeletal muscle phosphorylase kinase, and the molecular basis of its activation reactions, *Eur. J. Biochem.,* 34, 1, 1973.

198a. **Cooper, R. H., Sul, H. S., McCullough, T. E., and Walsh, D. A.,** Purification and properties of the cardiac isoenzyme of phosphorylase kinase, *J. Biol. Chem.,* 255, 11794, 1980.

199. **Graves, D. J., Carlson, G. M., Skuster, J. R., Parrish, R. F., Carty, T. J., and Tesmer, G. W.,** Pyridoxal phosphate-dependent conformational states of glycogen phosphorylase as probed by interconverting enzymes, *J. Biol. Chem.,* 250, 2254, 1975.

200. **Chrisman, T. D., Vandenheede, J. R., Khandelwal, R. L., Gella, F. J., Clayton, J. D., and Krebs, E. G.,** Purification and properties of liver phosphorylase kinase, *Adv. Enzymol.,* 18, 145, 1980.

201. **Hayakawa, T., Perkins, J. P., Walsh, D. A., and Krebs, E. G.,** Physicochemical properties of rabbit skeletal muscle phosphorylase kinase, *Biochemistry,* 12, 567, 1973.

202. **Jennissen, H. P. and Heilmeyer, L. M. G.,** Multiple forms of phosphorylase kinase in red and white skeletal muscle, *FEBS Lett.,* 42, 77, 1974.

203. **Cohen, P. T. W., Burchell, A., and Cohen, P.,** The molecular basis of skeletal muscle phosphorylase kinase deficiency, *Eur. J. Biochem.,* 66, 347, 1975.

204. **Horl, W. H., Jennissen, H. P., and Heilmeyer, L. M. G.,** Evidence for the participation of a Ca^{++}-dependent protein kinase and a protein phosphatase in the regulation of the Ca^{++}-transport ATPase of the sarcoplasmic reticulum. I. Effects of inhibitors of Ca^{++}-dependent protein kinase and protein phosphatase. *Biochemistry,* 17, 759, 1978.

205. **Horl, W. H. and Heilmeyer, M. G.,** Evidence for participation of a calcium-dependent protein kinase and protein phosphatase in the regulation of the calcium-transport ATPase of the sarcoplasmic reticulum. II. Effect of phosphorylase kinase and phosphorylase phosphatase, *Biochemistry,* 17, 766, 1978.

206. **Wray, H. L. and Gray, R. R.,** Cyclic AMP stimulation of membrane phosphorylation and Ca^{++}-activated, Mg^{++}-dependent ATPase in cardiac sarcoplasmic reticulum, *Biochim. Biophys. Acta,* 461, 441, 1977.

207. **LePeuch, C. J., Haiech, J., and Demaille, J. G.,** Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cAMP-dependent and calcium-calmodulin-dependent phosphorylations, *Biochemistry,* 18, 5150, 1979.

208. **Kirchberger, M. A. and Tada, M.,** Effects of adenosine 3':5'-monophosphate-dependent protein kinase on sarcoplasmic reticulum isolated from cardiac and slow and fast contracting skeletal muscle, *J. Biol. Chem.,* 251, 725, 1976.

209. **Tada, M., Ohmori, F., Yamada, M., and Abe, H.,** Mechanism of the stimulation of Ca^{2+}-dependent ATPase of cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase, *J. Biol. Chem.,* 254, 319, 1979.

210. **Cohen, P.,** The role of calcium ions, calmodulin and troponin in the regulation of phosphorylase kinase from rabbit skeletal muscle, *Eur. J. Biochem.,* 111, 563, 1980.

211. **Grand, R. J. A., Shenolikar, S., and Cohen, P.,** The amino acid sequence of the δ subunit (calmodulin) of rabbit skeletal muscle phosphorylase kinase, *Eur. J. Biochem.,* 113, 359, 1981.

212. **Skuster, J. R., Cahn, K. F. J., and Graves, D. J.,** Isolation and properties of catalytically active γ subunit of phosphorylase *b* kinase, *J. Biol. Chem.,* 255, 2203, 1980.

213. **Lyon, J. B. and Porter, J.,** The regulation of phosphorylase and glycogenolysis in skeletal muscle and heart of mice, *J. Biol. Chem.,* 238, 1, 1963.

214. **Cohen, P. T. W. and Cohen, P.,** Skeletal muscle phosphorylase kinase deficiency: detection of a protein lacking any activity in ICR/IAn mice, *FEBS Lett.,* 29, 113, 1973.

215. **Malthus, R., Clark, D. G., Watts, C., and Sneyd, J. G. T.,** Glycogen-storage disease in rats, a genetically determined deficiency of liver phosphorylase kinase, *Biochem. J.,* 188, 99, 1980.

216. **Meyer, W. L. and Fischer, E. H.,** Activation of skeletal muscle phosphorylase *b* kinase by Ca^{++}, *Biochemistry,* 3, 1033, 1964.

217. **Ozawa, E., Hosoi, K., and Ebashi, S.,** Reversible stimulation of muscle phosphorylase kinase by low concentrations of calcium ions, *J. Biochem. (Tokyo),* 61, 531, 1967.

218. **Yeaman, S. J. and Cohen, P.,** The hormonal control of activity of skeletal muscle phosphorylase kinase, phosphorylation of the enzyme at two sites *in vivo* in response to adrenalin, *Eur. J. Biochem.*, 51, 93, 1975.
219. **Clerch, L. B. and Huijing, F.,** The role of magnesium in muscle phosphorylase kinase activity, *Biochim. Biophys. Acta*, 268, 654, 1972.
220. **Wang, J. H., Stull, J. T., Huang, T. S., and Krebs, E. G.,** A study on the autoactivation of rabbit muscle phosphorylase kinase, *J. Biol. Chem.*, 251, 4521, 1976.
221. **Tu, J. and Graves, D. J.,** Inhibition of the phosphorylase kinase catalyzed reaction by glucose-6-phosphate, *Biochem. Biophys. Res. Commun.*, 53, 59, 1973.
222. **Singh, T. J. and Wang, J. H.,** Stimulation of glycogen phosphorylase kinase from rabbit skeletal muscle by organic solvents, *J. Biol. Chem.*, 254, 8466, 1979.
223. **Cohen, P.,** Phosphorylation of rabbit skeletal muscle phosphorylase kinase by cyclic GMP-dependent protein kinase, *FEBS Lett.*, 119, 301, 1980.
224. **Picton, C., Klee, C. B., and Cohen, P.,** Phosphorylase kinase from rabbit skeletal muscle: identification of the calmodulin-binding subunits, *Eur. J. Biochem.*, 111, 553, 1980.
225. **Cohen, P., Picton, C., and Klee, C. B.,** Activation of phosphorylase kinase from rabbit skeletal muscle by calmodulin and troponin, *FEBS Lett.*, 104, 25, 1979.
226. **Watterson, D. M., Harrelson, W. G., Keller, P. M. Sharief, F., and Vanaman, T. C.,** Structural similarities between the Ca^{++}-dependent regulatory proteins of 3′:5′-cyclic nucleotide phosphodiesterase bind actomyosin ATPase, *J. Biol. Chem.*, 251, 6501, 1976.
227. **Watterson, D. M., Sharief, F., and Vanaman, T. C.,** The complete amino acid sequence of the Ca^{++}-dependent modulator (calmodulin) of bovine brain, *J. Biol. Chem.*, 255, 962, 1980.
228. **Baker, P. F., Hodgkin, A. L., and Ridgeway, E. B.,** Depolarization and calcium entry in squid giant axons, *J. Physiol.*, 218, 709, 1971.
229. **Kretsinger, R. H.,** The informational role of calcium in the cytosol, *Adv. Cyc. Nuc. Res.*, 11, 1, 1979.
230. **Klee, C. B., Crouch, T. H., and Richmann, P. G.,** Calmodulin, *Annu. Rev. Biochem.*, 49, 489, 1980.
231. **Adelstein, R. S. and Eisenberg, E.,** Regulation of kinetics of the actin-myosin-ATP interaction, *Annu. Rev. Biochem.*, 49, 921, 1980.
232. **Brostrom, C. O., Hunkeler, F. L., and Krebs. E. G.,** The requirement of phosphorylase kinase for calcium, *J. Biol. Chem.*, 246, 1961, 1971.
233. **Kim, G. and Graves, J.,** On the hysteretic response of rabbit skeletal muscle phosphorylase kinase, *Biochemistry*, 12, 2090, 1973.
234. **Carlson, G. M. and Graves, D. J.,** Stimulation of phosphorylase kinase autophosphorylation by peptide analogs of phosphorylase, *J. Biol. Chem.*, 251, 7480, 1976.
235. **Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S., and Krebs, E. G.,** Catalysis of the phosphorylase kinase activation reaction, *J. Biol. Chem.*, 246, 1968, 1971.
236. **Horl, W. H., Jennissen, H. P., Groschel-Stewart, U., and Heilmeyer, L. M. G.,** in *Calcium Transport in Contraction and Secretion*, North-Holland, Amsterdam, 1975, 535.
237. **DeLange, R. J., Kemp, R. T., Riley, W. D., Cooper, R. A., and Krebs, E. G.,** Activation of skeletal muscle phosphorylase kinase by ATP and adenosine 3′:5′-monophosphate, *J. Biol. Chem.*, 243, 2200, 1968.
238. **Huang, T. S., Bylund, D. B., Stull, J. T., and Krebs, E. G.,** The amino acid sequences of the phosphorylated sites in troponin-I from rabbit skeletal muscle, *FEBS Lett.*, 42, 249, 1974.
239. **Stull, J. T., Brostrom, C. O., and Krebs, E. G.,** Phosphorylation of the inhibitor component of troponin by phosphorylase kinase, *J. Biol. Chem.*, 247, 5272, 1972.
240. **Schlender, K. K., Weig, S. H., and Villar-Pilasi, C.,** UDP-glucose: glycogen α-4-glucosyl transferase I kinase. Activity of purified muscle protein kinase cyclic nucleotide specificity, *Biochim. Biophys. Acta*, 191, 272, 1969.
241. **Perry, S. V. and Cole, H. A.,** Phosphorylation of troponin and the effects of interactions between the components of the complex, *Biochem. J.*, 141, 733, 1974.
242. **Schwartz, A., Entman, M. L., Komike, K., Lane, L. K., Moir, A. J. G., Cole, H. A., and Perry, S. V.,** The phosphorylation sites of troponin T from white skeletal muscle and the effects of interaction with troponin C on their phosphorylation by phosphorylase kinase, *Biochem. J.*, 161, 371, 1977.
243. **Krebs, E. G., Stull, J. T., England, P. J., Huang, T. S., Brostrom, C. O., and Vandenheede, J. R.,** The regulation of muscle metabolism and function by protein phosphorylation in *Protein Phosphorylation in Control Mechanisms*, Huijing, F. and Lee, E. Y. C., Eds., Academic Press, New York, 1973, 31.
244. **Schwartz, A., Entman, M. L., Komike, K., Lane, L. K., Van Winkle, W. B., and Bornet, E. P.,** The rate of calcium uptake into sarcoplasmic reticulum of cardiac muscle and skeletal muscle. Effects of cAMP-dependent protein kinase and phosphorylase *b* kinase, *Biochim. Biophys. Acta*, 426, 57, 1976.
245. **Sevilla, C. L. and Fischer, E. H.,** The purification and properties of rat muscle glycogen phosphorylase, *Biochemistry*, 8, 2161, 1969.

246. **DePaoli-Roach, A. A., Roach, P. J., and Larner, J.,** Rabbit skeletal muscle phosphorylase kinase: comparison of glycogen synthase and phosphorylase as substrates, *J. Biol. Chem.,* 254, 4212, 1979.
247. **Soderling, T. R., Srivastava, A. K., Bass, M., and Khatra, B. S.,** Phosphorylation and inactivation of glycogen synthase by phosphorylase kinase, *Proc. Natl. Acad. Sci. U.S.A.,* 76, 2536, 1979.
248. **Soderling, T. R., Sheorain, V. S. and Ericsson, L. H.,** Phosphorylation of glycogen synthase: stoichiometry, specificity and site of phosphorylation, *FEBS Lett.,* 106, 181, 1979.
249. **Walsh, K. X., Millikin, D. M., Schlender, K. K., and Reimann, E. M.,** Calcium-dependent phosphorylation of glycogen synthase by phosphorylase kinase, *J. Biol. Chem.,* 254, 6611, 1979.
249a. **Embdi, N., Rylatt, D. B., and Cohen, P.,** Glycogen synthase kinase 2 and phosphorylase kinase are the same enzyme, *Eur. J. Biochem.,* 100, 339, 1979.
250. **Gulyaeva, N. B., Guskova, R. A., Baranova, L. A., Gulyaev, N. N., Val'fson, P. L., and Severin, E. S.,** *Dokl. Akad. Nauk. U.S.S.R.,* 235, 696, 1977.
251. **Fischer, E. H., Blum, H. E., Byers, B., Heizmann, C., Kerrick, G. W., Lehky, P., Malencik, D. A., and Pocinwong, S.,** in *Metabolic Interconversion of Enzymes, 1975,* Shaltiel, E., Ed., Springer-Verlag, New York, 1976, 1.
252. **Dickneite, G., Jennissen, H. P., and Heilmeyer, L. M. G., Jr.,** Differentiation of two catalytic sites on muscle phosphorylase kinase for phosphorylase *b* and troponin T phosphorylation, *FEBS Lett.,* 87, 297, 1978.
253. **Tessmer, G. W., Skuster, J. R., Tabatabai, L. B., and Graves, D. J.,** Studies on the specificity of phosphorylase kinase using peptide substrates, *J. Biol. Chem.,* 252, 5666, 1977.
254. **Tabatabai, L. B. and Graves, D. J.,** Kinetic mechanism and specificity of the phosphorylase kinase reaction, *J. Biol. Chem.,* 253, 2196, 1978.
255. **Tessmer, G. W. and Graves, D. J.,** The phosphorylase kinase reaction on a peptide derived from glycogen phosphorylase, *Biochem. Biophys. Res. Commun.,* 50, 1, 1973.
256. **Graves, D. J., Uhing, R. J., Janski, A. M., and Viriya, J.,** Use of a double-headed peptide substrate to study the specificity of cAMP-dependent protein kinase and phosphorylase kinase, *J. Biol. Chem.,* 253, 8010, 1978.
257. **Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G.,** Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase, *J. Biol. Chem.,* 252, 4888, 1977.
258. **Shizuta, Y., Khandelwal, R. L., Maller, J. L., Vandenheede, J. R., and Krebs, E. G.,** Reversibility of phosphorylase kinase reaction, *J. Biol. Chem.,* 252, 3408, 1977.
259. **Hartshorne, D. J. and Pereschini, A. J.,** Phosphorylation of myosin as a regulatory component in smooth muscle, *Ann. N.Y. Acad. Sci.,* 356, 130, 1980.
260. **Adelstein, R. S., Conti, M. A., and Pato, M. D.,** Regulation of myosin light chain kinase by reversible phosphorylation and calcium-calmodulin, *Ann. N.Y. Acad, Sci.,* 356, 142, 1980.
261. **Ebashi, S., Mikawa, T., Hirata, M., Toyo-oka, T., and Nonomura, Y.,** Regulatory proteins of smooth muscle, in *Excitation-Contraction Coupling in Smooth Muscle,* Casteels, R., Godfraind, T., and Ruegg, J. C., Eds., Elsevier/North-Holland, Amsterdam, 1977, 325.
262. **Mikawa, T., Nonomura, Y., Hirata, M., Ebashi, S., and Kakiuchi, S.,** Involvement of an acidic protein in regulation of smooth muscle contraction by the tropomyosin-leiotonin system, *Biochem. (Tokyo),* 86, 1633, 1978.
263. **Scordilis, S. P. and Adelstein, R. S.,** A comparative study of the myosin light chain kinases from myoblast and muscle sources: studies on the kinases from proliferative rat myoblasts in culture, rat thigh muscle and rabbit skeletal muscle, *J. Biol. Chem.,* 253, 9041, 1978.
264. **Hartshorne, D. J. and Gorecka, A.,** *Handbook of Physiology,* Berne, R. M., Somlyo, A. P., and Sparks, H. V., Eds., American Physiology Society, Bethesda, Md., 1980, 93.
265. **Dabrowska, R., Sherry, J. M. F., Aramatorio, D., and Hartshorne, D. J.,** Chicken gizzard myosin light chain kinase depends on calmodulin for activity, *Biochemistry,* 17, 253, 1978.
265a. **Adelstein, R. S. and Conti, M. A.,** Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity, *Nature,* 256, 597, 1975.
266. **Yerna, M. J., Dabrowska, R., Hartshorne, D. J., and Goldman, R. D.,** Calcium-sensitive regulation of actin-myosin interactions in baby hamster kidney (BHK-21) cells, *Proc. Natl. Acad, Sci. U.S.A.,* 76, 184, 1979.
267. **Pires, E. M. V. and Perry, S. V.,** Purification and properties of myosin light-chain kinase from fast skeletal muscle, *Biochem. J.,* 167, 137, 1977.
268. **Aksoy, M. O., Williams, D., Sharkey, E. M., and Hartshorne, D. J.,** A relationship between calcium sensitivity and phosphorylation of gizzard actomyosin, *Biochem. Biophys. Res. Commun.,* 69, 35, 1976.
269. **Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M., and Uenishi, K.,** Identification of an activator protein for myosin light chain kinase as calmodulin, *J. Biol. Chem.,* 253, 1338, 1978.

270. **Walsh, M. P., Vallet, B. V., Autric, F., and Demaille, J. G.,** Purification and characterization of bovine cardiac CDR-dependent myosin light chain kinase, *J. Biol. Chem.*, 254, 12136, 1979.
271. **Wolf, H. and Hofmann, F.,** Purification of myosin light chain kinase from bovine cardiac muscle, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 5852, 1980.
272. **Adelstein, R. S., Conti, M. A., Hathaway, D. R., and Klee, C. B.,** Phosphorylation of smooth muscle myosin light chain kinase by the catalytic subunit, *J. Biol. Chem.*, 253, 8347, 1978.
273. **Hathaway, D. R. and Adelstein, R. S.,** Human platelet myosin light chain kinase requires calmodulin for activity, *Proc. Natl., Acad. Sci. U.S.A.*, 76, 1653, 1979.
274. **Dabrowska, R., Sherry, J. M. F., and Hartshorne, D. J.,** in *Motility in Cell Function,* John M. Marshall Symp. Cell Biol., Academic Press, New York, 1978.
275. **Dabrowska, R. and Hartshorne, D. J.,** A Ca^{++}-and modulator-dependent myosin light chain kinase from nonmuscle cells, *Biochem. Biophys. Res. Commun.*, 85, 1352, 1979.
276. **Adelstein, R. S., Conti, M. A., Rappaport, L., and Eaton, C. R.,** Regulation of myosin light chain kinase in muscle and nonmuscle cells, *Fed. Proc.*, 39 (Abstr.), 2702, 1980.
277. **Nairn, A. C. and Perry, S. V.,** Calmodulin and myosin light chain kinase of rabbit fast skeletal muscle, *Biochem. J.*, 179, 89, 1979.
278. **Barylko, B., Kuznicki, J., and Drabikowski, W.,** Identification of calcium-binding subunit of myosin light chain kinase from skeletal muscle with modulator protein, *FEBS Lett.*, 90, 301, 1978.
279. **Blumenthal, D. K. and Stull, J. T.,** Activation of skeletal muscle myosin light chain kinase by calcium (2^+) and calmodulin, *Biochemistry,* 19, 5608, 1980.
280. **Cheung, W. Y.,** Calmodulin plays a pivotal role in cellular regulation, *Science,* 207, 19, 1980.
281. **Wang, J. H. and Waisman, D. M.,** Calmodulin and its role in the second-messenger system, *Curr. Top. Cell. Reg.*, 15, 47, 1979.
282. **Wolff, D. J. and Brostrom, C. O.,** Properties and functions of the calcium-dependent regulator protein, *Adv. Cyc. Nuc. Res.*, 11, 27, 1979.
283. **Stull, J. T., Manning, D. R., High, C. W., and Blumenthal, D. K.,** Phosphorylation of contractile proteins in heart and skeletal muscle, *Fed. Proc.*, 39, 1552, 1980.
284. **Pemrick, S. M.,** The phosphorylated L_2 light chain of skeletal myosin is a modifier of the actomyosin ATPase, *J. Biol. Chem.*, 255, 8836, 1980.
285. **Barany, K. and Barany, M.,** Phosphorylation of the 18,000-dalton light chain of myosin during a single tetanus of frog muscle, *J. Biol. Chem.*, 252, 4752, 1977.
285a. **Kopp. S. J. and Barany, M.,** Phosphorylation of the 19,000-dalton light chain of myosin in perfused rat heart under the influence of negative and positive inotropic agents, *J. Biol. Chem.*, 254, 12007, 1979.
286. **Barany, K., Barany, M., Gillis, J. M., and Kushmerick, M. J.,** Phosphorylation-dephosphorylation of the 18,000-dalton light chain of myosin during the contraction-relaxation cycle of frog muscle, *J. Biol. Chem.*, 254, 3617, 1979.
287. **Stull, J. T. and High, C. W.,** Phosphorylation of skeletal muscle contractile proteins *in vivo, Biochem., Biophys. Res. Commun.*, 77, 1078, 1977.
288. **Adelstein, R. S., Conti, M. A., Hathaway, D. R., Eaton, R. C., and Klee, C. B.,** The role of calcium and phosphorylation in regulation of myosin light chain kinase, *Proc. 11th Int. Congr. Biochem.*, Canadian Biochemical Society, Toronto, 1979.
289. **Stull, J. T.,** Phosphorylation of contractile proteins in relation to muscle function, *Adv. Cyc. Nuc. Res.*, 13, 39, 1980.
290. **Suzuyama, Y., Umegane, T., Maita, T., and Matsuda, G.,** The amino acid sequence of the L-2 light chain of chicken skeletal muscle myosin, *Hoppe, Seyler's, Z. Physiol. Chem.*, 361, 119, 1980.
291. **Perrie, W. T., Smillie, L. B., and Perry, S. V.,** A phosphorylated light chain component of myosin from skeletal muscle, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 17, 1972.
292. **Collins, J. H.,** Homology of myosin DTNB light chain with alkali light chains, troponin C and parvalbumin, *Nature,* 259, 699, 1976.
293. **Matsuda, G., Maita, T., Suzuyama, Y., Setoguchi, Mr., and Umegane, T.,** Amino acid sequence of the L-2 light chain of rabbit skeletal muscle myosin, *J. Biochem. (Tokyo),* 81, 809, 1977.
294. **Jakes, R., Northrop, F., and Kendrick-Jones, J.,** Calcium binding regions of myosin regulatory light chains, *FEBS Lett.*, 70, 229, 1976.
295. **Noiman, E. S.,** Phosphorylation of smooth muscle myosin light chains by cAMP-dependent protein kinase, *J. Biol. Chem.*, 255, 11067, 1980.
296. **Hartshorne, D. J., Siemankowski, R. F., and Aksoy, M. O.,** in *Regulatory Mechanisms of Muscle Contraction,* Ebashi, S., Maruyama, K., and Endo, M., Eds., Springer-Verlag, Berlin, 1980.
297. **Yazawa, M. and Yagi, K.,** Purification of modulator-deficient myosin light-chain kinase by modulator protein-sepharose affinity chromatography, *J. Biochem. (Tokyo),* 84, 1259, 1978.
298. **Tanaka, T., Naka, M., and Hidaka, H.,** Activation of myosin light chain kinase by trypsin, *Biochem. Biophys. Res. Commun.*, 92, 313, 1980.

299. **Barany, M. and Barany, K.,** Phosphorylation of the myofibrillar proteins, *Ann. Rev. Physiol.,* 42, 275, 1980.
300. **Conti, M. A. and Adelstein, R. S.,** Phosphorylation by cyclic adenosine 3′:5′-monophosphate-dependent protein kinase regulates myosin light chain kinase, *Fed. Proc.,* 39, 1569, 1980.
301. **Kramer, G., Cimedevilla, J. M., and Hardesty, B.,** Specificity of the protein kinase activity associated with the hemin-controlled repressor of rabbit reticulocytes, *Proc. Natl. Acad. Sci. U.S.A.,* 73, 3078, 1976.
302. **Ranu, R. S. and London, I. M.,** Regulation of protein synthesis in rabbit reticulocyte lysates—purification and initial characterization of cyclic 3′:5′-AMP-independent protein kinase of the heme-regulated translational inhibitor (phosphorylation of Met-tRNA$_f$ binding factor), *Proc. Natl. Acad. Sci., U.S.A.,* 73, 4349, 1976.
303. **Farrel, P. J., Balkow, K., Hent, T., and Jackson, R.,** Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis, *Cell,* 11, 187, 1977.
304. **Trachsel, H., Ranu, R. S., and London, I. M.,** Regulation of protein synthesis in rabbit reticulocyte lysates: purification and characterization of heme-reversible translational inhibitor, *Proc. Natl. Acad. Sci. U.S.A.,* 75, 3654, 1978.
305. **Kramer, G. Henderson, A. B., Grankowski, N., and Hardesty, B.,** Translational control in eukaryotes in: *Ribosomes Structure, Function and Genetics,* Chambtis, G., Craven, G. R., Davis, J., Davies, K., Kahan, L., and Nomura, M., Eds., University Park Press, Baltimore, 1980, 825.
306. **Rabinovitz, M., Freedman, M. L., Fisher, J. M., and Maxwell, C. R.,** *Cold Spring Harbour Symp. Quant Biol.,* 34, 567, 1969.
307. **Trachsel, H., Ranu, R. S., and London, I. M.,** Purification of the heme-reversible form of the translational inhibitory protein kinase, *Methods Enzymol.,* 60, 485, 1979.
308. **Ranu, R. S. and London, I. M.,** Regulation of protein synthesis in rabbit reticulocyte lysates—preparation of efficient protein synthesis lysates and the purification and characterization of the heme-regulated translational inhibitory protein kinase, *Methods Enzymol.,* 60, 459, 1979.
309. **Gross, M. and Rabinovitz, M.,** Control of globin synthesis by hemin: factors influencing formation of an inhibitor of globin chain initiation in reticulocyte lysates, *Biochim. Biophys. Acta,* 287, 340, 1972.
310. **Maxwell, C. R., Kamper, C. S., and Rabinovitz, M.,** Hemin control of globin synthesis: an assay for the inhibitor formed in the absence of hemin and some characteristics of its formation, *J. Mol. Biol.,* 58, 317, 1971.
311. **Levin, D., Ernst, V., and London, I. M.,** Effects of the catalytic subunit of cAMP-dependent protein kinase (type II) from reticulocytes and bovine heart muscle on protein phosphorylation and protein synthesis in reticulocyte lysates, *J. Biol. Chem.,* 254, 7935, 1979.
312. **Ernest, V., Levin, D. H., and London, I. M.,** Evidence that glucose-6-phosphate regulates protein synthesis initiation, *J. Biol. Chem.,* 253, 7163, 1980.
313. **Ernst, V., Levin, D. H., and London, I. M.,** Inhibition of protein synthesis initiation by oxidized glutathione: activation of a protein kinase that phosphorylates the α-subunit of eukaryotic initiation factor 2, *Proc. Natl. Acad. Sci. U.S.A.,* 75, 4110, 1978.
314. **Grankowski, N., Kramer, G., and Hardesty, B.,** No effect of cAMP on protein synthesis in reticulocyte lysates, *J. Biol. Chem.,* 254, 3145, 1979.
315. **Ernst, V., Levin, D. H., Leroux, A., and London, I. M.,** Site-specific phosphorylation of the α-subunit of eukaryotic initiation factor eIF-2 by the heme-regulated and double-stranded RNA-activated eIF-2α kinases from rabbit reticulocyte lysates, *Proc. Natl. Acad. Sci. U.S.A.,* 77, 1286, 1980.
316. **Ranu, R. S.,** Regulation of protein synthesis in rabbit reticulocyte lysates—the heme-regulated protein kinase (HRI) and double-stranded RNA-induced protein kinase (dRI) phosphorylates the same sites on initiation factor 2, *Biochem. Biophys. Res. Commun.,* 91, 1437, 1979.
317. **Levin, D. and London, I. M.,** Regulation of protein synthesis: activation by double-stranded RNA of a protein kinase that phosphorylates eukaryotic initiation factor 2, *Proc. Natl. Acad. Sci. U.S.A.,* 75, 1121, 1978.
318. **Grosfeld, H. and Ochoa, S.,** Purification and properties of the double-stranded RNA-activated eukaryotic initiation factor 2 kinase from rabbit reticulocytes, *Proc. Natl Acad. Sci. U.S.A.,* 77, 6526, 1980.
319. **Ranu, R. S.,** Regulation of protein synthesis in rabbit reticulocyte lysates: purification and initial characterization of the double-stranded RNA-activated protein kinase, *Biochem. Biophys. Res. Commun.,* 97, 252, 1980.
320. **Levin, D. H., Petryshyn, R., and London, I. M.,** Characterization of double-stranded RNA-activated kinase that phosphorylates α subunit of eukaryotic initiation factor 2 (eIF-2α) in reticulocyte lysates, *Proc. Natl. Acad. Sci. U.S.A.,* 77, 832, 1980.
321. **Petryshyn, R., Levin, D. H., and London, I. M.,** Purification and characterization of a latent precursor of a double-stranded RNA-dependent protein kinase from reticulocyte lysates, *Biochem. Biophys. Res. Commun.,* 94, 1190, 1980.

322. **Petryshyn, R., Trachsel, H., and London, I. M.,** Regulation of protein synthesis in reticulocyte lysates: immune serum inhibits heme-regulated protein kinase activity and differentiates heme-regulated protein kinase from double-stranded RNA-induced protein kinase, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 1575, 1979.
323. **Lebleu, B., Sen, G. C., Shaila, S., Carbrer, B., and Lengyel, P.,** Interferon, double-stranded RNA, and protein phosphorylation, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 3107, 1976.
324. **Sen, G. C., Taira, H., and Lengyel, P.,** Interferon, double-stranded RNA, and protein phosphorylation, *J. Biol. Chem.*, 253, 5915, 1978.
325. **Zilberstein, A., Kimchi, A., Schmidt, A., and Revel, M.,** Isolation of two interferon-induced translational inhibitors: a protein kinase and oligo-isoadenylate synthetase, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 4734, 1978.
326. **Farrell, P. J., Sen, G. C., Dubois, M. F., Ratner, L., Slattery, E., and Lengyel, P.,** Interferon action: two distinct pathways for inhibition of protein synthesis by double-stranded RNA, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 5893, 1978.
327. **Lerch, K., Muir, L. W., and Fischer, E. H.,** Purification and properties of a yeast protein kinase, *Biochemistry*, 14, 2015, 1975.
328. **Dahmus, M. E.,** Stimulation of ascites tumor RNA polymerase II by protein kinase, *Biochemistry*, 15, 1821, 1976.
329. **Hathaway, G. M. and Traugh, J. A.,** Cyclic nucleotide-independent protein kinases from rabbit reticulocytes, *J. Biol. Chem.*, 254, 762, 1979.
330. **Dahmus, M. E. and Natzle, J.,** Purification of Novikoff ascites tumor protein kinase, *Biochemistry*, 16, 1901, 1977.
331. **Thornburg, W. and Lindell, T. J.,** Purification of rat liver nuclear protein kinase N II, *J. Biol. Chem.*, 252, 6660, 1977.
332. **Tuazon, P. T. and Traugh, J. A.,** Site-specific phosphorylation of β-casein by protein kinases from rabbit reticulocytes, *J. Biol. Chem.*, 253, 1746, 1978.
333. **Tuazon, P. T., Bingham, E. W., and Traugh, J. A.,** Cyclic nucleotide-independent protein kinases from rabbit reticulocytes, *Eur. J. Biochem.*, 94, 497, 1979.
334. **Traugh, J. A., Hathaway, G. M., Tuazon, P. T., Tahara, S. M., Floyd, G. A., Del Grande, R. W., and Lundak, T. S.,** Cyclic nucleotide-independent protein kinases from rabbit reticulocytes and phosphorylation of translational components, *ICN-UCLA Winter Symp.*, 3, 233, 1979.
335. **Tahara, S. M., Traugh, J. A., Sharp, S. B., Lundak, T. S., Safer, B., and Merrick, W. C.,** Effect of hemin on site-specific phosphorylation of eukaryotic initiation factor 2, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 789, 1978.
336. **Tuazon, P. T., Merrick, W. C., and Traugh, J. A.,** Site-specific phosphorylation of initiation factor 2 by three cyclic nucleotide-independent protein kinases, *J. Biol. Chem.*, 255, 10954, 1980.
337. **Lundak, T. S. and Traugh, J. A.,** Purification and characterization of the hemin-controlled repressor from rabbit reticulocytes, *Proc. Phosp. Bio-Reg.*, FMI-EMBO Workshop, 1979, 154.
338. **Hathaway, G. M., Lubben, T. H., and Traugh, J. A.,** Inhibition of casein kinase II by heparin, *J. Biol. Chem.*, 256, 27, 1980.
339. **Feige, J. J., Pirollet, F., Cochet, C., and Chambaz, E. M.,** Selective inhibition of a cyclic nucleotide-independent protein kinase (G-type casein kinase) by naturally occurring glycosaminoglycans, *FEBS Lett.*, 121, 139, 1980.
340. **Erikson, R. L., Collett, M. S., Erikson, E., and Purchio, A. F.,** Evidence that the avian sarcoma virus transforming gene product is a cyclic AMP-independent protein kinase, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 6260, 1979.
341. **Hunter, T. and Sefton, B. M.,** Transforming gene product of Rous sarcoma virus phosphorylates tyrosine, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 1311, 1980.
342. **Levinson, A. D., Oppermann, H., Varmus, H. E., and Bishop, J. M.,** The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine, *J. Biol. Chem.*, 255, 11973, 1980.
343. **Reynolds, F. H., Van de Ven, W. J. M., and Stephenson, J. R.,** Feline sarcoma virus P115-associated protein kinase phosphorylates tyrosine, *J. Biol. Chem.*, 255, 11040, 1980.
344. **Sefton, B. M., Hunter, T., and Raschke, W. C.,** Evidence that the Abelson virus protein functions in vivo as a protein kinase which phosphorylates tyrosine, *Virology*, in press.
345. **Collett, M. S., Brugge, J. S., and Erikson, R. L.,** Characterization of a normal avian cell protein related to the avian sarcoma virus transforming gene product, *Cell*, 15, 1363, 1978.
346. **Collett, M. S., Erikson, E., Purchio, A. F., Brugge, J. S., and Erikson, R. L.,** A normal cell protein similar in structure and function to the avian sarcoma virus transforming gene product, *Proc. Natl. Acad. Sci. U.S.A.*, 76, 3159, 1979.
347. **Sefton, B. M., Hunter, T., and Beemon, K.,** Relationship of polypeptide products of the transforming gene of Rous sarcoma virus and the homologous gene of vertebrates, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 2059, 1980.

348. **Erikson, E. and Erikson, R. L.,** Identification of a cellular protein substrate phosphorylated by the avian sarcoma virus-transforming gene product, *Cell,* 21, 829, 1980.
349. **Radke, K. and Martin, G. S.,** Transformation of Rous sarcoma virus: effects of *src* gene expression on the synthesis and phosphorylation of cellular polypeptides, *Proc. Natl. Acad. Sci. U.S.A.,* 76, 5212, 1979.
350. **Sefton, B. M. and Hunter, T.,** Vinculin: a cytoskeletal substrate of the transforming protein of Rous sarcoma virus, *Cell,* in press.
351. **Collett, M. S., Purchio, A. F., and Erikson, R. L.,** Avian sarcoma and virus-transforming protein, pp60^{src} shows protein kinase activity specific for tyrosine, *Nature,* 285, 167, 1980.
352. **Smart, J. E., Oppermann, H., Czernilofsky, A. P., Purchio, A. P., Erikson, R. L., and Bishop, J. M.,** Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus and its normal cellular homologue, *Proc. Natl. Acad. Sci. U.S.A.,* 78, 6013, 1981.
353. **Krzyzek, R. A., Mitchell, R. L., Lau, A. F., and Faras, A. J.,** Association of pp60^{src} and *src* protein kinase activity with the plasma membrane of nonpermissive and permissive avian sarcoma virus-infected cells, *J. Virol.,* 36, 805, 1980.
354. **Willingham, M. C., Jay, G., and Pastan, I.,** *Cell,* 18, 125, 1979.
355. **Courtneidge, S. A., Levinson, A. D., and Bishop, J. M.,** The protein encoded by the transforming gene of avian sarcoma virus (pp60^{src}) and homologous protein in normal cells (pp60$^{proto-src}$) are associated with the plasma membrane, *Proc. Natl. Acad. Sci. U.S.A.,* 77, 3783, 1980.
356. **Maness, P. F., Engeser, H., Greenberg, M. E., O'Farell, M. O., Gall, W. E., and Edelman, G. M.,** Characterization of the protein kinase activity of avian sarcoma virus *src* gene product, *Proc. Natl. Acad. Sci. U.S.A.,* 76, 5028, 1979.
356a. **Levinson, A. D., Courtneidge, S. A., and Bishop, J. M.,** Structural and functional domains of the Rous sarcoma virus transforming protein (pp60^{src}), *J. Virol.,* in press.
357. **Hunter, T. and Sefton, B. M.,** Protein kinase and viral transformation, in *Molecular Aspects of Cellular Regulation,* Volume II: The molecular action of toxins, viruses and interferons, 1980.
358. **Erikson, R. L., Purchio, A. F., Erikson, E., Collett, M. S., and Brugge, J. S.,** Molecular events in cells transformed by Rous sarcoma virus, *J. Cell Biol.,* 87, 319, 1980.
359. **Collett, M. S., Erikson, E., and Erikson, R. L.,** Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation, *J. Virol.,* 29, 770, 1979.
360. **Carpenter, G., King, L., and Cohen, S.,** Epidermal growth factor stimulates phosphorylation in membrane preparations *in vitro, Nature,* 276, 409, 1978.
361. **Carpenter, G. L., King, L., and Cohen, S.,** Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor, *J. Biol. Chem.,* 254, 4884, 1979.
362. **Cohen, S., Carpenter, G., and King, L.,** Epidermal growth factor-receptor-protein kinase interactions, *J. Biol. Chem.,* 255, 4834, 1980.
363. **Fabricant, R. N., DeLarco, J. E., and Todaro, G. J.,** Nerve growth factor receptors on human melanoma cells in culture, *Proc. Natl. Acad. Sci. U.S.A.,* 74, 565, 1977.
364. **Haigler, H., Ash, J. F., Singer, S. J., and Cohen, S.,** Hormone-receptor topology and dynamics: morphological analysis using ferretin-labeled EGF, *Proc. Natl. Acad. Sci. U.S.A.,* 75, 3317, 1978.
365. **Ushiro, H. and Cohen, S.,** Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes, *J. Biol. Chem.,* 255, 8363, 1980.
366. **Linn, T. C., Pettit, F. H., and Reed, L. J.,** α-Keto acid dehydrogenase complexes. X. Regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation and dephosphorylation, *Proc. Natl. Acad. Sci. U.S.A.,* 62, 234, 1969.
367. **Linn, T. C., Pettit, F. F., Hucho, F., and Reed, L. J.,** α-Keto acid dehydrogenase complexes. XI. Comparative studies of regulatory properties of the pyruvate dehydrogenase complexes from kidney, heart and liver mitochondria, *Proc. Natl. Acad. Sci. U.S.A.,* 64, 227, 1969.
368. **Denton, R. M. and Halestrap, A. P.,** Regulation of pyruvate metabolism in mammalian tissues, *Essays Biochem.,* 15, 37, 1979.
369. **Reed, L. J., Pettit, F. H., Yeaman, S. J., Teague, W. M., and Bleile, D. M.,** Structure, function and regulation of the mammalian pyruvate dehydrogenase complex, *FEBS Lett.,* 60, 47, 1980.
370. **Yeaman, S. J., Hutcheson, E. T., Roche, T. E., Pettit, F. H., Brown, J. R., Reed, L. J., Watson, D., C., and Dixon, G. H.,** Sites of phosphorylation on pyruvate dehydrogenase from bovine kidney and heart, *Biochem. J.,* 17, 2364, 1978.
371. **Sugden, P. H., Kerbey, A. L., Randle, P. J., Waller, C. A., and Reid, K. B. M.,** Amino acid sequences around the sites of phosphorylation in the pig heart pyruvate dehydrogenase complex, *Biochem. J.,* 181, 419, 1979.
372. **Davis, P. E., Pettit, F. H., and Reed, L. J.,** Peptides derived from pyruvate dehydrogenase as substrates for pyruvate dehydrogenase kinase and phosphatase, *Biochem. Biophys. Res. Commun.,* 75, 541, 1977.

373. **Walsh, D. A., Cooper, R. H., Denton, R. M., Bridges, B. J., and Randel, P. J.,** The elementary reactions of the pig heart pyruvate dehydrogenase complex, *Biochem. J.,* 157, 41, 1976.
374. **Sugden, P. H. and Randle, P. J.,** Regulation of pig heart pyruvate dehydrogenase by phosphorylation. Studies on the subunit and phosphorylation stoichiometries, *Biochem. J.,* 173, 659, 1978.
375. **Teague, W. M., Pettit, F. H., Yeaman, S. J., and Reed, L. J.,** Function of phosphorylation sites on pyruvate dehydrogenase, *Biochem. Biophys. Res. Commun.,* 87, 244, 1979.
376. **Sugden, P. H., Hutson, N. J., Kerbey, A. L., and Randle, P. J.,** Phosphorylation of additional sites on pyruvate dehydrogenase inhibits its reactivation by pyruvate dehydrogenase phosphate phosphatase, *Biochem. J.,* 169, 433, 1978.
377. **Cooper, R. H., Randle, P. J., and Denton, R. M.,** Stimulation of phosphorylation and inactivation of pyruvate dehydrogenase by physiological inhibitors of the pyruvate dehydrogenase reaction, *Nature,* 257, 808, 1975.
378. **Pettit, F. H., Pelley, J. W., and Reed, L. J.,** Regulation of pyruvate dehydrogenase kinase by acetyl CoA/CoA and NADH/NAD ratios, *Biochem. Biophys. Res. Commun.,* 65, 575, 1975.
379. **Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T., and Denton, R. M.,** Regulation of pyruvate dehydrogenase in rat heart, *Biochem. J.,* 154, 327, 1976.
380. **Hansford, R. G.,** Studies on the effects of CoA:acetyl-CoA, NAD:NADH and ADP:ATP ratios on the interconversion of active and inactive pyruvate dehydrogrenase in isolated rat heart mitochondria, *J. Biol. Chem.,* 251, 5483, 1976.
381. **Payne, M. E. and Soderling, T. R.,** Calmodulin-dependent glycogen synthase kinase, *J. Biol. Chem.,* 255, 8054, 1980.
382. **Shichi, H. and Somers, R. L.,** Light-dependent phosphorylation of rhodopsin, *J. Biol. Chem.,* 253, 7040, 1978.
383. **Takai, Y., Kishimoto, A., Inoue, M., and Nishizuka, Y.,** Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues, *J. Biol. Chem.,* 252, 7603, 1977.
384. **Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y.,** Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues, *J. Biol. Chem.,* 252, 7610, 1977.
385. **Vardanis, A.,** A unique cyclic nucleotide-dependent protein kinase, *J. Biol. Chem.,* 255, 7238, 1980.
386. **Anderson, C. M., Zucker, F. H., and Steitz, T. A.,** Space-filling models of kinase clefts and conformation changes, *Science,* 204, 375, 1979.
387. **Pickover, C. A., McKay, D. B., Engelman, D. M., and Steitz, T. A.,** Substrate binding closes the cleft between the domains of yeast phosphoglycerate kinase, *J. Biol. Chem.,* 254, 11323, 1979.