MECHANISTIC STUDIES OF cAMP-DEPENDENT PROTEIN KINASE ACTION

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ABBREVIATIONS

Ac	Acetyl
ADP	Adenosine 5′-diphosphate
ADPβS	Adenosine 5′-O-(2-thiodiphosphate)
AMP	Adenosine 5′-monophosphate
AMP-PCP	Adenosine 5′β,γ-methylenetriphosphate
AMP-PNP	Adenosine 5′-β,γ-imido triphosphate
ATP	Adenosine 5′-triphosphate
[γ-^{32}P]ATP	Adenosine 5′-[γ-^{32}P]triphosphate
ATPβS	Adenosine 5′-O-(2-thiotriphosphate)
ATPγS	Adenosine 5′-O-(3-thiotriphosphate)
BSA	Bovine serum albumin
cAMP	Adenosine 3′,5′-monophosphate
cGMP	Guanosine 3′,5′-monophosphate
DEAE	Diethylaminoethyl
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EPPES	4-(2-Hydroxyethyl)-1-piperazine-propanesulfonic acid
Homoarg	L-(+)-Homoarginine: $H_2NC(=NH)NH(CH_2)_4CH(NH_2)CO_2H$
HPLC	High Pressure Liquid Chromatography
Hyp	*cis*-4-Hydroxy-L-proline
MES	2-(N-Morpholino)ethanesulfonic acid
MOPS	4-Morpholinopropanesulfonic acid
Nyps	3-Nitro-2-pyridinesulfenyl
Npys-SCH_3	S-Methyl-3-nitro-2-pyridinesulfenyl
oATP	2′,3′-Dialdehyde derivative of ATP
Peptide *1*	Leu-Arg-Arg-Ala-Ser-Leu-Gly
Peptide *2*	Leu-Arg-Arg-Ala-Ser-Pro-Gly

Peptide *3*	Leu-Arg-Arg-Ala-Ser-(N-methyl)Leu-Gly
Peptide *4*	Leu-Arg-Arg-Ala-Cys(Npys)-Leu-Gly
Peptide *5*	AcLeu-Arg-Arg-Ala-Cys(Nyps)-Leu-Gly-OEt
Peptide *6*	AcLeu-Arg-Arg-Ala-Cys(Nyps)-Leu-[^{14}C]Gly-OEt
Peptide *7*	Leu-Arg-Arg-(*o*-NO_2)Tyr-Ser-Leu-Gly
Pys	2-Pyridinesulfenyl
Pys-SCH_3	S-Methyl-3-nitro-2-pyridinesulfenyl
TLCK	N^{α}-Tosyl-L-lysine choloromethylketone
TPCK	N^{α}-Tosyl-L-phenylalanine chloromethylketone
Tris	Tris(hydroxy methyl)amino methane

I. INTRODUCTION

Cyclic 3′,5′-adenosine monophosphate-dependent protein kinase (EC 2.7.1.37; ATP: protein phosphotransferase) is an enzyme which, in the presence of cAMP, catalyzes the transfer of the γ-phosphoryl group of ATP to serine or threonine residues of peptide or protein substrates.[1*] In addition, like certain other kinases such as creatine kinase and hexokinase, protein kinase catalyzes the slow hydrolysis of APT.[7] Both reactions require a divalent metal ion, and examples of each are shown in Figure 1. Early in the study of protein kinase it was found that the enzyme could be separated into several fractions by ion exchange chromatography.[8,9] The two largest fractions of protein kinase resolved using the anion exchanger DEAE-cellulose were designated "type I" or "type II" on the basis that the type-I enzyme is eluted first with a salt gradient.[10] The most extensively studied protein kinases have been the rabbit skeletal type-I[11] and the bovine heart type-II enzymes.[12] Both occur as catalytically inactive tetramers, consisting of two catalytic subunits and a regulatory dimer, each of which can be isolated by gel filtration after the addition of excess cAMP. The catalytic[13] and the regulatory subunit[14] from the type-II holoenzyme have both been sequenced. No X-ray crystallographic structures of protein kinase or its subunits from any source have been reported.

The free catalytic subunits of type-I and -II protein kinases are indistinguishable in terms of molecular weight (42,000 by SDS gel electrophoresis), catalytic activity, and substrate specificity.[15] Antibodies raised against the type-II catalytic subunit from bovine heart had an equal affinity for the type-I catalytic subunit from the same source.[16] In another study, both type-I and -II catalytic subunits were digested exhaustively with trypsin. The peptides from each enzyme were found to be largely homologous, but not identical.[17]

Most of the differences between the type-I and -II holoenzymes reside in the regulatory dimers. The molecular weight of the type-II bovine heart regulatory subunit that was calculated from the amino acid sequence data is 45,084.[14] While this molecular weight is about 10,000 less than that predicted by sodium dodecyl sulfate gel electrophoresis, it is still noteworthy that the latter method indicates that the type-II is larger than the type-I regulatory subunit (55,000 vs. 48,000 molecular weight). Also, only the type-II regulatory subunit can be phosphorylated by the catalytic subunit in the presence or absence of cAMP.[10,18,19] Antibodies raised against the type-II regulatory dimer from bovine heart did not react with the type-I regulatory dimer from the same source.[20] Two-dimensional maps of tryptic peptides generated from the two types of regulatory dimers showed that the proteins are not very homologous.[17]

A. Biological Role

Discovery of the cyclic 3′,5′-adenosine monophosphate-dependent protein kinase in rabbit

* The literature on cAMP dependent protein kinase has been reviewed before.[1-6]

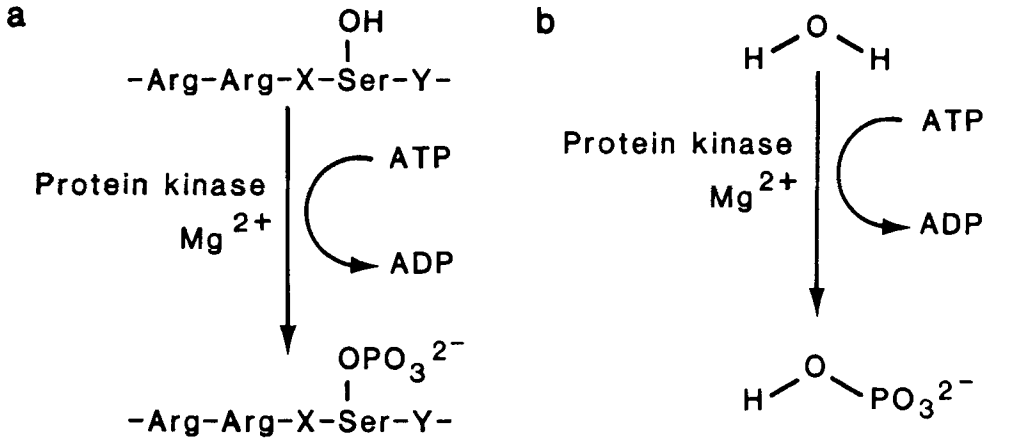


FIGURE 1. Phosphorylation by protein kinase. (a) A typical amino acid sequence phosphorylated by protein kinase; (b) ATP hydrolysis by protein kinase.

skeletal muscle completed what some investigators have called "the final link" in the process of 3′,5′-adenosine monophosphate (cAMP) activation of glycogenolysis, which is shown in Figure 2.[11,21,22] Soon afterward, the presence of this enzyme was demonstrated in a broad, if not the total, spectrum of mammalian tissues,[23] as well as in a variety of other sources, including lobster tail muscle,[24] silkworm pupae,[25,26] and the lower eukaryote, *Neurospora crassa*.[27] Effects of protein kinases are not limited to glycogenolysis. Many, if not all, of the effects of cAMP are thought to be mediated through regulation of cAMP-dependent protein kinases, which catalyze the transfer of the γ-phosphoryl group from MgATP to a serine or threonine residue at a specific site in a substrate protein.[28] cAMP has been found to be a "second messenger" for a large number of hormones, some of which are listed in Table 1.[29] This hypothesis for the expression of cAMP is supported by studies which have shown that all specific cAMP-binding activity in extracts of rabbit skeletal muscle or bovine heart can be accounted for by protein kinases,[20,30] and that when the photoaffinity analog of cAMP, 8-N_3-[^{32}P]cAMP, was photolyzed in a variety of tissues only protein kinases incorporated the label.[31] If cAMP serves to activate certain protein kinases, then the specificity of the process is mediated by the specificities of protein kinases for their respective substrate proteins and the localization of these phosphotransferase activities in the various tissues.[32,33]

B. Interactions between Regulatory and Catalytic Subunits

The major role of both types of regulatory subunits is to inhibit catalytic subunit in the absence of cAMP, and, in addition, the type-I regulatory subunit serves to increase the affinity of the type-I catalytic subunit for ATP.[10] Using magnetic resonance techniques, Granot and co-workers were able to investigate directly the interactions of type-II catalytic subunits and regulatory dimers.[34] What was shown in this study was that the holoenzyme binds ADP and MgADP with a fivefold greater affinity than does the catalytic subunit. However, while the catalytic subunit binds peptide *1*, a heptapeptide substrate of protein kinase, the holoenzyme does not in the absence of cAMP. This may indicate that the regulatory dimer binds to the protein binding site of the catalytic subunit, as suggested previously by Demaille et al.[35]

In order to understand better the process of holoenzyme activation, isolated regulatory dimers as well as the holoenzyme itself have been studied. It was found that both type-I and -II regulatory dimers bind 4 mol of cAMP per dimer at saturation.[36,37] Each regulatory subunit contains a higher affinity (site I) and a lower affinity (site II) cAMP binding site.[37,38]

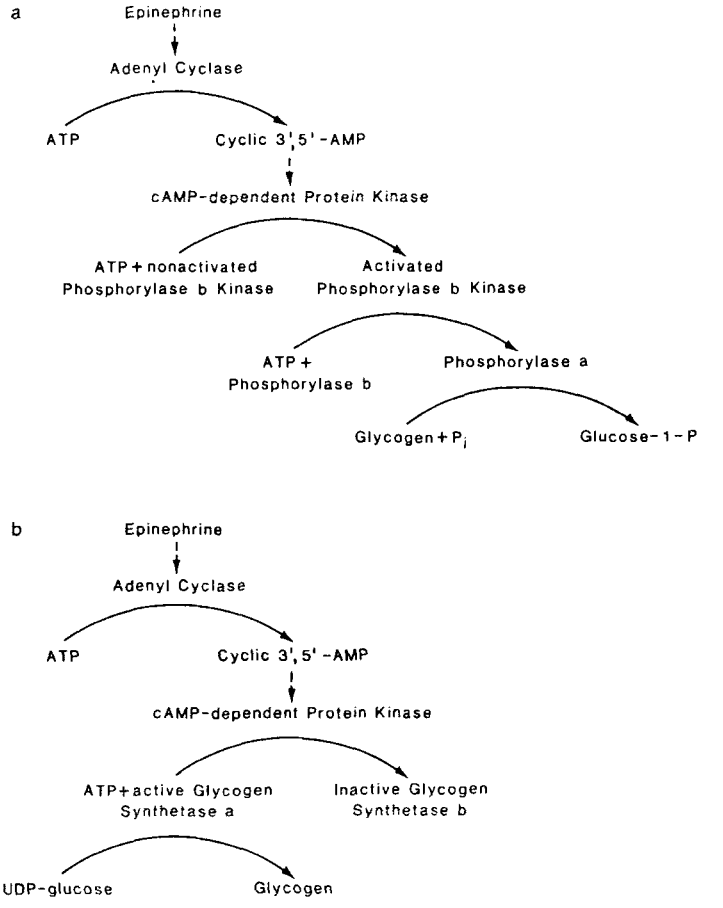


FIGURE 2. The control of glycogen metabolism by epinephrine. Protein kinase both activates glycogen degradation (a) and inhibits glycogen synthesis (b).

Table 1
HORMONES USING cAMP AS A SECOND MESSENGER[29]

Calcitonin
Chorionic gonadotropin
Corticotropin
Epinephrine
Follicle-stimulating hormone
Glucagon
Luteinizing hormone
Lipotropin
Melanocyte-stimulating hormone
Norepinephrine
Parathyroid hormone
Thyroid-stimulating hormone
Vasopressin

The interactions of the type-II regulatory dimer and catalytic subunits from bovine heart in the absence and presence of saturating cAMP were studied by Armstrong and Kaiser.[39] Isolated catalytic subunit is inactivated by reaction with DTNB, a reagent specific for the

modification of free sulfhydryls.[39,40] Not surprisingly, the regulatory subunit protects the catalytic subunit from DTNB inactivation in the absence of cAMP.[39] In the presence of saturating cAMP, DTNB inactivates the holoenzyme at a rate considerably faster than the DTNB inactivation of catalytic subunit alone. These results are best interpreted as evidence for interactions between the catalytic and regulatory subunits in the presence of saturating cAMP.[39] To account for this, Armstrong and Kaiser postulated the existence of an intermediary complex containing cAMP, regulatory dimer, and catalytic subunits, as shown in Equation 1.[39]

$$R_2C_2 + 4\ cAMP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R_2(cAMP)_4C_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R_2(cAMP)_4 + 2C \qquad (1)$$

Attempts to isolate the intermediate $R_2(cAMP)_4C_2$ were not successful,[39] but $R_2(cAMP)_4C_2$ has been detected by NMR in solutions containing 23 μM R_2C_2.[34] The kinetics for the interactions of cAMP, catalytic subunits, and regulatory subunits have been measured in several studies under a variety of conditions.[41-43] These data were all interpreted as providing evidence for the postulate of an intermediate of the type shown in Equation 1.[41-43]

Just how many cAMP molecules must bind to the regulatory subunit to activate protein kinase is unclear. Øgreid and Døskeland have presented kinetic evidence that the binding of one cAMP to type-II holoenzyme from bovine myocardium produces an unstable complex which is then relieved by what the authors suggest may be the release of one or both catalytic subunits.[44] In another study, the equilibrium expression Equation 2, which is derived from Equation 1, was assumed to be correct.[45]

$$[C]^2 = \frac{K[R_2C_2]\ [cAMP]^4}{[(cAMP)_4 \cdot R_2]} \qquad (2)$$

Since the holoenzyme is inactive[46] and the concentration of catalytic subunit is proportional to its activity, examination of Equation 2 shows that a plot of log (catalytic activity) vs. the log[cAMP] should have a slope of one if one cAMP for each regulatory subunit is required to activate the holoenzyme, or a slope of two if two are necessary. Smith et al. reported that the slope of this log-log plot was 1.89.[45]

II. THE CATALYTIC SUBUNIT

The catalytic subunit is thought to be responsible for all of the known catalytic activity and substrate specificity exhibited by the cAMP-dependent protein kinase. Because of this, and to avoid the complicating effects of regulation, most studies of this enzyme's action are performed using isolated catalytic subunit. Peters et al. found that this enzyme is composed of three major isozymes (pI = 7.01, 7.48, 7.78) which seem very similar structurally, and reported that each has three cysteines that could be titrated with the sulfhydryl reagent DTNB.[40] From the following two observations these authors concluded that none of the cysteine side chains were directly involved in the catalysis of phosphoryl group transfer. First, the titration of two cysteine residues was necessary to inactivate the protein kinase. Second, when the catalytic subunit cysteines were percyanylated, 3.0 equivalents of [^{14}C]CN were incorporated per subunit while 63% of the activity of the native enzyme was retained. Full activity was regenerated by treatment of DTNB modified or percyanylated enzyme with either DTT or β-mercaptoethanol. Unlike the native enzyme, the percyanylated protein was not inactivated by treatment with iodoacetamide.[40] These results, which support the contention that only cysteine residues were modified, are summarized in Table 2.[40] The modification of three sulfhydryls in the catalytic subunit from rabbit skeletal muscle has also been reported.[15,47] However, Armstrong and Kaiser reported that only two sulfhydryls were

Table 2
THE EFFECT OF CYSTEINE MODIFICATIONS OF ENZYME ACTIVITY[40]

Enzyme species[a]	Modifications/enzyme	Activity (%)
Native	0	100
Modified with DTNB	3.0[b]	1
Modified with DTNB then treated with 1 m*M* DTT at 30° for 1 hr	—	100
Cyanylated enzyme	3.0	63
Cyanylated enzyme after thiolysis (0.15 *M* β-mercaptoethanol for 75 min at 30°)	0.7	100
Cyanylated enzyme after thiolysis (as above) in the presence of 6 *M* Gdn·HCl	0.07	—

[a] The excess modifying reagent was removed prior to the measurement of activity.
[b] According to Armstrong and Kaiser, Reference 39, however, two sulfhydryl groups are modified.

available for modification in the bovine heart type-II enzyme,[39] a result later confirmed by elucidation of the sequence of the bovine heart type-II catalytic subunit.[13] The reason that in some studies three equivalents of DTNB were found to react with the catalytic subunit not understood.

A. Substrate Specificity

The sites of many proteins phosphorylated by cAMP-dependent protein kinases have been identified and sequenced; a partial list is provided in Table 3.[4] Kemp et al. postulated that local primary structure is an important determinant for specificity, and, in particular, arginine might be present in all local sequences recognized by the enzyme.[46] To test this possibility, many investigators have synthesized small peptides with varying primary structures and have studied their interactions with protein kinase. One of the first examples was a study of Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg phosphorylation.[48] This sequence is equivalent to amino acid residues 106 to 113 in myelin basic protein, and Daile et al. showed that only the serine residue between the two leucyl residues was phosphorylated in both the peptide and the protein.[48] Kinetic studies indicated that K_m was 240 μ*M* for the peptide and 53 μ*M* for protein, while V_{max} values were 0.3 and 0.07 mol of ^{32}P incorporated per minute, respectively, when [γ-^{32}P]ATP was the phosphoryl group donor.[48] Another peptide, Leu-Arg-Arg-Ala-Ser-Val-Ala, duplicated the phosphorylatable site of pyruvate kinase (type II) from rat liver and was a better substrate than the peptide of Daile et al.[49] Kinetic studies indicated that the apparent K_m for this peptide was less than 10 μ*M*, while V_{max} was about 20 μmol of ^{32}P incorporated per minute per milligram of kinase. Both terminal residues of this peptide could be eliminated, decreasing V_{max}/K_m only 20-fold, but further deletions of amino acids from either side created peptides that were not good substrates for protein kinase.[49]

Following the discovery that Leu-Arg-Arg-Ala-Ser-Val-Ala is phosphorylated by protein kinase with a low apparent K_m and a high V_{max}, a large number of peptides have been synthesized in order to determine the structural features of such peptides important for recognition by protein kinase. It has been reported that Leu-Arg-Arg-Ala-Ser-Leu-Gly (peptide *I*) is phosphorylated with an apparent K_m of 16 μ*M* and a V_{max} of 20 μmol/min mg enzyme.[50] Even substitutions of lysine, histidine, or homoarginine in place of an arginine increased the apparent K_m of this peptide 15- to 80-fold.[50] The optimal number of amino acids between the basic residues and serine was explored by synthesizing the complete series of dodecapeptides with the form $(Gly)_{6-n}$-Arg-Arg-$(Gly)_n$-Ala-Ser-Leu-Gly, where n = 0, 1, 2, 3, 4, or 5, and then determining the kinetic constants of each peptide's phosphorylation

Table 3
PROTEIN SUBSTRATES FOR PROTEIN KINASE AND SEQUENCES OF PHOSPHORYLATABLE SITES[4]

Substrate	Sequence
Phosphorylase kinase (β-subunit)	Ala-Arg-Thr-Lys-Arg-Ser-Gly-Ser(P)-Val-Thr-Glu-Pro-Leu
Phosphorylase kinase (α-subunit)	Phe-Arg-Arg-Leu-Ser(P)-Ile-Ser-Thr-Glu-Ser-Glx
Glycogen synthase (site 1)	Lys-Ser-Asn-Ser(P)-Val-Asp-Thr-Ser-Ser-Leu-Arg
Glycogen synthase (site 2)	Lys-Arg-Ala-Ser(P)-Arg
Pyruvate kinase	Gly-Val-Leu-Arg-Arg-Ala-Ser(P)-Val-Ala-Glx-Leu
Histone H1	Ala-Lys-Arg-Lys-Ala-Ser(P)-Gly-Pro-Pro-Val-Ser
Histone H2a	Ala-Lys-Thr-Arg-Ser-Ser(P)-Arg-Ala
Histone H2b	Arg-Lys-Arg-Ser(P)-Arg-Lys-Glu-Ser(P)-Tyr-Ser-Val-Tyr Pro-Lys-Lys-Gly-Ser(P)-Lys-Ala
Protein phosphatase inhibitor	Ile-Arg-Arg-Arg-Arg-Pro-Thr(P)-Ala-Thr
Troponin I (rabbit skeletal muscle)	Val-Arg-Met-Ser(P)-Ala-Asp-Ala-Met Arg-Gln-His-Leu-Lys-Ser(P)-Val-Met-Gln-Leu
Troponin I (rabbit heart)	Val-Arg-Arg-Ser(P)-Asp-Arg-Ala-Tyr-Ala Arg-Val-Arg-Ile-Ser(P)-Ala-Asp-Ala-Met-Met
Myelin basic protein	Pro-Ser-Gln-Arg-His-Gly-Ser(P)-Lys-Tyr-Leu-Ala Gly-Arg-Gly-Leu-Ser(P)-Leu-Ser-Arg Arg-His-Arg-Asp-Thr(P)-Gly-Ile
β-Casein-B	Phe-Thr-Glu-Arg-Gln-Ser(P)-Leu-Thr-Leu-Thr-Asp
Protamine (site 1)	Arg-Arg-Arg-Arg-Ser-Ser-Ser(P)-Arg-Pro-Ile-Arg
Protamine (site 2)	Arg-Arg-Ala-Ser(P)-Arg-Pro-Val-Arg
Protamine (site 3)	Arg-Arg-Arg-Arg-Ser(P)-Arg-Arg-Ala

by protein kinase.[51] $(Gly)_6$-Arg-Arg-Ala-Ser-Leu-Gly, with a similar sequence as peptide *1*, Leu-Arg-Arg-Ala-Ser-Leu-Gly, is the best substrate of this series.[51] All of these peptides, as well as others not mentioned here, are listed in Table 4 along with the kinetic constants for their phosphorylation by protein kinase, when available.[48-57]

A naturally occurring heat-stable protein inhibitor of protein kinase was discovered by Walsh et al.[58] and purified from rabbit skeletal muscle.[35] This inhibitor was found to have a molecular weight of 11,300, and it binds to the catalytic subunit with a K_I of about 2 n*M*.[35] Peptide inhibitors have also been synthesized. Feramisco and Krebs chose the sequence of the peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly (peptide *1*) as the basis for designing inhibitors since it is phosphorylated with a low K_m and a high V_{max}.[59] They synthesized a number of peptides, replacing the serine of peptide *1* with various other amino acids.[59] A list of these peptides is shown in Table 5.[35,59] The best inhibitor of this series was Leu-Arg-Arg-Ala-Ala-Leu-Gly, which competitively inhibited the phosphorylation of peptide *1* with a K_I of 490 μ*M*.[59] Since this is much greater than the K_m for the phosphorylation of peptide *1* (16 μ*M*), it is probable that either the hydroxyl group of serine is critical for binding, or K_d for peptide *1* is considerably higher than K_m for its phosphorylation.[59] For a two-substrate reaction, if K_d is greater than K_m it is indicative that the binding of one substrate affects the binding of the other. This would occur in mechanisms such as a steady-state sequential or random mechanism, but not a rapid equilibrium random mechanism. In order to determine K_d for a peptide substrate, Feramisco and Krebs synthesized N^{α}-[^{3}H]acetyl Leu-Arg-Arg-Ala-Ser-Leu-Gly and used equilibrium dialysis to determine that K_d is about 250 μ*M*, compared to an apparent K_m equal to 3 μ*M* that was determined from the peptide phosphorylation kinetics.[59]

III. THE ORIENTATION OF SUBSTRATES IN THE PROTEIN KINASE ACTIVE SITE

The catalytic subunit requires the presence of a divalent cation in order to catalyze

Table 4
PEPTIDE SUBSTRATES FOR PROTEIN KINASE

Peptide	$K_{m,app}$(μ*M*)	V_{max}(μmol/min mg)	Ref.
Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg	240	0.3	48
Arg-Gly-Tyr-Ser-Leu-Gly	4,200 ± 200	16.4 ± 0.3	52
Gly-Gly-Tyr-Ser-Leu-Gly	12,400 ± 1,300	0.41 ± 0.02	52
His-Gly-Tyr-Ser-Leu-Gly	13,400 ± 500	0.95 ± 0.02	52
Lys-Gly-Tyr-Ser-Leu-Gly	14,700 ± 400	0.86 ± 0.01	52
Leu-Arg-Arg-Ala-Ser-Val-Ala	10 or less	—	49
Arg-Arg-Ala-Ser-Val-Ala	20	—	49
Arg-Arg-Ala-Ser-Val	80	—	49
Leu-Arg-Arg-Ala-Ser-Leu-Gly	16.0 ± 0.9	20.0 ± 0.5	50
Leu-Ala-Arg-Ala-Ser-Leu-Gly	4,900 ± 700	8.7 ± 0.6	50
Leu-Arg-Ala-Ala-Ser-Leu-Gly	6,300 ± 400	5.3 ± 0.2	50
Leu-Lys-Arg-Ala-Ser-Leu-Gly	1,400 ± 100	17.1 ± 0.4	50
Leu-Arg-Lys-Ala-Ser-Leu-Gly	260 ± 10	16.9 ± 0.3	50
Leu-His-Arg-Ala-Ser-Leu-Gly	415 ± 22	12.1 ± 0.3	50
Leu-Arg-His-Ala-Ser-Leu-Gly	1,340 ± 50	6.5 ± 0.1	50
Leu-Homoarg-Arg-Ala-Ser-Leu-Gly	350 ± 20	10.0 ± 0.3	50
Leu-Arg-Homoarg-Ala-Ser-Leu-Gly	440 ± 40	7.3 ± 0.7	50
Arg-Arg-Ala-Ser-Leu-Gly	26.0 ± 2	17.9 ± 0.5	50
Arg-Ala-Ser-Leu-Gly	4,400 ± 400	10.2 ± 0.5	50
Leu-Arg-Arg-Ala-Ser-Leu	57.0 ± 4	18.1 ± 0.4	50
Leu-Arg-Arg-Ala-Ser	—	Negligible	50
Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu	3,900 ± 800	4.1 ± 0.8	50
Lys-Arg-Ala-Gln-Ile-Ser-Val-Arg-Gly-Leu	1,400 ± 300	0.05 ± 0.01	50
Lys-Ala-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu	2,200 ± 900	0.04 ± 0.02	50
Lys-Arg-Lys-Gln-Ile-Ser-Val-Ala-Gly-Leu	36 ± 1	21.4 ± 0.3	50
Lys-Arg-Lys-Gln-Ile-Ser-Val-Gly-Gly-Leu	300 ± 30	15.4 ± 1.2	50
Lys-Arg-Lys-Gln-Ile-Ser-Gly-Arg-Gly-Leu	960 ± 70	1.3 ± 0.05	50
Lys-Arg-Lys-Gln-Gly-Ser-Val-Arg-Gly-Leu	300 ± 30	14.3 ± 0.7	50
Arg-Lys-Gln-Ile-Ser-Val-Arg	3,500 ± 200	2.5 ± 0.1	50
Arg-Lys-Glu-Ile-Ser-Val-Arg	5,200 ± 500	2.5 ± 0.2	50
Arg-Lys-Gln-Ile-Thr-Val-Arg	4,000 ± 1,000	0.04 ± 0.01	50
Lys-Lys-Gln-Ile-Ser-Val-Arg	6,000 ± 2,000	0.14 ± 0.04	50
Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu	17,000 ± 8,000	0.13 ± 0.08	50
Lys-Gln-Gly-Ser-Gly-Arg-Gly-Leu	—	Negligible	50
Leu-Arg-Arg-Ala-Thr-Leu-Gly	590 ± 80	5.6 ± 0.3	50
Val-Arg-Arg-Ser-Asp-Arg-Ala-amide	1,100 ± 200	0.037 ± 0.002	53
Leu-Arg-Arg-Ala-Ser-Leu-Gly	3.6 ± 0.5	23.2 ± 1.1	53
Leu-Arg-Arg-Ala-Ser-Leu-Gly-amide	2.5 ± 0.4	30.1 ± 1.7	53
Arg-Arg-Arg-Ser-Asp-Arg-Ala-amide	66 ± 3	0.81 ± 0.03	53
Leu-Arg-Arg-Ala-Ser-Leu-Gly	6.5 ± 0.4	17.3 ± 0.4	51
Gly-Gly-Gly-Leu-Arg-Arg-Ala-Ser-Leu-Gly	4.6 ± 0.5	19.0 ± 0.7	51
Gly-Gly-Gly-Gly-Gly-Leu-Arg-Arg-Ala-Ser-Leu-Gly	5.2 ± 0.6	19.3 ± 0.7	51
G-G-G-G-G-G-G-Arg-Arg-Ser-Leu-Gly*	1,080 ± 47	14.7 ± 0.3	51
G-G-G-G-G-G-Arg-Arg-Ala-Ser-Leu-Gly	2.4 ± 0.3	18.2 ± 0.5	51
G-G-G-G-G-Arg-Arg-G-Ala-Ser-Leu-Gly	400 ± 38	8.4 ± 0.021	51
G-G-G-G-Arg-Arg-G-G-Ala-Ser-Leu-Gly	4,070 ± 413	2.7 ± 0.3	51
G-G-G-Arg-Arg-G-G-G-Ala-Ser-Leu-Gly	290 ± 30	0.05 ± 0.01	51
G-G-Arg-Arg-G-G-G-G-Ala-Ser-Leu-Gly	960 ± 61	0.26 ± 0.01	51
G-G-G-G-G-G-G-Lys-Arg-Ser-Leu-Gly	11,800 ± 1,900	3.96 ± 0.31	51
G-G-G-G-G-G-Lys-Arg-Ala-Ser-Leu-Gly	394 ± 30	10.93 ± 0.28	51
G-G-G-G-G-Lys-Arg-G-Ala-Ser-Leu-Gly	5,590 ± 1,050	3.89 ± 0.31	51
G-G-G-G-Lys-Arg-G-G-Ala-Ser-Leu-Gly	6,440 ± 790	1.26 ± 0.10	51
G-G-G-Lys-Arg-G-G-G-Ala-Ser-Leu-Gly	10,200 ± 1,040	0.70 ± 0.05	51
Arg-Lys-Ala-Ser-Gly-Pro	1,020 ± 120	4.1 ± 0.3	51
Arg-Arg-Lys-Ala-Ser-Gly-Pro	2,310 ± 150	22.1 ± 150	51

Table 4 (continued)
PEPTIDE SUBSTRATES FOR PROTEIN KINASE

Peptide	$K_{m,app}$(μ*M*)	V_{max}(μmol/min mg)	Ref.
Leu-Arg-Arg-Ala-Ser-Leu-Gly	63 ± 4	50.6 ± 3	51
Ser-Arg-Lys-Leu-Ser-Asn-Phe-Gly	100	—	55
Ser-Arg-Lys-Leu-Ser-Asp-Phe-Gly	300	—	55
Leu-Arg-Arg-Ala-Hyp-Leu-Gly	18,000	1	56
Leu-Arg-Arg-Pro-Ser-Leu-Gly	6.0	20	56
Arg-Lys-Arg-Ser-Arg-Lys-Glu	100	1.1	57
Arg-Lys-Glu-*Ser*-Tyr-Ser-Val	100	16.5	57

[a] The one-letter abbreviation for glycine is G.

Table 5
INHIBITORS OF PROTEIN KINASE[a]

Sequence	K_I (μ*M*)	Source
Leu-Arg-Arg-Ala-Ala-Leu-Gly	490 ± 10	59
Leu-Arg-Arg-Ala-Val-Leu-Gly	810 ± 70	59
Leu-Arg-Arg-Ala-His-Leu-Gly	2150 ± 350	59
Leu-Arg-Arg-Ala-Asp-Leu-Gly	2150 ± 320	59
Leu-Arg-Arg-Ala-Gly-Leu-Gly	1670 ± 130	59
Leu-Arg-Arg-Ala-Asn-Leu-Gly	1090 ± 580	59
Arg-Arg	3080 ± 400	59
AcLeu-Arg-Arg-Ala-Ala-Leu-Gly	620 ± 50	59
Protein kinase heat-stable inhibitor	0.002	35

[a] All of these inhibitors inhibit protein kinase catalyzed phosphoryl group transfer competitively with respect to peptide or histone substrates.

phosphoryl group transfer.[60] Mg^{2+}, Mn^{2+}, and Co^{2+} can all be utilized by protein kinase, but the effects of each vary depending on the experimental conditions and the source of enzyme.[61] One reason for this variance was explained by the work of Armstrong et al.[62] Using kinetic, nuclear magnetic resonance (NMR), and electron paramagnetic resonance (EPR) techniques, Armstrong et al.[62] investigated the interactions of the protein kinase catalytic subunit with Mg^{2+} and Mn^{2+}. Their results have shown that in the absence of nucleotides, the enzyme binds Mn^{2+} very weakly. The binding of one ADP or AMP-PCP per catalytic subunit caused the appearance of two tight Mn^{2+} binding sites, the first complexed to oxygens on the nucleotide, and the other in an inhibitory site bridging the nucleotide to the enzyme.[62,63] Kinetic studies indicated that Mn^{2+} or Mg^{2+} bound in the inhibitory site inhibited the phosphotransferase reaction up to 50–fold and fivefold, respectively.[62]

A. The Conformation of Tetraaminecobalt (III)-ATP Bound in the Protein Kinase Active Site

Because Mn^{2+} is paramagnetic, NMR spectroscopy can be used to investigate the effects of Mn^{2+} on hydrogen and phosphorus nuclei within about 25 Å of the ion. From paramagnetic effects on the longitudinal relaxation rates of protons and phosphorous, the distance between the metal and these nuclei can be calculated. Using this technique, Granot et al. determined the distances between Mn^{2+} and nine atoms of the molecule $Co(NH_3)_4ATP$ while these molecules were bound in the protein kinase active site[63]. By molecular model building and computer analysis, these data were interpreted to yield two possibilities for the conformation of $Co(NH_3)_4ATP$ in the enzyme active site.[63] The major difference in the two solutions is

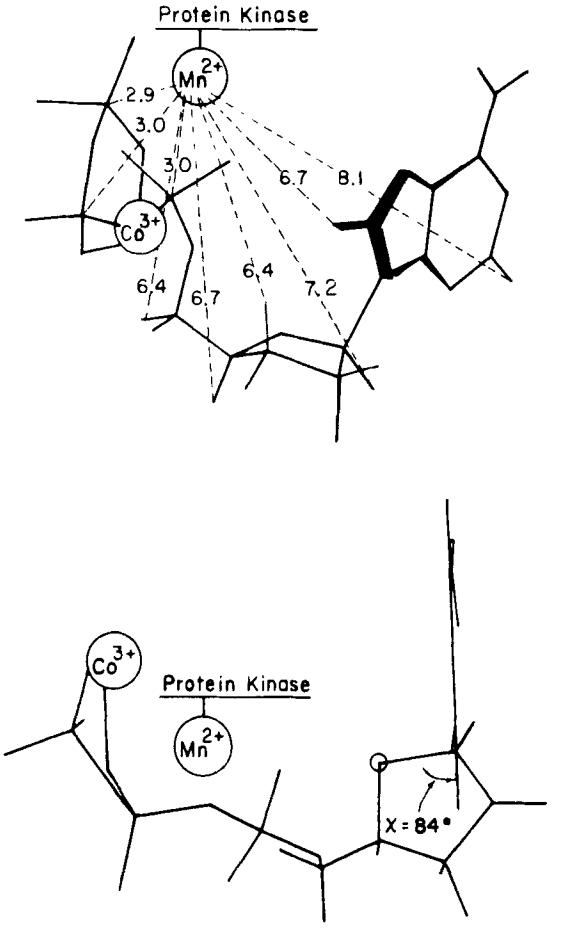


FIGURE 3. The conformation of $Co(NH_3)_4ATP$ bound in the protein kinase active site with Mn^{2+}. Distances are in Å and are from reference 63.

the torsion angle at the glycosidic bond: one solution yielded $\chi = 84 \pm 10°$ (*anti*-conformation) and the other yielded $\chi = 284 \pm 10°$ (*syn*-conformation). To distinguish between these possibilities additional NMR measurements were made, this time employing the Nuclear Overhauser Effect (NOE). This technique is based on the fact that when a proton nucleus is selectively irradiated it will measurably change the intensity or integrated area of nuclear resonances of proton nuclei within about 5 Å of the irradiated nucleus. Mildvan et al.[64] report that for enzyme-bound $Co(NH_3)_4ATP$ the only NOE observed from a ribose proton to an adenine proton was from ribose H'_2 to adenine H_8.[64] From their measurements these workers concluded that ribose H'_2 is at least 52% farther from adenine H_2 than from adenine H_8 and, therefore, $Co(NH_3)_4ATP$ must have an *anti*-conformation when bound in the catalytic subunit active site.[64] This *anti*-conformation is shown in Figure 3.

B. Stereochemistry of the Reactive Metal-Ion-Nucleoside-Triphosphate Complex

In analogy to MgATP, β,γ-bidentate, $Co^{3+}(NH_3)_4ATP$ has two stereoisomers. Unlike MgATP the complexes of $Co(NH_3)_4ATP$ are substitution-inert, and the stereoisomers are, therefore, separable. The cAMP-dependent protein kinase catalytic subunit was shown to catalyze phosphoryl group transfer from the Δ isomer of β,γ-bidentate, $Co^{3+}(NH_3)_4ATP$ to

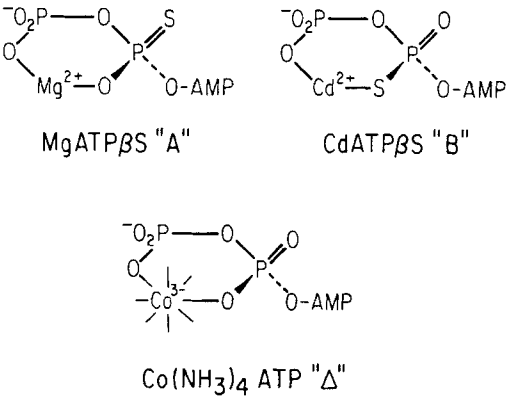
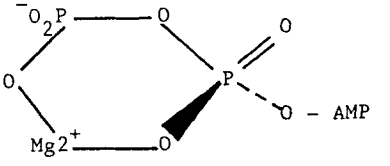


FIGURE 4. The geometries of MgATPβS "A", CdATPβS "B", and $Co(NH_3)_4ATP$ "Δ".

the heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly at a rate 0.1% the rate seen with MgATP, while the λ isomer had no detectable substrate activity.[65] Additionally, the enzyme was shown to utilize the "A" isomer of MgATPβS as well as it did MgATP (k_{cat} = 640 min^{-1} and $K_{m,ATP}$ = 10.7 μ*M*), a rate 500 times that using the "B" isomer.[66] When Cd^{2+} was used in place of Mg^{2+}, then ATPβS "B" was used 20-fold faster than the "A" isomer.[66] Jaffe and Cohn have shown from ^{31}P NMR studies that Cd(II) chelates ATPβS and ATPγS through the sulfur atom while Mg(II) chelates the same ATP analogs through the oxygen atom of the thiophosphoryl groups.[67] Because of these metal ion preferences, β,γ-bidentate MgATPβS "A" and β,γ-bidentate CdATPβS "B" are expected to be geometrically equivalent,[67] as shown in Figure 4. In addition, both complexes are thought to have the same geometry as the Δ isomer of β,γ-bidentate, $Co^{3+}(NH_3)_4ATP$ (Figure 4).[66,67] From these facts, it may be concluded that protein kinase utilizes the MgATP isomer that has the geometry shown below.



C. The Conformation of Peptide Substrates in the Protein Kinase Catalytic Site

The importance of a peptide or protein's primary structure in determining the interactions of these molecules with the protein kinase catalytic subunit has already been discussed. In addition, the question must be raised whether this enzyme phosphorylates only peptides and proteins that can assume a specific secondary structure. To investigate this possibility, Granot et al. used NMR spectroscopy to study the conformation of the peptide substrates Leu-Arg-Arg-Ala-Ser-Leu-Gly and Leu-Arg-Arg-Tyr-Ser-Leu-Gly, in addition to the inhibitor Leu-Arg-Arg-Ala-Ala-Leu-Gly when each was bound in the catalytic subunit active site.[68] Distances measured from carbon-bound protons on these peptides to the paramagnetic metals Mn^{2+} in the complex

peptide-enzyme-(β,γ-bidentate, $Co^{3+}(NH_3)_4ATP$)
Mn^{2+}

or Cr^{3+} in the complex peptide-enzyme-(β,γ-bidentate,Cr^{3+}-β,γ-methylene ATP) were not

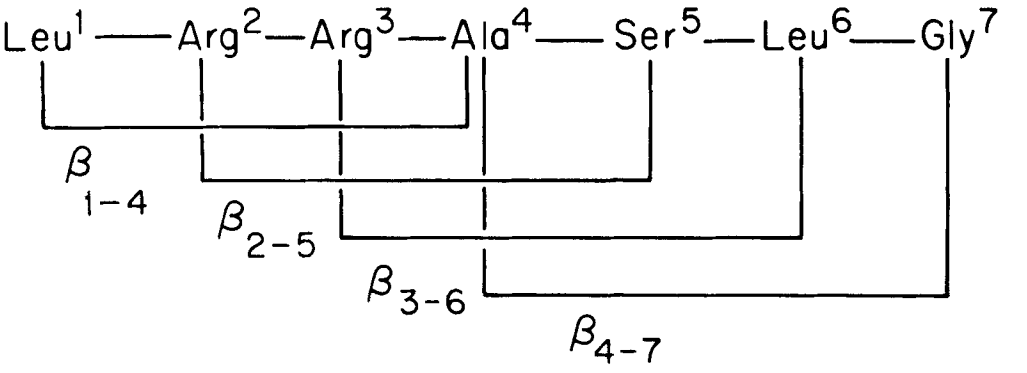


FIGURE 5. Four possible β turns within the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly.

compatible with either an α-helical or a β-pleated-sheet conformation for the enzyme-bound heptapeptides. Additionally, Granot et al. argued from the sequences of the peptides which have been reported in the literature as being phosphorylated by protein kinase, that there can be no obligatory requirement for a β turn for peptides to be substrates of this enzyme.[68]

The β turn is a conformation that involves a sequence of four consecutive amino acid residues and has a hydrogen bond between the carbonyl oxygen in the first amino acid residue and the N-H of the fourth residue (Figures 5 and 6A). For the enzyme-bound peptide *1* four β turns are possible (see Figure 5). Neither the β_{1-4} or the β_{4-7} turns can be obligatory since peptides without Leu[1] or Gly[7] are good substrates for protein kinase.[49,50] A peptide in which Ser[5] has been replaced by hydroxyproline[5] is phosphorylated by protein kinase with very poor kinetic parameters,[56] so a β_{2-5} turn is probably not required. Granot et al. have reported that peptide *2*, with Pro[6] in place of Leu[6], was found not to be a substrate of protein kinase, within the limits of detection.[68] Analogous kinetic studies showed that the substrate peptide *1*, Leu-Arg-Arg-Ala-Ser-Leu-Gly, was phosphorylated at 1000 times the minimal detectable rate. Further, no inhibition of the protein kinase catalyzed phosphorylation of peptide *1* (32 μ*M*) by the addition of up to 400 μ*M* peptide *2* was observed, indicating the $K_{I,\ peptide\ 2}$ is greater than 1 m*M*.[68] Proline is a secondary amine, so amides made from this amine cannot donate an N–H for a hydrogen bond. In addition, the cyclic structure of proline is relatively rigid, so it is possible that peptide *2* is unable to assume a particular conformation that is "recognized" by cAMP-dependent protein kinase. To investigate this latter possibility peptide *3*, AcLeu-Arg-Arg-Ala-Ser-(N-methyl)Leu-Gly, was synthesized, and the interactions of the protein kinase catalytic subunit with peptide *3* were studied.[69] Like proline, the amine of N-methyl leucine is a secondary amine, but N-methyl leucine does not have a cyclic structure. No phosphorylation of this peptide was observed under the conditions where peptide *1* reacted at a rate that was 2×10^4 times the minimal detectable rate.[69] Inhibition studies done under similar conditions employing 25 μ*M* peptide *1* and 0 to 5 m*M* peptide *3* indicated that peptide *3*, if it inhibits the phosphorylation reactions, does so with a K_I significantly higher than 5 m*M*.[69]

It is possible that peptides *2* and *3* are not good substrates for the protein kinase catalytic subunit simply due to steric hindrance around the site of phosphoryl group transfer. Alternatively, the amide proton they lack may be part of a hydrogen bond between a substrate and the enzyme. There is precedence for this type of behavior. Ingles and Knowles have suggested that a hydrogen bonding interaction between a substrate amido –NH– group other than that of the scissile bond and an enzymic group of α-chymotrypsin is necessary for effective catalysis by this enzyme.[70] It is also possible that a β_{3-6} turn is required for these peptides to be substrates of protein kinases. Peptides *2* and *3* do not contain the amide proton necessary to make a β_{3-6} turn favorable. However, Granot et al.[68] argue that a β_{3-6} turn

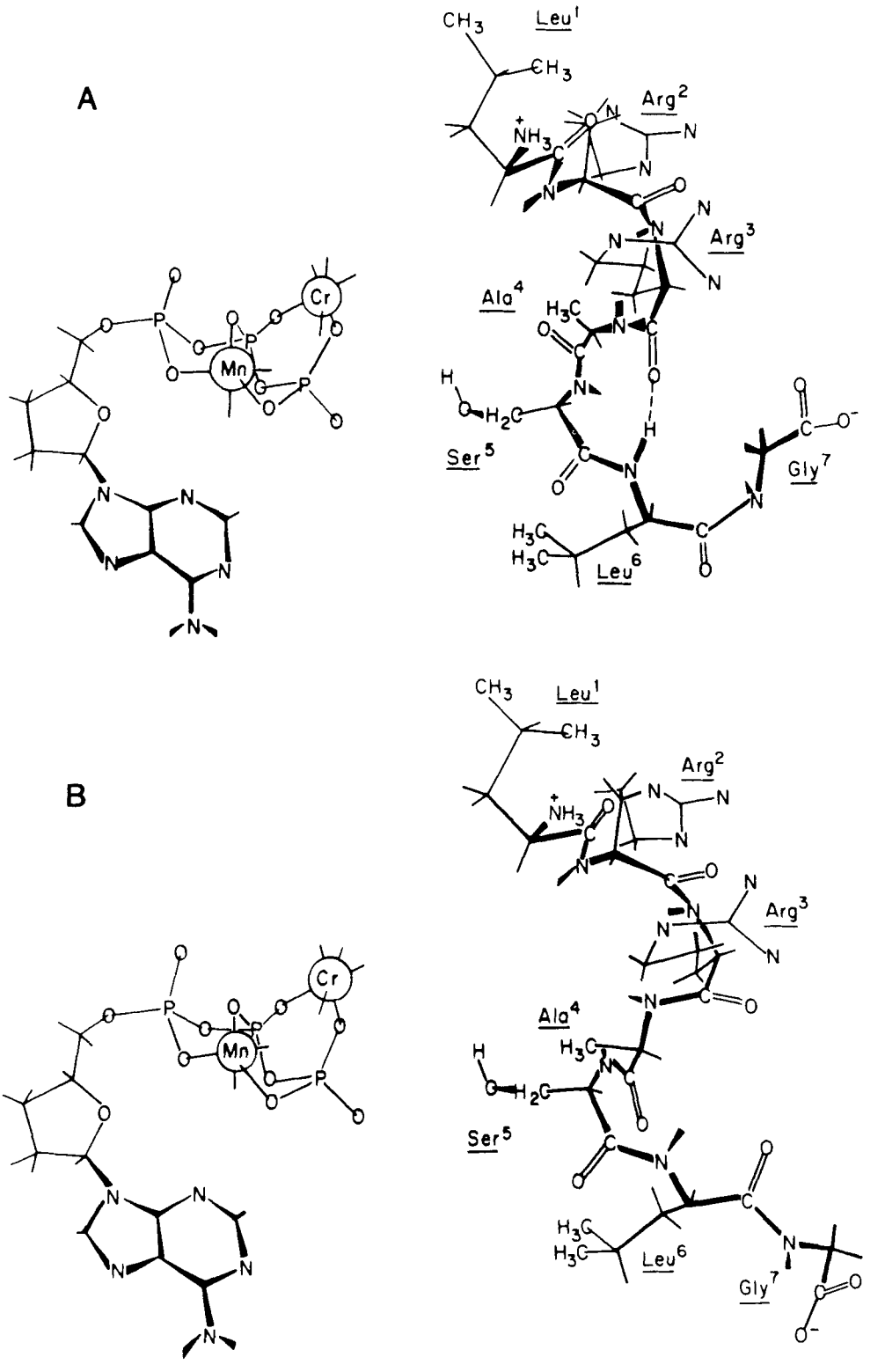


FIGURE 6. Arrangement and conformations of substrates at the active site of protein kinase concomitant with distances determined by NMR. (A) $\beta_{3\text{-}6}$ turn conformation for the peptide; (B) coil conformation for the peptide.

Table 6
MODIFICATIONS OF THE PROTEIN KINASE CATALYTIC SUBUNIT

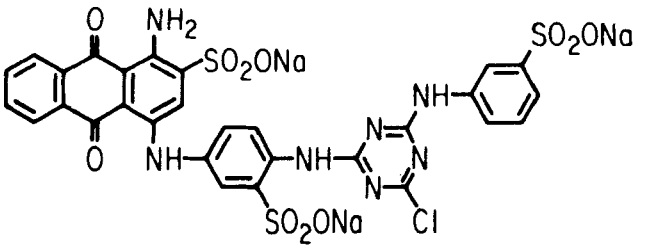
Modifying reagent	Source of kinase	Number of residues modified	Residues modified	Ref.
Cibacron Blue F3GA	Bovine brain type II	?	?	78
DTNB	Bovine heart type II	2	Cysteines	39, 40
DTNB	Rabbit skeletal type I	2	Cysteines	47, 80–82
TLCK	Rabbit skeletal type I	1?	Cysteine?	83
Iodoacetic acid	Porcine heart type I	3	Cysteines + ?	84
Ethoxyformic anhydride	Bovine brain types I and II	1?	Tyrosine?	85
Glycine ethyl ester	Rabbit skeletal type I	2	Glutamic acid	86
oATP	Pig brain	1	Lysine	87
p-Fluorosulfonyl-[^{14}C]-benzoyl 5′-adenosine	Pig skeletal type II	1	Lysine 72	76, 88, 89
AcLeu-Arg-Arg-Ala-Cys(Nyps)-[^{14}C]Leu-Gly-OEt	Bovine heart type I	1	Cysteine 199	77

cannot be required since Kemp et al.[71] have reported that synthetic peptides containing a phosphorylatable Thr residue followed by a proline are substrates, although not good ones, for cAMP-dependent protein kinase. In addition, two protein substrates have been found that contain similarly located prolines.[72,73] Furthermore, the peptides Leu-Arg-Arg-Ala-Ser-Leu-Gly,[50] Leu-Arg-Arg-Pro-Ser-Leu-Gly,[56] Leu-Arg-Arg-Tyr-Ser-Leu-Gly,[68] and Leu-Arg-Arg-Trp-Ser-Leu-Gly[74] have all been shown to be phosphorylated by protein kinase with virtually the same kinetic parameters. According to the Chou and Fasman parameters for predicting β turns,[75] these peptides have widely varying potentials for forming $\beta_{3\text{-}6}$ turns. If a $\beta_{3\text{-}6}$ turn structure were required in the enzyme-bound peptide, then the above peptides would probably not all have the same reactivity toward phosphorylation catalyzed by the enzyme. Considering these facts, the possibility that protein kinase requires substrates to assume a particular β turn does not seem likely. Therefore, Granot et al. concluded by a process of elimination that if this enzyme has an obligatory conformational requirement for peptide and protein substrates in the active site, this structure is probably a coil (Figure 6B).[68]

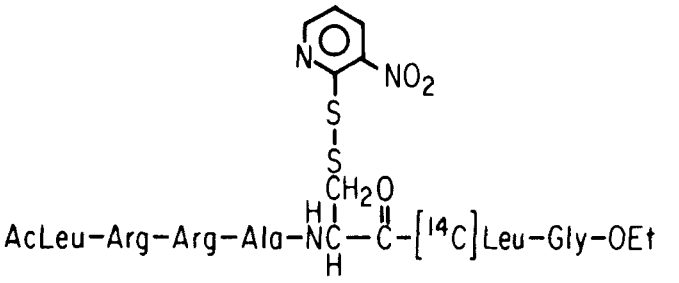
IV. CHEMICAL MODIFICATIONS

A lack of sufficient information about the nature of crucial residues in the active site of protein kinase precludes a detailed discussion of the molecular mechanism of enzyme action. Many chemical modifications of the protein kinase catalytic subunit have been performed. In this manner, both lysine-72[76] and cysteine 199[77] have been identified as being at or near the active site. Several of these modifications are listed in the following pages and are summarized in Table 6.[39,40,47,76-89] The structures of the modifying reagents used in these studies are shown in Figure 7.

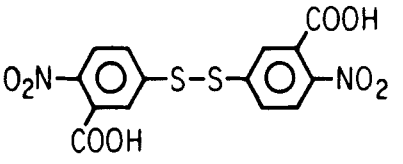
Cibacron blue F3GA, containing a triazine ring chloride, was shown to modify covalently the type-II protein kinase catalytic subunit from bovine brain.[78] The inactivation of the catalytic subunit under neutral conditions employing excess modifying agent follows pseudo first-order kinetics, and the dissociation constant for the dye was determined to be 100 μM. Since Cibacron Blue F3GA was observed to saturate the enzyme prior to modification and because the substrates ATP, peptide *1*, and histone all protected the enzyme from inactivation, the authors concluded that the dye was an active site-directed reagent. In addition, holoen-



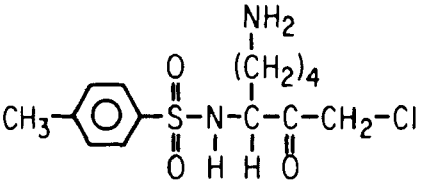
Cibacron Blue F_3GA



Peptide 6

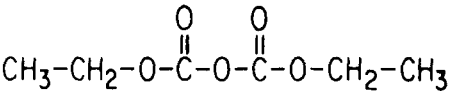


DTNB

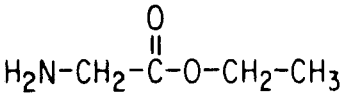


TLCK

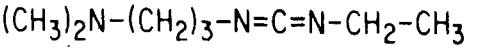
Iodoacetic acid



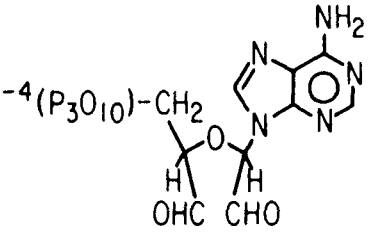
Ethoxyformic anhydride



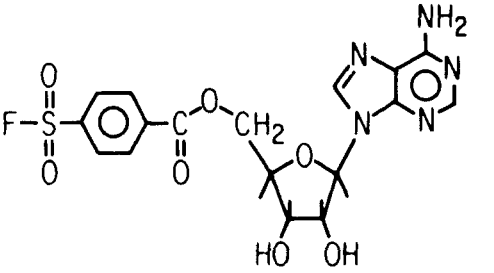
Glycine ethyl ester



1-(3-dimethylaminopropyl)-3-ethylcarbodimide



oATP



p-Fluorosulfonyl-benzoyl 5'-adenosine

FIGURE 7. Structures of the modifying reagents listed in Table 6.

zyme was found to be resistant to dye inactivation in the absence but not the presence of cAMP.[78] This is in agreement with previous results that indicate the regulatory subunit shields the catalytic subunit active site.

Due to the high reactivity of the sulfhydryl groups of protein kinase, cysteine residues have been modified more than any other residues of the enzyme. As mentioned earlier, the modification of catalytic subunit with DTNB has been reported by several laboratories.[39,40,47,79-83] Armstrong and Kaiser found that two cysteines in the bovine heart protein kinase catalytic subunit can be modified with DTNB, and the inactivation of enzyme with this reagent is a biphasic process.[39] The latter result was in agreement with those of Peters et al., also for the bovine heart catalytic subunit.[40] However, Jiménez et al. report that modification of the type-I catalytic subunit from rabbit skeletal muscle is dependent on ionic strength, and while the kinetics of the reaction are biphasic at "low" ionic strength (μ = 0.03), they become monophasic at "high" ionic strength (μ = 0.22).[47] Furthermore, the same workers also reported that the inactivation of the rabbit skeletal muscle catalytic subunit was a monophasic process and occurred concomitantly with the modification of a kinetically characterized sulfhydryl group, referred to as SH_{II}, at both "low" and "high" ionic strength.[80,81] The conditions of modification at high ionic strength are similar to those of Peters et al.[40] and at low ionic strength are similar to those of Armstrong and Kaiser,[39] who both observed biphasic modification and inactivation kinetics, making the observation of monophasic kinetics surprising. In order to explain the salt-induced change in the relative rates at which both sulfhydryl groups react with DTNB, Jiménez and co-workers investigated the ionic strength dependence of the protein kinase-catalyzed phosphorylation of histone H2b. These investigators found that increasing the ionic strength of the assay system from μ = 0.03 to μ = 0.13 caused the K_m values measured for both the histone and the MgATP to increase from three- to fourfold, while V_{max} remained essentially unchanged.[47,81] Similarly, increases of ionic strength had previously been reported to result in the inhibition of the phosphorylation of various protein substrates catalyzed by cAMP-dependent protein kinase.[90,91] Jiménez et al. proposed that these results indicate that the conformation of the catalytic subunit was not the same at μ = 0.03 as at μ = 0.13, under the conditions employed in their study.[81]

MgATP protected the catalytic subunit of protein kinase from inactivation by reaction with DTNB and did so by specifically preventing the modification of SH_{II}, while not affecting the reactivity of SH_I.[81] In the presence of magnesium acetate, saturating concentrations of either ATP or AMP-PNP afforded better protection from inactivation by DTNB modification than either ADP or AMP thus, establishing the importance of the γ–phosphoryl group for the protective effect.[81] The protein substrates histone H2b and protamine, instead of protecting enzyme, considerably enhanced the reactivity of both SH_I and SH_{II} toward DTNB.[82] Peptide *1* accelerated the rate of SH_{II} modification with DTNB, without affecting the reactivity of SH_I.[82] Protamine, histone H2b, and peptide *1* are all positively charged; therefore, it is plausible that in binding to the catalytic subunit these substrates could "channel" the negatively charged DTNB into the active site.[82] Conversely, MgATP is negatively charged and might repel the disulfide. To test this postulate, Kupfer et al. modified the catalytic subunit using the two uncharged disulfides, 2,2′-dithiodipyridine and 2,2′-dithiobis(5-nitropyridine) and the negatively charged 6,6′-dithiodinicotinic acid, in addition to DTNB.[82] The structures of these reagents are shown in Figure 8. Each of these four reagents was shown to inactivate the enzyme, and in all cases the activity was quantitatively regenerated by treatment with 2-mercatoethanol.[82] It was found that MgATP protected enzyme from inactivation by any of the above disulfide reagents, while the stimulatory effects of histone H2b and protamine were restricted only to the negatively charged disulfides.[82] Since the reactivity of SH_{II} is affected by the presence of substrates, the authors concluded that SH_{II} was part of an active site residue.[82]

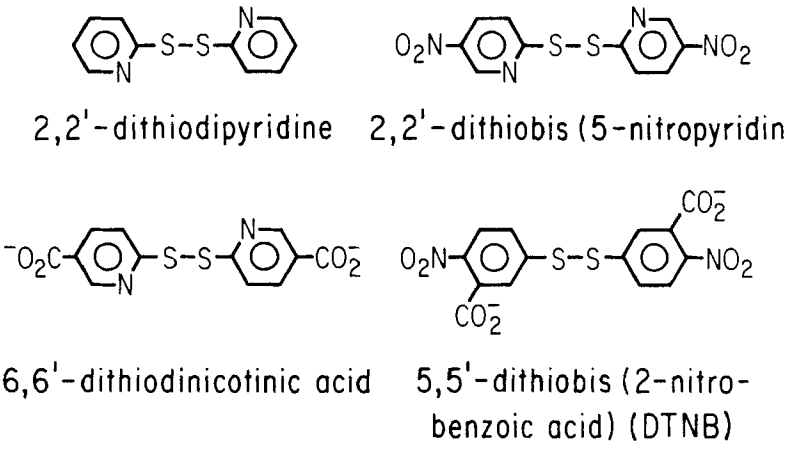


FIGURE 8. Symmetrical disulfide modifying reagents.

The rabbit skeletal muscle type-I protein kinase catalytic subunit was irreversibly inactivated by treatment with N^{α}-tosyl-L-lysine chloromethylketone (TLCK) or N^{α}-tosyl-L-phenylalanine chloromethylketone (TPCK), reagents originally designed for the modification of the proteolytic enzymes trypsin and chymotrypsin, respectively.[83] Under similar conditions, the alkylating reagents chloroacetic acid and chloroacetamide did not react with the catalytic subunit. MgATP and histone H2b protected the enzyme against inactivation by TLCK, but neither substrate effectively protects against inactivation by TPCK.[83] Prior treatment of the catalytic subunit with DTNB prevented this irreversible inactivation caused by modification with TLCK, and after treatment with TLCK one less sulfhydryl group of the enzyme reacted with DTNB; so these authors concluded that this reagent had modified a single active site cysteine residue.[83]

Another method of modifying cysteine residues is to alkylate the sulfhydryl groups with iodoacetic acid. Nelson and Taylor have labeled the porcine heart type-II catalytic subunit using [^{14}C]iodoacetic acid and found that the label was incorporated into both cysteine residues plus onto one other group that they could not identify, which was not a cysteine residue.[84] MgATP protected the catalytic subunit from alkylation by iodoacetamide, blocking the reaction of not only both sulfhydryls but of the third group as well. This result suggests that both of the cysteine residues and perhaps the third group are located at or near the active site of the catalytic subunit.

Other types of amino acids presumably located in the vicinity of the active site have been identified. Both type-I and type-II bovine brain catalytic subunits were modified with ethoxyformic anhydride.[85] MgATP protected these enzymes from inactivation, and the enzyme activity was only partially restored by brief treatments with hydroxylamine. N-Ethoxyformylimidazole is the product of histidine modified with ethoxyformic anhydride, and this compound is deacylated to regenerate histidine within a few minutes by treatment with hydroxylamine.[92] Since hydroxylamine only partially regenerated the enzyme activity, Witt and Roskoski reasoned that a tyrosine residue had been modified.[85] The pK_a of the group modified was measured to be 7.8 ± 0.3, a somewhat low value for tyrosine.[85]

In another study, glycine ethyl ester was shown to modify covalently rabbit skeletal muscle type-I catalytic subunit, which had been activated by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide.[86] The former modification resulted in a total loss of phosphotransferase activity.[86] Using the methodology developed by Hoare and Koshland,[87] Matsuo et al. quantitated the incorporation of 1.7 ± 0.2 molecules of [1-^{14}C]glycine ethyl ester for each catalytic subunit.[86] The enzyme was protected from inactivation by the presence of histones or

polyarginine, a competitive inhibitor with respect to peptide, but not by MgATP. To identify the residues that were modified, the labeled catalytic subunit was digested exhaustively by proteolytic enzymes, and after purification of the remaining peptides, the modified residues were identified as derivatives of glutamic acid.[86]

oATP, the 2′,3′ dialdehyde derivative of ATP, is reported to modify porcine brain protein kinase.[87] ATP protected the catalytic subunit against inactivation by oATP. Following sodium borohydride reduction of the suspected aldimine bond between the enzyme and [^{14}C]oATP, modified catalytic subunit was digested exhaustively with trypsin. A labeled peptide was isolated by two-dimensional paper electrophoresis, first at pH 6.5 and again at pH 3.5. Amino acid analysis following hydrolysis with hydrochloric acid indicated that this peptide contained 1.0 valine, 0.9 proline, 1.1 glycine, 0.9 arginine, and an additional compound, which comigrated with authentic hydrochloric acid-treated α amino-ε-N-(2,3-dihydroxypropyl)-lysine during two-dimensional chromatography on polyamide plates.[87] The N-terminal amino acid of this peptide was determined to be valine.[87] Interestingly, residues 185 to 195 from the primary sequence of the bovine heart catalytic subunit[13] are

186		191		196
Gly-Phe-Ala-Lys-Arg-Val-Lys-Gly-Arg-Thr-Trp				

If lysine 192 is modified and the enzyme is digested by trypsin, the modified peptide would have valine at the N terminus and contain glycine and arginine as well, in addition to the modified amino acid. This is similar, but not identical to the composition of the peptide isolated by Kochetkov et al.[87] It is possible that this porcine brain protein kinase catalytic subunit is largely homologous, but not identical to the type–II catalytic subunit isolated from the bovine heart.

Zoller and Taylor used *p*-fluorosulfonyl-[^{14}C]benzoyl 5′-adenosine to modify irreversibly the catalytic subunit of the type-II protein kinase isolated from porcine skeletal muscle.[88] Catalytic activity was completely inhibited by the incorporation of one molecule of reagent per enzyme, and inactivation of the enzyme could largely be prevented by the addition of MgATP or MgADP, while the metal-free nucleotides afforded little protection.[88] This loss of enzyme activity followed pseudo first-order kinetics, and since the adenosine derivative was observed to saturate the enzyme at high concentrations of reagent, Zoller and Taylor described the reaction by the scheme of Equation 3 and analyzed the kinetics according to the equality in Equation 4.[88]

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} Ei \qquad (3)$$

$$k_{obs} = \frac{k_2}{\frac{K_I}{[I]} + 1} \qquad (4)$$

In these equations, $K_I = [E][I]/[EI]$, E is the catalytic subunit, I is the adenosine analog, and Ei is the modified enzyme. From a plot of k_{obs}^{-1} vs. $[I]^{-1}$, K_I was determined to be 0.057 m*M*, close to their value for the K_m for ATP (0.012 m*M*).[88] At saturation k_2 was determined to be 0.0364 min^{-1}.[88] Very similar values for these constants have been reported for the modification of bovine heart type-II catalytic subunit with the same reagent.[89] Following tryptic digestion of the [^{14}C]-labeled enzyme, Zoller and Taylor isolated a single radioactive peptide containing a modified lysine residue.[88] This residue was later identified as lysine-72.[76]

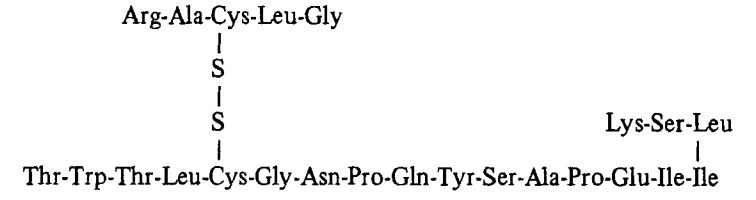
To identify additional residues in the protein kinase catalytic subunit active site, it would

be desirable to modify the enzyme with a series of affinity analogs based on the structure of the substrate peptide *1*. By the systematic replacement of amino acid residues in the peptide sequence Leu-Arg-Arg-Ala-Ser-Leu-Gly with potentially labile amino acid analogs, it might be possible to create a variety of affinity labels and suicide substrates for protein kinase. Using this approach, Leu-Arg-Arg-Ala-Cys(Npys)-Leu-Gly, peptide *4* was synthesized and used to modify the protein kinase catalytic subunit.[77] Peptide *4* contains the group 3-nitro-2-pyridinesulfenyl (Npys) which has been used previously as a protecting group in peptide chemistry.[94] The modification of the protein kinase catalytic subunit with peptide *4* was monitored spectrophotometrically by following the release of the Npys moiety.

Modification of the protein kinase catalytic subunit with peptide *4*, peptide *5*, Nyps-SCH_3, or Pys-SCH_3 resulted in the loss of over 99% of enzymic activity.[77,95] Spectrophotometric titrations of the catalytic subunit with these reagents indicated that the peptides reacted with a single enzymic group, while the thiomethylating reagents reacted with two.[77,95] Enzyme that had been modified with DTNB would not react with any of the above reagents, and thiomethylated enzyme did not react with DTNB. In contrast, enzyme modified with peptides *4* or *5* still reacted with one equivalent of DTNB in a kinetically monophasic process. The catalytic activity of any modified enzyme could be restored by treatment with dithiothreitol.[77,95]

The reactions of catalytic subunit with peptide *4*, peptide *5*, Pys-SCH_3, and Nyps-SCH_3 all followed kinetics which were pseudo first-order with respect to the release of the Npys or the Pys chromophores. In addition, at high concentrations peptides *4* and *5* were observed to saturate the enzyme, and using methods similar to those employed by Zoller and Taylor,[88] these peptides were found to modify 4.3 μM catalytic subunit with an apparent value of K_I of 40 μM and a k_2 value of 0.25 sec^{-1}.[77] These low K_I values suggest that peptides *4* and *5* are binding in the enzyme active site. Additional evidence that the peptides are active-site directed reagents is that either MgATP or peptide *1* in the presence of Mg^{2+} and β,γ-methylene ATP protect the enzyme against inactivation with peptide *4*.[77]

By radiolabel incorporation measurements, as well as by spectrophotometric analysis, peptide *6*, the radiolabeled analog of peptide *5*, has been shown to modify a single residue.[77] Modified enzyme was digested with trypsin and mapped by HPLC as described by Nelson and Taylor[84] and adapted by Bramson et al.[77] On the basis of amino acid analysis, dansylation of N-terminal residues, and automated solid-phase sequencing, the two radiolabeled peptides isolated in this manner were identified as



and the fragment resulting from the trypsin catalyzed cleavage of the remaining arginine from this peptide.[72] The enzymatic portion of these fragments correspond to amino acid residues 199 to 213 of the protein kinase catalytic subunit, indicating that cysteine 95 was the residue modified by peptide *6*.[13,77]

The protein kinase catalytic subunit has been reported to be inactive once thiomethylated[77] or modified with DTNB.[39,40,47,80] However, the enzyme retains about 60% of its activity when cyanylated.[40,96] This is interesting since the thiomethyl group is not very different in size from a cyano group. A simple explanation for this behavior is that a cysteine residue is in the protein kinase active site near the catalytic site of phosphoryl group transfer and that this residue is not catalytically functional.

Nucleotide complexes of the catalytic subunit bind two Mn^{2+} atoms per enzyme, one in

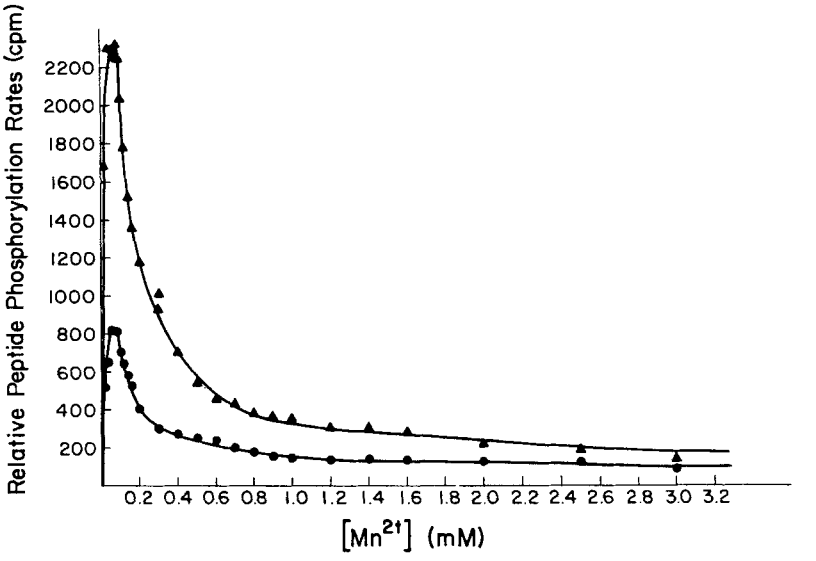


FIGURE 9. The dependence of the initial rate of peptide *1* phosphorylation on total Mn(II) concentration. These assays were performed using the [γ-^{32}P]ATP-peptide assay in 50 m*M* Tris buffer, pH 7.5, containing 100 μ*M* [γ-^{32}P]ATP, 675 μ*M* peptide *1*, 0.15 *M* KCl, 0.1 m*M* DTT, 0.125 mg/mℓ BSA, and 8.9 n*M* native enzyme (Δ) or cyanylated enzyme, omitting the DTT (o) at 25°C. The data represent the average of two activity determinations for each point. No DTT was included in the assays when cyanylated enzyme was used.

an activating site and the other in an inhibitory site bridging ATP to the enzyme.[62,63] One possible explanation for this observation might be that an active site cysteine of the enzyme coordinates to the metal in the inhibitory site.[96] This appears not to be the case since cyanylation of the catalytic subunit fails to prevent inhibition by excess Mn^{2+} (Figure 9).

V. MECHANISTIC PROPOSALS FOR PROTEIN KINASE MEDIATED PHOSPHORYL GROUP TRANSFERS

The mechanisms that have been suggested for the catalytic action of protein kinase may be classified into three groups: (1) those involving a phosphorylated enzyme intermediate, necessitating double displacement in order to transfer the phosphoryl group; (2) a single displacement "associative" mechanism requiring direct transfer of the phosphoryl group; and (3) a "dissociative" type mechanism, involving the formation of a metaphosphate intermediate (see Figure 10). In addition, mechanisms that blend features of 1, 2, and 3 must be considered. One such mechanism is an associative mechanism for which the transition state has substantial metaphosphate character. At the present time, the evidence is insufficient to eliminate any of these mechanisms. As shall be described below, some experimental results can be interpreted as being more consistent with a particular mechanism rather than another. As summarized in Table 7, the evidence to be considered includes studies of: (1) steady-state kinetics, (2) phosphoryl group transfer from [γ-^{32}P]ATP to [^{14}C]ADP, (3) the use of Co(III)ATP as a substrate, (4) the relative rates of ATPase and peptide kinase activities of the enzyme in the presence of Mg^{2+} or Mn^{2+}, and (5) intersubstrate distances measured using magnetic resonance techniques.

A. Steady-State Studies of the Protein Kinase Catalytic Mechanism

Moll and Kaiser[97] and Kochetkov et al.[87] have studied the kinetics of histone phosphorylation catalyzed by cAMP-dependent protein kinases derived from bovine and porcine

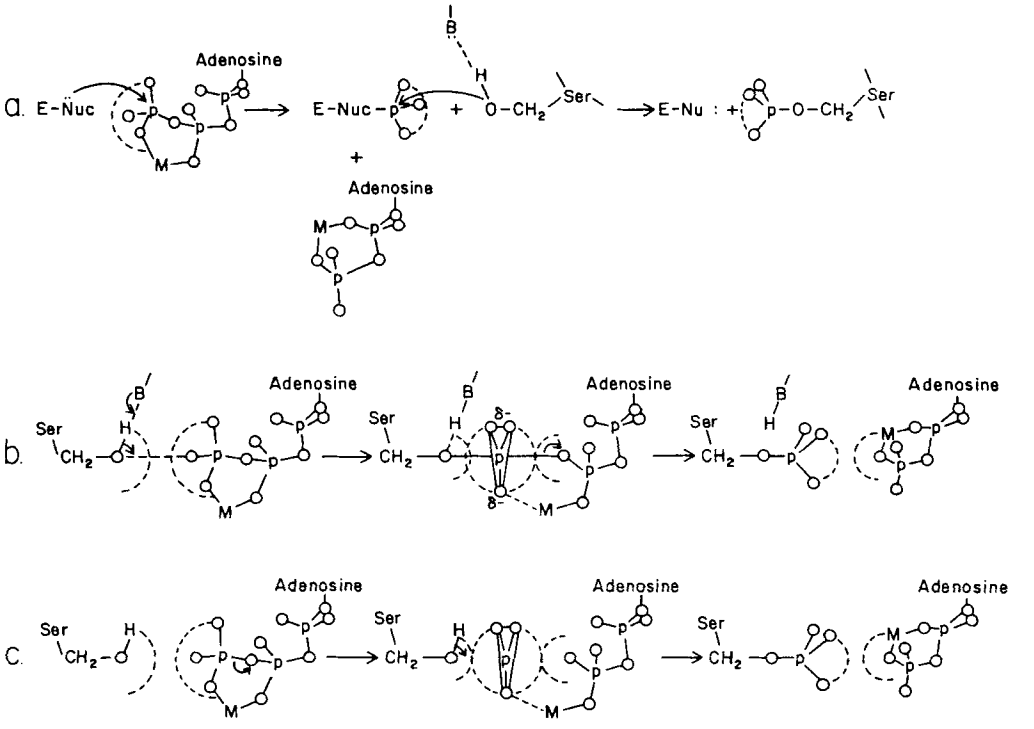


FIGURE 10. Alternate mechanisms for the protein kinase catalyzed phosphoryl group transfer. Both mechanisms a and b require a base (B), while mechanism c does not. (a) Ping-pong mechanism; the nucleotide product must dissociate before the second substrate can be bound. (b) Associative mechanism with substrates either binding sequentially or randomly. (c) Dissociative mechanism with substrates that can also bind sequentially or randomly.

Table 7
INTERPRETATIONS OF STUDIES 1—5[a]

	Most consistent with a			
Study[a]	**Double displacement mechanism**	**Associative mechanism**	**Dissociative mechanism**	**Associative mechanism with a transition state having metaphosphate character**
1	No	Yes	Yes	Yes
2	No	Yes	Yes	Yes
3	No	Yes	Yes	Yes
4	Yes	No	Yes	Yes
5	Yes	No	Yes	Yes

[a] 1, 2, 3, 4, and 5 are defined in the text.

brains, respectively. In both cases, double reciprocal plots of the initial rate data with either histone or ATP varied gave rise to parallel lines, so the authors suggested that the phosphoryl group transfer might occur via a double displacement "ping-pong" type mechanism.[87,97] Under the assay conditions of Moll and Kaiser, no long-lived reaction intermediate was kinetically detected,[97] but Kochetkov et al. reported that phosphorylated histidine was isolated after incubation of the enzyme with [γ-^{32}P]ATP.[98] Kinetic results from more recent work with protein kinases from other sources, however, are compatible with mechanisms that do not invoke a covalent intermediate. Double reciprocal plots of initial rate data from the

phosphorylation of peptides by catalytic subunits from calf thymus[54] or rabbit skeletal muscle[99] were found to intersect on the x axis, consistent with the single displacement mechanisms b and c (Figure 10). In addition, dead-end inhibitors[99] and the products ADP or phosphohistone[100] were all observed to inhibit the phosphotransferase reaction catalyzed by the rabbit or bovine skeletal muscle enzymes in a manner consistent with mechanisms b and c, but not with mechanism a.

B. Isotopic Exchange Studies

Even though no phosphoenzyme intermediate has been demonstrated for any protein kinase catalyzed reaction, the evidence against such an intermediate is not compelling. Among the evidence is the observation that the bovine heart enzyme does not catalyze phosphoryl group transfer from γ-^{32}P-labeled MgATP to ^{14}C-labeled MgADP,[66] although the enzyme has ATPase activity in the absence of peptide or protein substrates.[77] If a phosphoenzyme intermediate is formed, such an intermediate might be very short lived; NMR studies have shown that the time necessary for one nucleotide to dissociate from the enzyme and another to bind is very short (certainly less than 1 μsec) and exchange experiments suggest that the phosphoenzyme must decompose before a second nucleotide can bind. An alternate possibility that cannot be ruled out at the present time is that a phosphoenzyme intermediate is formed in the reaction of MgATP with protein kinase but that the MgADP product is not released into solution until the phosphoenzyme decomposes.

C. Protein Kinase Uses Co(III)ATP as a Substrate

The substitution inert $Co(NH_3)_4ATP$ complex functions as a substrate of protein kinase in a phosphoryl group transfer reaction with peptide *I* acting as the phosphoryl group acceptor.[65] This argues against a double displacement mechanism with a phosphoenzyme intermediate, since Co(III) cannot readily dissociate ADP or acquire new ligands.

D. Enzyme Catalyzed ATPase and Peptide Kinase Partitioning Ratios in the Presence of Mn^{2+} or Mg^{2+}

If a phosphorylated intermediate is not formed, then this leaves the dissociative and associative processes as possibilities for the mechanism of the phosphoryl group transfers. An associative mechanism may either proceed through a penta coordinate intermediate or by way of concerted bond breaking and bond making, whereas in a dissociative mechanism bond breakage occurs before bond formation begins. Armstrong et al. have reported that the cAMP-dependent ATPase and peptide kinase activities of protein kinase decrease by a similar factor (~20-fold) when Mg^{2+} is replaced by Mn^{2+} as the activating cation.[7] This suggests both that the activating metal is involved in the transition state of the rate-determining step and that in this rate-determining step bond breaking between the γ phosphoryl group and ADP is not concerted with bond formation between the phosphoryl group and the entering nucleophile. The justification for the latter statement is that if the ATPase and peptide kinase reactions were to proceed by an associative mechanism, then the transition states for these two reactions which involve different phosphoryl group-accepting nucleophiles are expected to be different enough that the change from Mg^{2+} to Mn^{2+} is expected to affect those transition states to different extents.

E. Intersubstrate Distances in the Protein Kinase Active Site: Mechanistic Implications

To have a detailed understanding of the mechanism of protein kinase catalysis, it would be useful to know the distance between the phosphorous atom in the γ phosphorous group of enzyme-bound ATP and the hydroxyl oxygen of the accepting amino acid residue. For example, the distance predicted for van der Waal's contact between a phosphorous atom and an oxygen atom is 3.3 Å. Such a reaction coordinate distance has been found by NMR

Table 8
INTERSUBSTRATE DISTANCES DETERMINED USING NMR AND ESR MEASUREMENTS[68,102]

Technique used	Substrates or substrate analogs bound	From	To	Distance (A)
NMR	Mn^{2+}, $Co(NH_3)_4$AMP-PCP	Mn^{2+}	Methylene protons of $Co(NH_3)_4$AMPCP	5.0 ± 0.9
ESR	Mn^{2+}, CrAMP-PCP	Mn^{2+}	Cr^{3+}	4.8 ± 0.4
NMR	CrAMP-PCP, Leu-Arg-Arg-Ala-Ser-Leu-Gly	Cr^{3+}	The C_β-H_2 of serine in the peptide	8.1 ± 0.8
NMR	Mg^{2+}, CrAMP-PCP, Leu-Arg-Arg-Ala-Ser-Leu-Gly	Cr^{3+}	The C_β-H_2 of serine in the peptide	8.1 ± 0.8
NMR	Mn^{2+}, $Co(NH_3)_4$AMP-PCP, Leu-Arg-Arg-Ala-Ser-Leu-Gly	Mn^{2+}	The C_β-H_2 of serine in the peptide	9.1 ± 0.9
NMR	Mn^{2+}, $Co(NH_3)_4$ATP, Leu-Arg-Arg-Ala-Ser-Leu-Gly	Mn^{2+}	The C_β-H_2 of serine in the peptide	9.5 ± 1.3
NMR	Mn^{2+}, $Co(NH_3)_4$ATP	Mn^{2+}	The nearest Ala methyl group	10.5 ± 1.2

methods in the active site of pyruvate kinase, suggesting that an associative mechanism operates.[101] Indeed, a distance less than 4.9 Å would allow some associative character to a phosphoryl transfer mechanism since new bond formation would begin before old bond cleavage was complete.[101] Therefore, if protein kinase catalyzes phosphoryl transfer through an associative mechanism (Figure 10, scheme b) the distance along the reaction coordinate should be less than 4.9 Å. The distance between the phosphorous and the oxygen atoms necessary to permit a dissociative mechanism (Figure 10, scheme c) or a phosphoenzyme intermediate (Figure 10, scheme a) is 4.9 Å.[101] To determine the phosphorous to oxygen distance, Granot and co-workers used NMR and ESR measurements to determine the distances between and from the enzyme-bound paramagnetic probes Mn^{2+} (bound in the inhibitory site) and Cr^{3+} (in the activating site) to AMP-PCP and the peptides Leu-Arg-Arg-Ala-Ser-Leu-Gly and Leu-Arg-Arg-Ala-Ala-Leu-Gly (Table 8).[68,102] From a Dreiding molecular model constructed consistent with the measurements listed in Table 8, the distance between the γ phosphorous of AMP-PCP and the hydroxyl oxygen of the peptide was estimated to be 5.3 ± 0.7 Å (Figure 11).[102]

While a distance of 5.3 ± 0.7 Å along the protein kinase catalyzed reaction coordinate the existence of a metaphosphate intermediate, other explanations are possible. The distance measurements were generally made in the presence of a strongly inhibitory metal (Mn^{2+}), and this could deform the "normal" substrate conformation. This probably does not occur since the presence of Mg^{2+}, which inhibits the phosphotransferase activity of protein kinase less strongly than Mn^{2+}, in the inhibitory site did not significantly alter the intersubstrate distances or arrangement (Table 8).[102] Also, assuming that the more active substrates and their analogs bind in the same manner, it is still possible that the substrates move toward each other as the transition state for the reaction is approached.[102] If this does not happen, the long reaction coordinate distance is consistent with either a dissociative mechanism (Figure 10, scheme c) or a phosphoenzyme intermediate (Figure 10, scheme a). The evidence against the latter was summarized above. While a dissociative mechanism is an interesting possibility worthy of further study, it is certainly not established since the lower limit reaction coordinate distance of 4.6 Å would allow a mechanism with some associative character but with an elongated phosphorane transition state.[102]

In summary, the structural and kinetic evidence, while not conclusive, suggest either a dissociative mechanism (Figure 10, scheme b) or an associative mechanism with an elongated transition state (Figure 10, scheme c).[102,103]

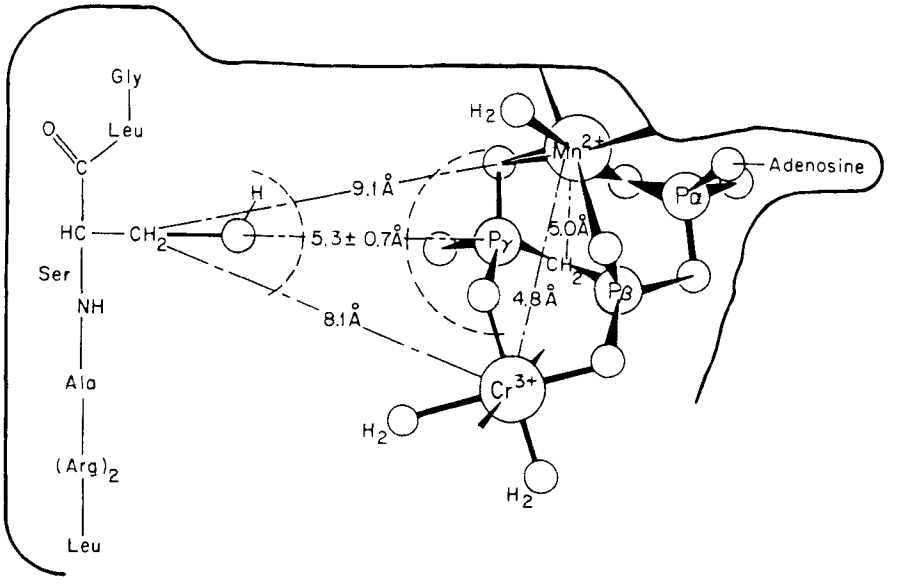


FIGURE 11. Intersubstrate and intermetallic distances in the protein kinase active site as determined by NMR. The van der Waal's radii of the γ-phosphate and the Ser oxygen are shown as dashed arcs.[102]

F. A Continuous Assay for Protein Kinase Activity

Prior to the development of the spectrophotometric assay to be described, the activity of protein kinase was most commonly measured using a [γ-^{32}P]ATP-peptide assay.[104] Phosphorylation of the serine residue in peptide *7*, Leu-Arg-Arg-(*o*-NO_2)Tyr-Ser-Leu-Gly, is catalyzed by cAMP-dependent protein kinase, and this process can be monitored spectrophotometrically.[105] Absorption spectra of a solution containing MgATP and peptide *7*, before and after phosphoryl group transfer had been catalyzed by protein kinase, are shown in Figure 12. The magnitude of the decrease in ϵ_{430} is at a maximum ($\gtrsim$350 M^{-1}) in MOPS buffer, pH 7.0. In Tris buffer at pH 7.5, the average values for k_{cat}, $K_{m,peptide}$, and $k_{cat}/K_{m,peptide}$ calculated from the total time courses of the protein kinase catalyzed phosphorylation of peptide *7* were 3000 ± 400 min^{-1}, 41 ± 11 μ*M*, and 73 ± 22 μM^{-1} min^{-1}, respectively, while in MOPS buffer at pH 7.0 these values were 2400 ± 200 min^{-1}, 15 ± 2 μ*M*, and 160 ± 10 μM^{-1} min^{-1}, respectively.[96,106]

In a preliminary study, the kinetics of peptide *7* phosphorylation were measured in MES and EPPES buffer solutions ranging from pH 6 to 9, and the results obtained are shown in Figure 13.[96] The pH dependence of the kinetic parameter $k_{cat}/K_{m,peptide}$ can be described by a bell-shaped curve with a $(k_{cat}/K_{m,peptide})_{lim}$ value of 183 ± 12 μM^{-1} min^{-1} and controlling pK_as of 6.16 ± 0.12 and 8.32 ± 0.13.[96] From the conditions of the experiment, these pK_a values could, in principle, reflect dissociating groups in either the enzyme-ATP-Mg^{2+} complex or in the free peptide. However, neither of these pK_a values correspond to those of the peptide since the pK_a of the nitrotyrosine residue was determined spectrophotometrically to be 7.01 ± 0.03 when the peptide is phosphorylated and 6.83 ± 0.02 when it is not.[105] If the pK_as of 6.16 and 8.32 represent dissociation constants for ionizing groups affecting the phosphorylation reaction catalyzed by protein kinase, then the following conditions must be met: (1) these groups must titrate as do acids and bases, (2) protein kinase must be active in only one ionic form, (3) all proton transfers must be fast compared to the chemical steps, and (4) no change in the rate-determining step can occur over the pH range 6 to 9.[106] In general, pK_as measured from the pH dependence of an enzyme's activity are assigned to particular groups in that enzyme thought to be involved in the catalytic process by showing

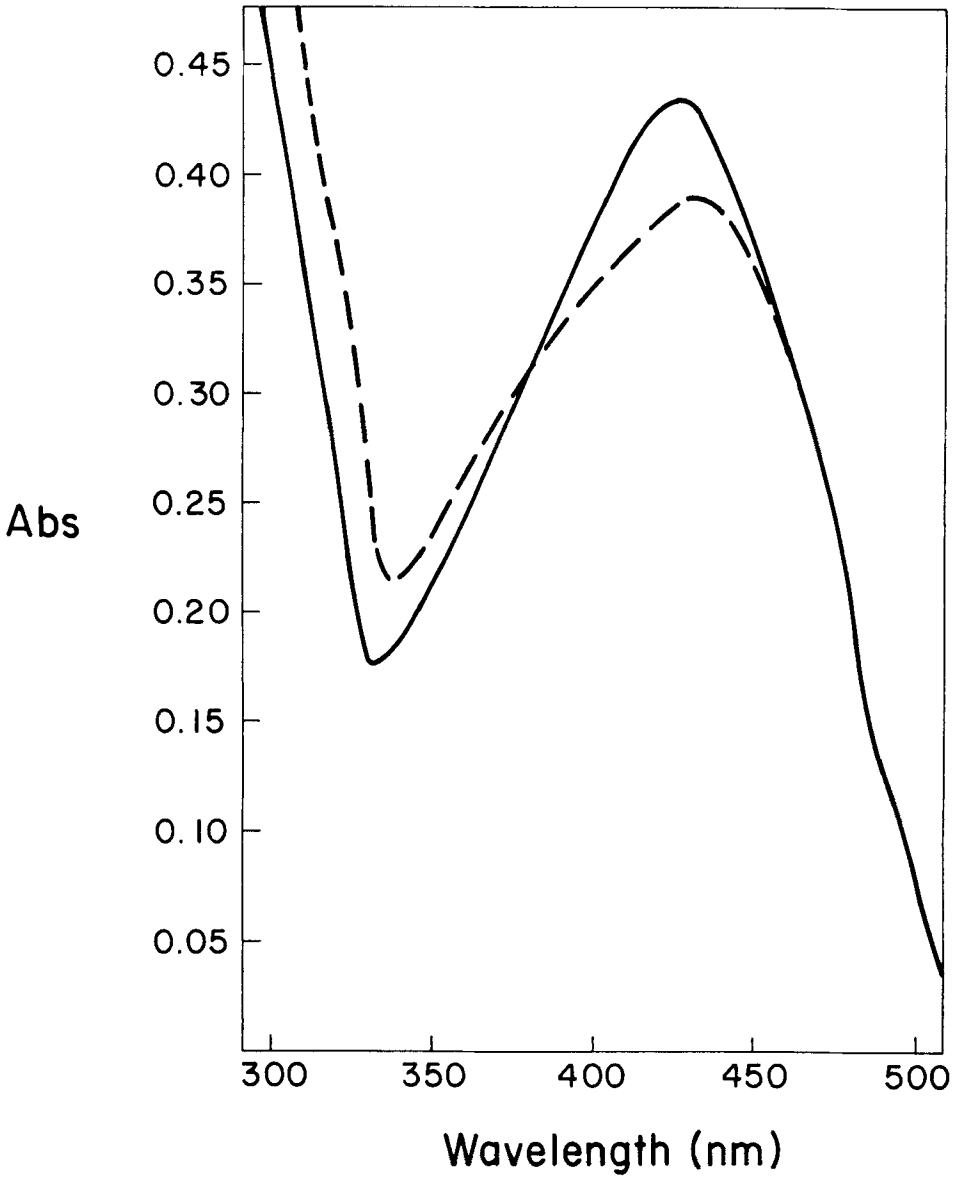


FIGURE 12. Substrate and product spectra for peptide 7. These spectra were measured in a mixture containing 200 μ*M* peptide 7, 256 μ*M* ATP, 10 m*M* MOPS, at pH 6.5 before (solid line) and after (dashed line) the addition of 500 n*M* protein kinase catalytic subunit. The volume of added enzyme was 1% of the total solution volume, and so the resulting dilution does not account for a major portion of the absorbance change.

to the pK_a calculated from the pH dependence of enzyme activity. Unfortunately, very little is known about the amino acid residues in the active site of protein kinase. Only two active site residues of protein kinase, lysine 72[76] and cysteine 199,[77] have been identified by group-specific modifications and inactivations of enzymic activity. No data for the pH dependence of these modifications are available at this time. While assignments of the pK_as measured for the protein kinase catalyzed phosphorylation of peptide *7* to specific residues are not currently possible, from observations of the pK_a values measured for amino acid residues in various proteins or small peptides, some possibilities can be considered. The pK_a of 6.16

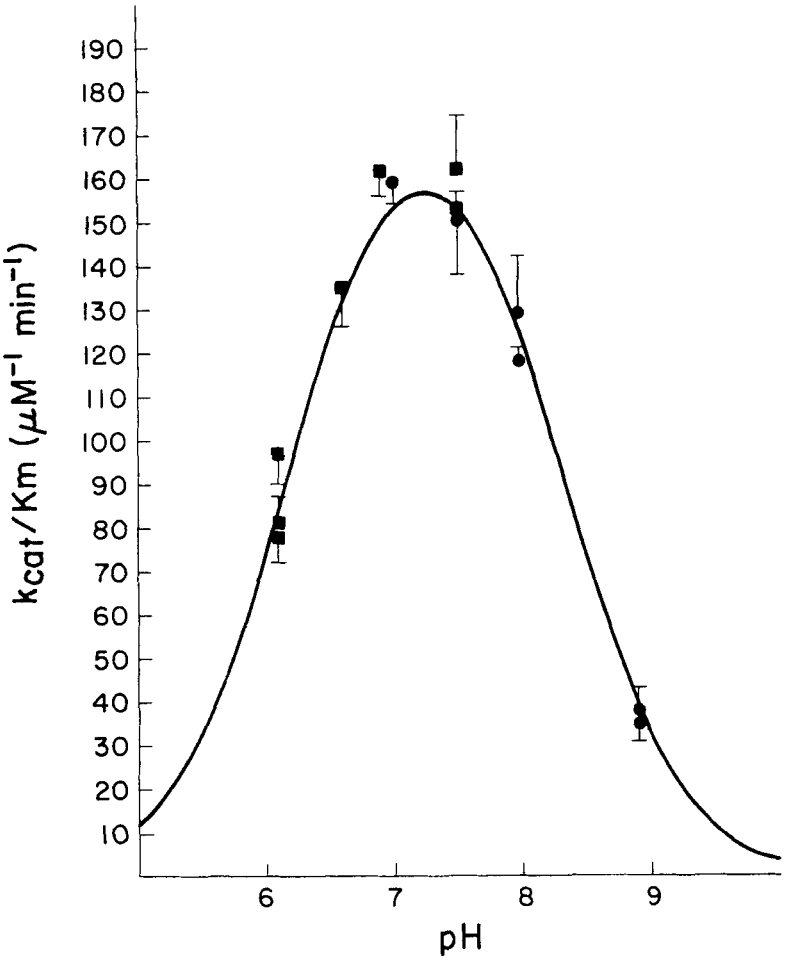


FIGURE 13. The pH dependence of the kinetic parameter $k_{cat}/K_{m,peptide}$. Enzyme activity was measured using the spectrophotometric assay in 50 m*M* MES (■) or EPPES (●) buffer with 0.15 *M* KCl, 0.2 mg/mℓ BSA, 1 m*M* DTT, 10 m*M* Mg^{2+}, 2 m*M* ATP, and 100 μ*M* peptide 7, all at 30°C. The solid line was calculated using the equation.

$$k_{cat}/K_{m,peptide} = \frac{(k_{cat}/K_{m,peptide})_{lim}}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}}$$

± 0.12 is a typical value for a histidine residue, but could also be caused by the ionization of the free carboxyl group of a glutamic or an aspartic acid residue as in carboxypeptidase A and lysozyme. This pK_a may also be due to the second ionization of an enzyme phosphate group (protein kinase has two[40]), or, possibly, MgATP. The pK_a of 8.32 ± 0.13 is compatible with a cysteine, perhaps cysteine 199, and is rather low for lysine or tyrosine or high for histidine, but these residues may still be possible ionizing groups that could affect enzymic activity.

After the results of the peptide 7 spectrophotometric assay had been published,[105] Wright et al. reported the synthesis of Leu-Arg-Arg-Trp-Ser-Leu-Gly and that the phosphorylation of this peptide caused a 20% increase in the tryptophan fluorescence intensity measured at 358 nm.[74] The kinetic parameters for the protein kinase holoenzyme catalyzed phosphorylation of this tryptophan-containing peptide are 84 ± 6 min^{-1} and 2.7 ± 0.5 μ*M* for k_{cat}

and $K_{m,peptide}$, respectively, when measured in 25-m*M* MOPS (pH 7.0 with 10 m*M* $MgCl_2$, 2 μ*M* cAMP, between 0.7 and 9.1 μ*M* peptide, and 0.2 to 1.0 m*M* ATP at 25°C).[74] These values of k_{cat} and $K_{m,peptide}$ were determined by initial rate kinetics and were reported to be very similar to those of peptide *1* when measured under the same conditions. This fluorometric assay and the spectrophotometric assay utilizing peptide *7* together offer a choice in spectrophotometric assay.

VI. SUMMARY

The details of the process by which protein kinase catalyzes phosphoryl group transfers are beginning to be understood. Early work that explored the primary specificity of cAMP-dependent protein kinase action enabled the synthesis of small peptide substrates for the enzyme. Enzyme-peptide interactions seem simpler to understand than protein-protein interactions, so peptide substrates have been used in most protein kinase studies. In most investigations the kinetics for the phosphorylation of small peptides have been interpreted as being consistent with mechanisms which do not invoke phospho-enzyme intermediates (see, for example, Bolen et al.[66]). Protein kinase has been shown to bind two metal ions in the presence of a nucleotide.[62] Using magnetic resonance techniques the binding of these ions has been utilized to elucidate the conformation of nucleotide[63] and peptide[68] substrates or inhibitors when bound in the enzymic active site. Also, two new peptides with the form Leu-Arg-Arg-Ala-Ser-Y-Gly, where Y was either Pro or (N-methyl)Leu, were synthesized and found not to be substrates, within the limits of detection, for protein kinase.[69] The striking lack of affinity that protein kinase has for such peptides which are unlikely to form a $\beta_{3\text{-}6}$ turn has not been reported before. Our results may indicate that this type of turn is a requirement for protein kinase catalyzed phosphorylation or that these peptides lack the ability to form a particular hydrogen bond with the enzyme.

Magnetic resonance techniques have indicated that the distance between the phosphorous in the γ-phosphoryl group of MgATP and the hydroxyl oxygen of serine in the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly is 5.3 ± 0.7 Å.[96] This, together with certain kinetic evidence, suggests that the mechanism by which protein kinase catalyzes phosphoryl group transfer has considerable dissociative character. Chemical modifications, including one using a peptide-based affinity label, have identified two residues at or near the active site, lysine-72[76] and cysteine 199.[77] While neither of these groups has been shown to be catalytically essential, similar studies may help to identify groups that are directly involved in the catalytic process. Finally, a spectrophotometric assay for cAMP-dependent protein kinase has been described.[105] Using this assay the preliminary results of an in-depth study of the pH dependence of protein kinase catalyzed phosphoryl group transfer have been obtained.[69] This study shall aid in the identification of active site residues and should contribute to the elucidation of the enzyme's catalytic mechanism. The spectrophotometric assay has been shown to succeed because phosphorylation of the serine residue in the peptide sequence Leu-Arg-Arg-(*o*-NO_2)Tyr-Ser-Leu-Gly perturbs the environment of the neighboring *o*-nitrotyrosine residue enough to cause a measurable change in the absorbance of the phenolic chromophore.[69] This assay technique can be adapted for kinetic studies on other enzymes. By the selection of appropriate reporter groups and the placement of these moieties adjacent to sites in specific sequences that are phosphorylated or dephosphorylated by particular enzymes, it may be possible to develop continuous assays for these enzymes. Indeed, cGMP-dependent protein kinase has been assayed in this manner,[107] as has phosphoprotein phosphatase.[74]

ACKNOWLEDGMENT

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NOTE ADDED IN PROOF

Since the submission of our article in December, 1982 two important papers on the kinetic mechanism of cAMP protein kinase action have appeared. They are listed below.

1. **Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., and Roskoski, R. Jr.,** Adenosine cyclic 3′,5′-monophosphate dependent protein kinase:kinetic mechanism for the bovine skeletal muscle catalytic subunit, *Biochemistry*, 21, 5794, 1982.
2. **Whitehouse, G. and Walsh, D. A.,** Studies on the kinetic mechanism of the catalytic subunit of the cAMP-dependent protein kinase, *J. Biol. Chem.*, 258, 3693, 1983.