Studies on the Kinetic Mechanism of the Catalytic Subunit of the cAMP-dependent Protein Kinase*

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The kinetic mechanism of the catalytic subunit of the cAMP-dependent protein kinase has been investigated employing the heptapeptide Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate. Initial velocity measurements were performed over a wide range of ATP and Kemp-tide concentrations indicated that the reaction follows a sequential mechanistic pathway. In line with this, the results of product and substrate inhibition studies, the patterns of dead end inhibition obtained employing the nonhydrolyzable ATP analogue, AMP·PNP (5'-adenylylimidodiphosphate), and equilibrium binding determinations taken in conjunction with the patterns of inhibition observed with the inhibitor protein of the cAMP-dependent protein kinase that are reported in the accompanying paper (Whitehouse, S., and Walsh, D. A. (1983) J. Biol. Chem. 258, 3682-3692), are best fit by a steady state Ordered Bi-Bi kinetic mechanism.

Although the inhibition patterns obtained employing the synthetic peptide analogue in which the phosphorylatable serine was replaced by alanine were apparently incompatible with this mechanism, these inconsistencies appear to be due to some element of the structure of this latter peptide such that it is not an ideal dead end inhibitor substrate analogue. The data presented both here and in the accompanying paper suggest that both this substrate analogue and the ATP analogue, AMP·PNP, do not fully mimic the binding of Kemptide and ATP, respectively, in their mechanism of interaction with the protein kinase. It is proposed that, as with some other kinase reactions, the configuration of the terminal anhydride bond of ATP assumes a conformation once the nucleotide is bound to the protein kinase that assists in the binding of either Kemptide or the inhibitor protein but not the alanine-substituted peptide and that AMP·PNP, because of its terminal phosphorylimido bond, cannot assume this conformation which favors protein (or peptide) binding.

Although the cAMP-dependent protein kinase is of central importance in hormonal regulation of metabolism, its exact mechanism has yet to be elucidated. Initial velocity studies reported for the holoenzyme and the catalytic subunit have been inconsistent, especially with histone as substrate (1-4). Parallel double reciprocal plots, indicative of a ping-pong mechanistic pathway, were obtained when histone was employed as substrate for the bovine brain holoenzyme (1), whereas converging plots, diagnostic of a sequential pathway, were obtained when the same substrate was used with the rabbit skeletal muscle catalytic subunit (2). The phosphorylation of histone H1 (and peptides derived from H1) catalyzed by calf thymus catalytic subunit was reported to follow sequential kinetics (3), although the mechanism was suggested to be ping-pong when the reaction was catalyzed by porcine brain catalytic subunit (4) and in that case a putative phosphoprotein intermediate was even identified.

A number of studies have been reported employing the heptapeptide, Kemptide, which closely corresponds to the amino acid sequence around the phosphorylatable serine in porcine liver pyruvate kinase (5). The use of this peptide eliminates a number of possible problems associated with the use of protein substrates, e.g. multiple phosphorylation sites and possible contamination of the substrate preparation with other kinases, both of which can compromise the interpretation of kinetic data. Indeed, the results obtained using this peptide have been more consistent; converging initial velocity plots have been reported in studies employing the catalytic subunit from bovine thymus (3), cardiac (6), and skeletal muscle (7) protein kinases. Interestingly, with ATP/P (A' diastereomer, the reaction with Kemptide appeared to follow a ping-pong mechanism (6).

Consistent with a sequential pathway and based upon a series of physical and kinetic measurements using the bovine cardiac muscle catalytic subunit with Kemptide as substrate, Gronot, Mildvan and coworkers (8-12) concluded that there were two probable kinetic mechanisms for the protein kinase, the steady state ordered and the steady state random. Since neither of these mechanisms can be eliminated with data published to date, the mechanism has been investigated further. This paper contains the product and dead end inhibition patterns, together with equilibrium binding of substrates and inhibitors to the catalytic subunit of the protein kinase. From this, and from data in an accompanying report (13), a mechanism of the protein kinase is suggested and this mechanism may help to explain the apparent inconsistencies of the data that have been reported by the various laboratories.

1 The histone used was a commercial mixture but contained predominantly H1.

2 The abbreviations used are: Kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Boc, t-butyloxycarbonyl; AMP·PNP, 5'-adenylylimidodiphosphate; Ala-peptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid.

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Experimental Procedures

Phosphorylation Reactions—Kemptide was the phosphate acceptor in all kinase assays. Phosphorylation reaction mixtures contained: 62.5 mM HEPES (pH 7.1), 12.5 mM magnesium acetate, 0.25 mM EGTA, 0.3 mg/ml of bovine serum albumin, 5-120 ng/ml of protein kinase catalytic subunit, [γ-32P]ATP (19,000 cpm/pmol in Fig. 1, 200-200 cpm/pmoll in all other experiments) and peptide substrate in a total volume of 80 μl. Substrate and inhibitor concentrations were as indicated in the figures and figure legends. Reactions were initiated by the addition of [γ-32P]ATP (Fig. 1) or enzyme (all other experiments) and conducted for 1-5 min at 30 °C. Product formation was linear with respect to time and to amount of enzyme, less than 10% of the limiting substrate was consumed during the reaction, and hydrolysis of ATP (14) and protein kinase catalytic subunit concentrations employed in these kinetic studies. Reactions were terminated by the addition of acetic acid to final concentrations of 33% (Figs. 1, 4c, and 5) or 25% (all other experiments). Phosphopeptide formation was quantified with the use of phosphocellulose paper (Whatman P81) squares (15) and the procedure was shown to be valid even with the very high peptide concentrations that were used in the experiment presented in Fig. 5.

Ligand-binding Measurements—Equilibrium binding of [2,8-3H]ADP (New England Nuclear), [2,8-3H]AMP-PNP (ICN), [3H]Leu-Arg-Arg-Ala-Ala-Leu-Gly, [3H]Ac-Leu-Arg-Ala-Ser-Leu-Gly and [3H]Leu-Arg-Arg-Ala-Ser-Phe-Leu-Gly were measured in 0.2 ml microdialysis chambers (Hoefer Scientific Instruments) at 4 °C. Protein kinase catalytic subunit was constrained to one chamber by a semipermeable membrane (M, cutoff 12,000-14,000). Incubations were carried out in the presence of 20 mM HEPES (pH 7.2), 50 mM NaCl, and 7 mM 2-mercaptoethanol. Aliquots (10-20 μl) were taken from each side of the membrane after equilibrium was attained (10-24 h) and dissolved in 15 ml of Aquosol (New England Nuclear) for liquid scintillation counting. Less than 20% of the initial protein kinase activity was lost during the incubation period as determined in several of the experiments. [3H]ADP was purified by Dowex AG 1-X2 (Formate) column chromatography before use (16). [3H]AMP-PNP was lyophilized from H2O before use. Analysis of nucleotide or peptide radioactivity was determined by thin layer chromatography (17) or high voltage electrophoresis, respectively, indicated that no major changes in the radioabeled compounds occurred during the binding experiments.

Data Processing—Enzyme kinetic data were analyzed using a Hewlett Packard desk top computer (model 8253-A). Two substrate enzyme kinetic data were fitted to the equations for (a) sequential and (b) ping-pong mechanisms (18),

\[ V = \frac{V_{\max}}{K_{a} + [A]} + \frac{K_{a} + K_{s}}{K_{a} + [A]} \]

\[ V = \frac{V_{\max}}{K_{a} + [B]} + \frac{1}{K_{a} + [B]} \]

by using the weighting factors described by Cieland (19); the most significant fit was calculated on the basis of the variance ratio (F) test.

Enzyme inhibition data were analyzed by fitting the data to the equation for mixed noncompetitive inhibition (20),

\[ V = \frac{V_{\max}}{K_{a} + [A] + \frac{1}{K_{s} + [S]}} \frac{1 + \frac{1}{K_{s} + [S]}}{K_{s} + [S]} \]

(where \( K_{a} \) and \( K_{s} \) are the competitive and uncompetitive inhibitor constants, respectively) and comparing the significance of fit (F test) with that obtained by using the equations for competitive, simple linear noncompetitive, and uncompetitive inhibition. For each plot the lines in the figures represent the calculated lines after fitting the data to the appropriate equations. The points represent the actual experimental values. The kinetic constants were calculated from the solution of the respective equations.

For binding studies, the \( K_{d} \) values, standard errors, and stoichiometry of binding were determined from linear regression analysis of Scatchard plots (21) of [bound ligand]/[free ligand] versus [bound ligand].

Peptide Synthesis and Purification—The synthesis of Kemptide and Ala peptide has been described previously (22). In the experiment of Figs. 1 and 4c, the Kemptide used was purchased from Peninsula Laboratories, San Carlos, CA. For peptides obtained commercially or prepared, the amino acid analyses were, within experimental error, identical with the stoichiometric ratios. The tritiated peptide, Leu-Arg-Arg-Ala-Ala-[3H]Leu-Gly, was synthesized in an identical manner with that described (22) with the exception that Boc-[4,5-3H]leucine was employed to provide the radioactive residue. Boc-[4,5-3H]leucine was prepared by reaction between [4,5-3H]leucine (ICN, 46 C/mm01) and 2,4-butanediol diisocyanate (Aldrich Chemical Company) (23). Peptides were cleaved from the resin with HBr in trifluoroacetic acid, and the amino acid side chains were deprotected by catalytic dehydrogenation (24). [3H]Ac-Leu-Arg-Arg-Ala-Ser-Leu-Gly was prepared by the reaction of purified Kemptide with [3H]acetic anhydride (ICN, 20 C/mm01) (25). Leu-Arg-Arg-Ala-Ser(P)-Leu-Gly was prepared in 100-ml reaction volumes with 10-100 μg/ml of protein kinase, using reaction conditions described for phosphorylation reactions. The purification and assessment of purity of the peptides and the determination of the concentration of stock solutions have been described previously (22).

RESULTS AND DISCUSSION

Two-substrate Kinetics of the Forward Reaction of the Protein Kinase

Initial velocity studies, in the absence of products, were performed over a wide range of ATP and Kemptide concentrations (0.09 to 60 μM and 0.25 to 50 μM, respectively), in order to study both the kinetic mechanism and the kinetic constants of the reaction. In Fig. 1 reciprocal velocity is plotted versus the reciprocal of either ATP (a and c) or Kemptide (b and d) concentration, with the data points at high substrate concentration shown on the expanded scales in Fig. 1, c and d, respectively. The data were fitted to the equation for sequential kinetic with the lines in the figures being calculated from all of the data points, i.e. the lines in a are the best fit of the data in both a and c and vice versa.

The degree of fit obtained between the actual data points and the theoretical lines at both low (a and b) and high (c and d) substrate concentrations strongly indicates, in concurrence with the more limited data of others (3, 6, that with Kemptide as substrate the protein kinase follows a sequential, rather than a ping-pong, mechanistic pathway. That is, both of the substrates bind to the enzyme prior to product release. The kinetic constants obtained from these data are listed in Table 1 (A).

Product Inhibition

The results of initial velocity measurements made in the presence of the products of the reaction (phospho-Kemptide and Mg·ADP(33)) are presented in Fig. 2. As indicated, ADP was a competitive inhibitor versus ATP and a simple linear noncompetitive inhibitor versus Kemptide; phospho-Kemptide gave mixed noncompetitive inhibition patterns versus either Kemptide or ATP. A high degree of fit is observed between the data points and the calculated lines obtained from the equations for the respective inhibition patterns. The inhibition constants obtained from these data are summarized in Table 1 (B).

The product inhibition patterns observed are entirely consistent with a steady state Ordered Bi-Bi kinetic mechanism.

As illustrated in Fig. 1d, the interpretation of parallel lines, and in consequence a ping-pong mechanism, may appear likely if the substrate concentration range used is not sufficiently extensive, this error being more difficult to avoid because of the specific kinetic constants of this enzyme.
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Kemptide

\[ \frac{1}{v} = \frac{k_{-1} + k_{2} [A] + k_{3} [B]}{k_{1} k_{2} k_{3}} \]

\[ E + \text{Mg-ATP} \rightarrow E \text{Mg-ATP} \]

\[ E \text{Mg-ATP} + \text{Kemptide} \rightarrow E \text{Mg-ATP} - \text{Kemptide} \]

\[ E \text{Mg-ATP} - \text{Kemptide} \rightarrow E \text{Mg-ADP} + \text{Pi} \]

where \( A \) and \( B \) represent Mg-ATP and Kemptide, and \( P \) and \( Q \) the products, phospho-Kemptide, and Mg-ADP.

**Table I**

**Summary of kinetic data**

| Substrate                  | \( K_a \)        | \( V_{max} \)   | \( K_m \)        | \( K_a \)        |
|----------------------------|------------------|-----------------|------------------|------------------|
| Mg-ATP\(^{-}\)             | 7.6 ± 0.9 \( \mu M \) | 16.8 ± 0.7 \( \mu mol/min/mg \) | 4.4 ± 1.9 \( \mu M \) | 2.7 ± 1.1 \( \mu M \) |
| Kemptide                   | 4.7 ± 0.7 \( \mu M \) |                |                  |                  |

**A. Kinetic constants from two-substrate kinetics\(^a\)**

**B. Constants from product inhibition experiments\(^b\)**

**C. Constants from dead end inhibition experiments\(^c\)**

**Footnotes:**

\(^a\) Experimental plots presented in Fig. 1, Fig. 2, and Figs. 3 and 4, respectively.

\(^b\) NC, noncompetitive.

\(^c\) Comp., Competitive.

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**Fig. 1.** Initial velocity patterns for the protein kinase with varying ATP and Kemptide. Double reciprocal plots of \( 1/v \) versus \( 1/[\text{Mg-ATP}] \) (a and c) or versus \( 1/[\text{Kemptide}] \) (b and d). Conditions are as described under “Experimental Procedures.” In c and d are presented expanded scales to indicate the data points for high concentrations of ATP and Kemptide, respectively. The lines plotted in each figure are those obtained by fitting all of the data points for initial velocities at both high and low substrate concentrations (i.e. a and c or b and d, respectively) to the equation for sequential kinetics. Thus, for example, the lines in b which have no data points on them are for the data points in d.
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Kemptide-P

-0.41 h

Kemptide-P

4.5 mM

2.3 mM

1.1 mM

0 mM

ADP

100 μM

50 μM

20 μM

0 μM

-0.4- (c) 3.2 mM

1 h 6 mM

0.8 mM

0 mM

0.05

0.01

0.10

0.15

0.20

0.25

0.30

0.35

0.40

0.45

0.50

0.55

0.60

0.65

0.70

0.75

0.80

0.85

0.90

0.95

1.00

1/[Kemptide] (μM)^{-1}

1/[ATP] (μM)^{-1}

1/[Kemptide] (μM)^{-1}

1/[ATP] (μM)^{-1}

Fig. 2. Product inhibition patterns of the protein kinase by phospho-Kemptide and ADP. a, reciprocal velocity versus 1/[Kemptide] at 35 μM Mg-ATP^2− with varying phospho-Kemptide; b, reciprocal velocity versus 1/[Kemptide] at 3.74 μM Mg-ATP^2− with varying ADP; c, reciprocal velocity versus 1/[ATP] at 34 μM Kemptide with varying phospho-Kemptide; and d, reciprocal velocity versus 1/[ATP] at 34 μM Kemptide with varying ADP. All other conditions are as under “Experimental Procedures.” The lines are those fit to the data points, using the equations for: a and c, mixed noncompetitive; b, simple linear noncompetitive; and d, competitive inhibitor, as indicated under “Experimental Procedures.”

Kemptide-P

3.2 mM

1.6 mM

0.8 mM

0 mM

1/[ATP] (μM)^{-1}

1/[ATP] (μM)^{-1}

Fig. 3. Dead end inhibition patterns of the protein kinase by AMP-PNP. a, reciprocal velocity versus 1/[Kemptide] at 3.91 μM Mg-ATP^2− with varying AMP-PNP; b, reciprocal velocity versus 1/[ATP] at 34 μM Kemptide with varying AMP-PNP. All other conditions are as under “Experimental Procedures.” The lines are those fit to the data points, using the equations for: a, simple linear noncompetitive; and b, competitive inhibition, respectively.

respectively. The competitive inhibition by Mg-ADP^1−, when Mg-ATP^2− was the varied substrate, supports the assignment of Mg-ATP^2− as the first substrate to bind, and assuming that there are no unusual rate constants, suggests that the mechanism is not steady state random. The product inhibition patterns also exclude the possibility, as has been suggested (7), that the reaction mechanism might be nonrapid equilibrium random sequential since, if it were, both products should have been competitive inhibitors with their substrate counterparts (33).

Inhibition Patterns with AMP-PNP, the Inhibitor Protein of the cAMP-dependent Protein Kinase, and Ala-Peptide (the Alanine-Substituted Nonphosphorylatable Analog of Kemptide)

Dead end inhibitors often provide for a definitive assignment of a reaction mechanism (30, 31, 34). In the accompanying paper (13), the Inhibitor Protein was shown to be a competitive inhibitor versus Kemptide, to be an uncompetitive inhibitor versus Mg-ATP^2−, to require the presence of ATP in order to bind to the protein kinase and, in consequence, to increase the apparent affinity of ATP binding by the protein kinase. These observations are compatible with and at least partially diagnostic of, the reaction mechanism of the protein kinase being ordered with the nucleotide binding first; they specifically argue against the mechanism being steady state random, since this would require that inhibition versus ATP should be noncompetitive instead of, as observed, uncompetitive (13).

The interaction of a second dead end inhibitor, AMP-PNP, was also found to be compatible with an ordered sequential mechanism. As with the Inhibitor Protein (32), AMP-PNP was not a substrate for the protein kinase (data not shown). AMP-PNP was a noncompetitive inhibitor versus Kemptide and competitive with ATP (Fig. 3); the inhibition
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Constants obtained from these data are given in Table I (C). The data are compatible with the conclusion that AMP-PNP competes with ATP for free enzyme but does not interact with the same enzyme form as does Kemptide (i.e., E-ATP).

Whereas the inhibition patterns obtained with the dead end inhibitors, AMP-PNP and Inhibitor Protein, were indicative of a steady state ordered mechanism for the protein kinase, those obtained with Ala-peptide suggested an added complexity. Ala-peptide, which is not a substrate for the protein kinase (data not given), was a competitive inhibitor versus Kemptide at saturating ATP (Ref. 22 and Fig. 4b) and a noncompetitive inhibitor versus ATP (Fig. 4a). These patterns, for a dead end inhibitor, would be more consistent with a steady state random mechanism, however, at a low ATP concentration (data not given), was a competitive inhibitor versus ATP by equilibrium dialysis; an estimate of the dissociation constant for Mg-AMP-PNP was 2 mM when added to binding assay for acetylated Kemptide. Stoichiometry was calculated from the amount of enzyme initially present; less than 20% of the initial protein kinase activity was lost during the incubation period as determined in several of the experiments.

Equilibrium-binding Studies

A further elucidation of the interaction of nucleotides and peptides with the protein kinase was obtained by direct binding measurements using equilibrium dialysis. Binding affinities and stoichiometries were determined by Scatchard analysis. For each ligand examined, only a single class of binding sites was observed; the results are summarized in Table II.

**FIG. 4. Inhibition of the protein kinase by Ala-peptide.** a, reciprocal velocity versus 1/[ATP] at 4 μM Kemptide with varying Ala-peptide; b, reciprocal velocity versus 1/[Kemptide] at 30 μM Mg-ATP2− with varying Ala-peptide; c, reciprocal velocity versus 1/[Kemptide] at 1 μM Mg-ATP2− with varying Ala-peptide. All other conditions are as under "Experimental Procedures." The lines are those fit to the data points, using the equations for a, simple linear noncompetitive; b, competitive, and c, mixed noncompetitive inhibition, respectively.

**TABLE II**

| Ligand                      | Stoichiometry | Kd ± S.E. |
|-----------------------------|---------------|-----------|
| Mg-[2,8-'H]ADP2−           | 0.82          | 4 ± 1 μM  |
| Mg-[2,8-'H]AMP-PNP2−       | 0.70          | 35 ± 3 μM |
| ['H]Acetyl Kemptide        | 0.86          | 0.18 ± 0.02 mM |
| ['H]Acetyl Kemptide (plus) | 0.96          | 0.21 ± 0.06 mM |
| Mg-AMP-PNP2−               |               |           |
| ['H]Ala-peptide            | 0.73          | 0.23 ± 0.07 mM |

Other reaction mechanisms, both ADP and AMP-PNP compete with ATP for free enzyme. The observation that Mg-ADP2− binds to the catalytic subunit eliminates the possibility that the reaction mechanism for the protein kinase might be Iso Theorell-Chance (30); with such a mechanism the product inhibition patterns are the same as those predicted for a steady state ordered mechanism, but the product which yields competitive inhibition patterns (i.e. ADP, Fig. 2) should not bind to free E.

The ATPase activity of the catalytic subunit (14) prohibits the determination of the dissociation constant for Mg-ATP2− by equilibrium dialysis; an estimate of the Kd may, however, be made from a comparison with Mn-ATP2−. The kinetic constants for both Mg-ATP2− and Mn-ATP2− are very similar (6-13) and the Kd for Mn-ATP2− has been shown to be in the range of 6 to 30 μM by two approaches, one involving magnetic resonance (10, 12) and the other Millipore filtration (13). The Kd for Mn-ATP2− is also within this range (10), and thus it can be assumed that the Kd for Mg-ATP2− is in the low micromolar range. This value would be compatible with the proposed ordered reaction mechanism with the nucleotide binding first. For such a reaction, the Kd value (4.4 μM, Table I), which is also the negative of the concentration at the point of intersection of the initial velocity plots for the first reactant (i.e., Fig. 1, a and c), is a direct kinetic measurement of the Kd for this reactant, i.e. the Kd for ATP (35).

Binding of Peptides—In order to assess the affinity of the
protein kinase for peptide substrate, acetylated Kemptide ([3H]Ac-Leu-Arg-Arg-Ala-Ser-Leu-Gly) was employed. This peptide is an efficient substrate for the protein kinase with an apparent $K_\text{m}$ of 3 ± 0.5 μM (22). Acetylated Kemptide bound to the protein kinase with a stoichiometry close to 1:1; only a single class of binding sites was observed but the $K_b$ value for this binding (0.18 mM) was almost two orders of magnitude higher than the $K_b$ value for the peptide in the phosphorylation reaction. With a reaction mechanism that is predominantly ordered, only a weak or negligible binding of Kemptide in the absence of nucleotide would be anticipated but binding, were it testable, should be enhanced by the presence of the first substrate, namely ATP. This cannot be tested by equilibrium dialysis with ATP because of rapid product formation. It was tested with the ATP analog, AMP-PNP, but as indicated (Table II), the presence of AMP-PNP did not alter the observed, and high, $K_b$ value for acetylated Kemptide. An explanation for this could be that even though the reaction is ordered, AMP-PNP does not fully mimic the actions of ATP and in particular, as has been proposed also in the accompanying manuscript (13), AMP-PNP is not able to promote the conformation of the protein kinase that is necessary for enhanced peptide substrate binding. A similar difference in the interactions of ATP and AMP-PNP was also observed with the Inhibitor Protein (13), in that ATP was shown to be required for the interaction of the protein kinase with the Inhibitor Protein, but this requirement could not be fulfilled by AMP-PNP. Further, when tested kinetically, inhibitor protein did not bind to a protein kinase-AMP-PNP complex.

The observed $K_b$ value for Kemptide of the protein kinase (180 μM, Table II) provides evidence, in addition to that given by the product inhibition patterns (i.e., Fig. 2), that the reaction mechanism for the protein kinase is not rapid equilibrium random. With the latter mechanism, the ratio of

$$\frac{K_b(\text{ATP})}{K_b(\text{Kemptide})} = \frac{K_b(\text{Kemptide})}{K_b(\text{Kemptide})}$$

and, as a consequence, the $K_b$ value, as determined from the point of intersection of the reciprocal plots (2.7 μM, Table I and Fig. 1, b and d), would equal the $K_d$ for Kemptide. No binding of acetylated Kemptide was detectable at such a concentration, arguing strongly against the reaction being random.

In experiments using [32P]Leu-Arg-Ala-Ser(P)-Leu-Gly as the ligand in the presence or absence of Mg2+ (15 mM), little or no binding could be detected. Since the protein kinase concentration used in those experiments was ~100 μM, it indicates that, if it does bind, the $K_b$ for phospho-Kemptide must be substantially greater than 1.0 mM, since a 10% binding would have been readily detected.

Isotopically labeled Ala-peptide (Leu-Arg-Ala-Ala-[4,5,3H]Leu-Gly) was used to measure its binding to the protein kinase. Ala-peptide bound to the protein kinase with a $K_d$ value (0.23 mM, Table II) that is in the lower end of the range for the $K_b$ values as determined from the kinetic analyses (Table I) and is also very similar to the $K_d$ observed for acetylated Kemptide.

Because of the observation that Kemptide could bind to protein kinase in the absence of ATP, but that it did so with a $K_d$ that was two orders of magnitude higher than its $K_b$ and at a concentration in the range where Ala-peptide was an effective inhibitor, the ability of Kemptide to serve as a substrate for the protein kinase at these high concentrations was tested. As is indicated in Fig. 5, Kemptide displays substrate inhibition with a half-maximal inhibition observed at approximately 1 mM. This result has been observed with Kemptide obtained commercially or with the peptide synthesized as indicated under “Experimental Procedures.” The observation of inhibition of the protein kinase at high concentrations of Kemptide opens the possibility that the binding of Kemptide, as detected by equilibrium dialysis, is not that associated with the productive catalytic reaction, but is that which results in substrate inhibition. As may be noted, inhibition occurs at a concentration that is slightly higher than the measured $K_d$ value, but this is also what is observed with Ala-peptide (cf. Tables I and II); this probably reflects the difference in approach of the two measurements, i.e. kinetic versus direct binding.

**GENERAL CONCLUSION**

**Proposed Mechanism for the Protein Kinase**—A proposal for the mechanism of reaction of the protein kinase and of the sites of inhibition by nucleotides and peptides is depicted in Fig. 6. This model is consistent with the observations presented here and in the accompanying paper (13) and with the extensive reports of others (1-12, 14, 22); it can also provide a rationale for why with some substrates the protein kinase gives sequential kinetics (2, 3, 6) and with others apparently ping-pong (1, 4, 6). In particular, the proposed mechanism draws heavily upon the observations and ideas of Gronot, Mildvan and coworkers (8-12). The data of initial velocity reciprocal plots (Fig. 1), the patterns of product inhibition (Fig. 2), the patterns of dead end inhibition by AMP-PNP (Fig. 3) and by Inhibitor Protein (13), and the binding affinities of ATP and of ADP and AMP-PNP (Table II), are most consistent with the reaction mechanism for the protein kinase (with Kemptide as substrate) being predominantly, if not exclusively, ordered with ATP binding first with a $K_d$ of 4 μM. Upon the binding of ATP, it is proposed that a specific conformation (depicted in Fig. 6 as (E-ATP)+) is induced in the terminal anhydride bond either as a consequence of ATP binding per se or by the subsequent binding of Kemptide (or Inhibitor Protein). It is proposed that in the productive reaction, either Kemptide binds with high affinity to (E-ATP)+ or the consequential induction of this form by Kemptide enhances the formation of the (E-ATP)+-Kemptide complex. Catalysis ensues, leading to the sequential release of phospho-Kemptide and ADP.

This suggestion that the conformation of the terminal anhydride bond of ATP plays a pivotal role in (E-ATP)+ formation and in consequence the reaction kinetics, comes from competitive inhibition would also be observed if the Inhibitor Protein bound to E-ATP and Kemptide to (E-ATP)+.
our data with AMP·PNP and from the observations of Gronot, Mildvan and coworkers who have used magnetic resonance to map the co-ordinates in the active catalytic site of the protein kinase (8-12). As has recently been reviewed by Knowles (36), there is also similarity between what has been proposed here and what has been elucidated for some other kinases.

The interaction of AMP·PNP with the protein kinase is described by the data presented here (Fig. 3 and Tables I and II) and in the accompanying paper (13). AMP·PNP is a competitive inhibitor versus ATP, presumably binding at the same site; however, in addition to it not being a substrate, AMP·PNP does not fully mimic ATP in other ways. In the accompanying manuscript (13), it has been shown that the interaction of the Inhibitor Protein with the protein kinase requires the presence of ATP but that this requirement cannot be met by AMP·PNP. It is proposed that the high efficacy of the Inhibitor Protein (Kᵢ = 0.49 µM) results because it, like Kemptide, either binds preferentially to the (E·ATP)* complex or can induce its formation. The competitive inhibition pattern of Inhibitor Protein versus Kemptide is consistent, although not obligatory, with the conclusion that both Inhibitor Protein and Kemptide compete for the same enzyme form, namely (E·ATP)*. AMP·PNP does not mimic ATP in that no binding of Inhibitor Protein to protein kinase can be observed when AMP·PNP replaces ATP and, furthermore, when tested by multiple inhibition analyses (13, 37), there does not appear to be binding of Inhibitor Protein to an E·AMP·PNP complex. Thus, since the presence of AMP·PNP does not increase the affinity of the protein kinase for Kemptide (Table II) and result in an apparent Kᵢ of comparable value to the Kᵢ of Kemptide in the protein kinase reaction (i.e. in the low micromolar range), it is most likely that the terminal phosphorylimido bond cannot assume the same conformation as is proposed to occur in the (E·ATP)* complex.

Since AMP·PNP does not act like ATP it suggests that the terminal P-O-P bonding is critical to the formation of (E·ATP)*. From magnetic resonance measurements using cobalt(III) and chromium(III) complexes of adenosine-(βγ-methylene)triphosphate, Gronot et al. (9, 10) have calculated the intersubstrate distances at the active site of the protein kinase. Their observations indicated that the reaction co-

![Proposed mechanism for the protein kinase reaction](http://www.jbc.org/)

**Fig. 6. Proposed mechanism for the protein kinase reaction.** In this figure only, the abbreviations Kem, KemP, AlαK, and PKI refer to Kemptide, phospho-Kemptide, Ala peptide, and Inhibitor Protein, respectively.
observed were ping-pong rather than ordered (1-4), and similarly, one study with Kemptide reported sequential kinetics with ATP but ping-pong kinetics with ATP/βS. These apparent anomalies can be explained by a simple extension of the proposed reaction mechanism; namely, if [(E·ATP)*] is more correctly written as (E·ADP·P*) with P* denoting the m	exthophosphate intermediate, then Kemptide may bind to (E·ADP·P*) before ADP is released giving rise to sequential kinetics, whereas with histone, ADP release may occur first, giving rise to ping-pong kinetics. Similarly, even with Kemptide as substrate there may be a greater tendency for ADP/βS to dissociate from E than for ADP resulting in ping-pong kinetics with ATP/βS whereas sequential kinetics are observed with ATP.

In the reaction mechanism that is proposed for the protein kinase (Fig. 6), it is suggested that the high efficacy of Kemptide as a substrate, or Inhibitor Protein as an inhibitor, is because the affinity of each is enhanced due to the conformational change that occurs in the terminal anhydride bond that results in (E·ATP)* formation. Kemptide also binds to the enzyme in the absence of ATP, but with a low affinity. However, because of the close similarity between the $K_i$ for Kemptide measured in the absence of ATP (~0.2 mM, Table II) and the concentration of half-maximal substrate inhibition ($I_{0.5}$ ~ 1.0 mM, Fig. 5), it is suggested that substrate inhibition is due to the binding of Kemptide to free enzyme leading to an abortive complex that is not catalytically competent (Fig. 6). Comparison between the interaction of Kemptide and that of Ala-peptide suggests that the two have some similarities and some distinct differences. It is proposed that Ala-peptide can interact either with free enzyme or with E·ATP* (or (E·ATP)*) but, in contrast to Kemptide, the binding of Ala-peptide is not increased as a consequence of the conformational change that results in (E·ATP)* formation. Kemptide also binds to the enzyme in the absence of ATP, but with a low affinity.

The reaction mechanism that is proposed in Fig. 6 provides for a reasonable explanation of the data obtained to date. As is axiomatic of any kinetic study, such do not prove a reaction mechanism but simply provide for the framework of future studies.

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