Phosphorylation of Histone Catalyzed by a Bovine Brain Protein Kinase*

PROTEIN KINASE ASSAY EMPLOYING POLYETHYLENEIMINE-CELLULOSE THIN LAYER SHEETS

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GEORGE W. MOLL, JR. AND EMIL THOMAS KAISER
From the Departments of Biochemistry and Chemistry, University of Chicago, Chicago, Illinois 60637

The use of polyethyleneimine-cellulose thin layer sheets to follow the phosphorylation of histone and decomposition of ATP catalyzed by an adenosine 3'-5'-monophosphate (cyclic AMP)-stimulated protein kinase, protein kinase I, has made possible a more detailed analysis of the time course of these reactions than has been achieved previously by observing only recovered phosphorylated protein. When [$^{32}$P]ATP was employed, significant error was introduced by the presence of $^{32}$P, at the solvent front on these sheets, and this limited the accuracy of the available information. However, the analysis of assays performed with [$^{14}$C]ATP was straightforward and appeared to have an accuracy comparable to that of the present standard assay. This appears to be the first use of [$^{14}$C]ATP to assay protein kinases. Our physical characterization of protein kinase I showed it to be a homogeneous protein species by polyacrylamide gel electrophoresis, sodium dodecyl sulfate gel electrophoresis and analytical ultracentrifugation. Kinetic studies with protein kinase I indicated the absence of histone phosphatase and cyclic AMP phosphodiesterase activity. Furthermore, the ATPase activity seen is believed to be intimately associated with the protein kinase action, particularly in view of the observed dependence of the rate of P$_i$ production on the presence of cyclic AMP. The kinetic data for the phosphorylation of histone catalyzed by protein kinase I under full stimulation by cyclic AMP are consistent with a double displacement mechanism.

Problems of purification and incomplete characterization are commonly encountered in the study of cyclic AMP-dependent protein kinases. Thus, the bovine brain cyclic AMP-dependent protein kinases which have been studied in some detail with respect to substrate specificity as well as the influence of various factors upon the enzyme-catalyzed phosphorylation have been partially purified species (1, 2). Reports on two preparations of cyclic AMP-dependent protein kinases from bovine brain have appeared in the literature (2, 3). The stimulated and unstimulated catalytic activities of the enzyme obtained in the first preparation by Miyamoto et al. (2) were examined under varying conditions of temperature, pH, enzyme concentration, and in the presence of several different nucleotides, metal ions, and protein substrates. In their later report Miyamoto et al. (3) described the purification and partial characterization of several cyclic AMP-dependent protein kinases from bovine brain, but they did not perform an extensive kinetic analysis on any of these enzymes. In particular, the catalytic assay system was considerably different from that employed 4 years earlier by this group (2).

The assays which have been used in past work on the protein kinases have depended upon the recovery of the phosphorylated protein product or on measurements of the alteration in enzymic activity of a phosphorylated protein, e.g. phosphorylase or glycogen synthetase (4). It seemed worthwhile to us to explore the possibility that the PEI-cellulose thin layer method of Cashel et al. (5) for the preparation and identification of nucleotides might afford an excellent experimental approach for the observation of changes in nucleotide concentrations with time during phosphorylation reactions catalyzed by protein kinases. We present here an analysis of the kinetics of the phosphorylation of histone catalyzed by a cyclic AMP-dependent protein kinase (protein kinase I) obtained from bovine brain. A novel assay employing PEI-cellulose sheets is compared with a standard assay method, and the assay method with these sheets is used to obtain a more complete understanding of the phosphorylation of histone catalyzed by protein kinase I than was previously achieved by observing only recovered phosphorylated histone.

EXPERIMENTAL PROCEDURE

Materials

The following calf thymus histone fractions were obtained from Sigma: type II (calf thymus mixture), type IV (arginine-rich, $f_1$), type V (lysine-rich, $f_2$), and type VI (lysine-rich, $f_6$). Casein (salt-free enzymatic hydrolysate from milk), bovine serum albumin (crystallized and lyophilized), ovalbumin (90% electrophoretic purity), and chymo-
trypsinogen A (crystallized six times) were obtained from Sigma. 
Protamine sulfate (salmine) A grade and 2-(N-morpholinol)ethanesul-
monic acid (Mes) A grade were obtained from Calbiochem. Ultrapure 
cyclic AMP, cyclic GMP, and ATP were purchased from Schwarz/
Mann; and phosphorylase A was obtained from Worthington. Buffer 
chemicals were all of the highest grade obtainable. Rat liver cytos-
ribosomes were donated by Professor Ira Wool, Department of Bio-
chemistry, University of Chicago. \( \gamma^2P \)ATP of high specific activity 
(1 to 4 mCi/\mu mol) was prepared according to the method of Glynn 
and Chappell (6), and the product was greater than 97% radiochemically 
pure by PEI-cellulose chromatography, as described by Cashel et al. 
(5). [U-'\(^{14}C\) ]ATP, 40 to 60 mCi/mmol, 99% radiochemical purity, in 
2% ethanol and Triton X-100 was obtained from Amersham/Searle. 
Cylinders (0.6 x 10 cm) without stacker gels according to a modifica-

Methods

Standard Catalytic Assay—The quantitative standard assay system 
was a modification of that described by Miyamoto et al. (3). The incubation 
mixture contained 4 mCi \( \gamma^2P \)ATP (10 \muCi/\mu mol) to yield the final volume of 0.2 ml. Following the assay, an 
aliquot of the \( \gamma^2P \)ATP solution was placed in a scintillation vial to 
measure radioactivity, permitting the conversion of counts per min to 
picomoles. The tubes were immediately mixed with a Vortex stirrer 
and incubated 5 min at 30°C. The incubation was performed according to the 
method of Fairbanks et al. (9). Gels were electrophoresed at 
2 mA/gel in pH 8.5 Tris-glycine buffer. Following electrophoresis, the 
location of the bromphenol blue marker was fixed with a piece of 
copper wire and the gels were stained as described above for analytical 
gel electrophoresis. Tests for PAS-positive carbohydrate were made by 
the method of Arai and Wallace (10). All gels were scanned on a Gilford 
gel scanning system at 540 or 560 nm.

Analytical Ultra centrifugation—Analytical ultracentrifugation was 
performed according to the method of Yphantis (11) in a Beckman 
model E analytical ultracentrifuge. Interference optics were employed.

PEI-cellulose Thin Layer Analysis of Protein Kinase Activity—The 
method of Cashel et al. (5) was employed for the analysis of the 
products of histone phosphorylation assays by PEI-cellulose thin layer 
chromatography. For this purpose, 25 \mu l of the incubation mixture was 
removed with an Eppendorf pipette 15 s prior to the predesignated 
termination interval and then spotted onto designated origins on a 
PEI-cellulose thin layer sheet at room temperature. The thin layer 
sheet was developed in a chromatographic tank with 1 M KH\(_2\)PO\(_4\), pH 3.4. The solvent front was allowed to move to within 18 cm from the origin, 
and the thin layer sheet was then removed and allowed to dry at room 
temperature.

RESULTS

A cyclic AMP-stimulated protein kinase (protein kinase I) was 
prepared from bovine brain (obtained from Pel-Freez) as described 
elsewhere (12). The Superfine Sephadex G-200 profile from a typical preparation is shown in Fig. 1, and a summary of a representative preparative procedure is pre-

Ultraviolet Spectra—Spectral scans in the ultraviolet region were 
obtained using 1-cm path length quartz cuvettes in a Cary model 15 
dual beam spectrophotometer.

Analytical Gel Electrophoresis—Analytical gel electrophoresis was 
performed at 5 mA/gel in pH 9.0 Tris-borate buffer, according to the 
instructions given for a 7.5% polyacrylamide gel in the Ortec Instruc-
tion Manual 4290 Electrophoresis System. Gels were prepared as 
cylinders (0.6 x 10 cm). The gels were stained for 1 hour in 0.25% 
Coomassie brilliant blue R in 45% absolute methanol and 9% glacial 
acetic acid at room temperature. The gels were destained in 10% isopropanol alcohol/10% acetic acid and stored at 5°C in 10% acetic acid.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—
Sodium dodecyl sulfate gels were prepared as 10% polyacrylamide 
cylinders (0.6 x 10 cm) without stacker gels according to a modifica-
tion of the method of Fairbanks et al. (9). Gels were electrophoresed at 
2 mA/gel in pH 8.5 Tris-glycine buffer. Following electrophoresis, the 
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Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—
Sodium dodecyl sulfate gels were prepared as 10% polyacrylamide 
cylinders (0.6 x 10 cm) without stacker gels according to a modifica-
The standard catalytic assay was performed with 9 x 10^{-8} M protein kinase I (0.3 μg per assay) and increasing amounts of added cyclic AMP. For this concentration of protein kinase I, the rate of histone phosphorylation was independent of variations in the concentration of cyclic AMP in the range of 2 to 8 μM. The data obtained fit a double reciprocal plot with the rate and cyclic AMP concentration as the variables giving a dissociation constant of (2.6 ± 0.6) x 10^{-8} M for the binding of cyclic AMP as an activator in good agreement with the values reported by Shuman and co-workers (1, 15) of 0.9 to 5.0 x 10^{-8} M for partially purified bovine brain protein kinase. A maximum of 5% of the total cyclic AMP present in these assays was converted to AMP over a 5-day period at 5°C in the presence of 6 x 10^{-9} M protein kinase I as determined by thin layer sheets developed with Schwarz/Mann buffer system No. 26 (isopropyl alcohol/concentrated ammonium hydroxide/0.1 M H3BO3, 60/10/30, which gives RF = 0.13 for AMP and RF = 0.61 for cyclic AMP). At concentrations of 18 μM and 15 μM, cyclic GMP was able to stimulate protein kinase I to 98 and 88%, respectively, of the extent seen with 5 μM cyclic AMP.

To test the possibility that histone might act as an activator of protein kinase I, the enzyme (0.5 μg) was preincubated with 100 μg of histone in 125 μl of 50 mM sodium acetate solution, pH 6.0 for 5 min at 30°C. Then the standard assay was performed (addition of 10 mM magnesium acetate and 5 μM [γ-32P]ATP with or without 5 μM cyclic AMP added to the preincubated samples to yield a final volume of 200 μl) with this mixture and appropriate controls. The results shown in Table III indicated only a 2-fold stimulation of protein kinase I catalytic activity due to preincubation of the enzyme with histone.

Fig. 5 shows the relationship between enzyme concentration and the initial rate of histone phosphorylation (5-min standard assay). The rate is proportional to enzyme concentration throughout the range of minimal change in the initial substrate concentration. In the studies described in this article protein kinase I concentrations of 1.5 x 10^{-8} M (2.5 x 10^{-9} μg/pl) or less were employed unless otherwise indicated.

Figs. 6 and 7 show the results of the thin layer analysis of the standard assay system along with typical autoradiograms. The autoradiograms show that the products can be clearly separated. It should be noted that radioactivity occurs at the solvent front in the case of [γ-32P]ATP assays while it is absent in the case of [U-14C]ATP assay. This radioactivity is therefore assigned to P, and added to the other P, counts. This addition increases the error in the studies with [γ-32P]ATP. Background corrections for each species are made on the basis of counts obtained from a thin layer analysis of an incubation mixture without enzyme. The counts per min are converted to picomoles by calculating the total concentration of nucleotide present from the known initial amount added per assay. Product formation shows a linear dependence on time up to 6 min. Not surprisingly there is no apparent initial burst of P, or ADP within the limits of resolution since the amount of enzyme is only 2.4 pmol in these studies. The amount of phosphorylated histone measured by thin layer analysis at 5 min is within 10% of that obtained by a concurrently performed standard assay with trichloroacetic acid precipitation of phosphorylated histone, when corrected for the independently determined overall recovery of 66% in the precipitation procedure. When added to the amount of P, released at 5 min this amount of phosphorylated histone is within 10% of the amount of [U-14C]ADP released within 5 min. The cyclic AMP stimulation results are also in agreement with those obtained by the trichloroacetic acid precipitation approach.

Throughout the [γ-32P]ATP studies no [β-32P]ADP species was observed. Thus, there was no evidence for the presence of
an enzyme capable of catalyzing such a transfer reaction, as had been observed for a protein kinase preparation by Kemp and Murray (16). Also, the release of P$_i$ had not been observed before for cyclic AMP-dependent protein kinases due to the design of previous assays. This last observation suggested that it would be worthwhile to see if titration of the catalytic site could be carried out by the observation of a burst of P$_i$ at high enzyme concentration.

The results of experiments in which 75 pmol of protein kinase I were incubated with 1,160 pmol of [γ-$^{32}$P]ATP or 730 pmol of [U-$^{14}$C]ATP in 200 μl total volume with 10 mM magnesium acetate and 40 mM sodium acetate, pH 6.0, are shown in Fig. 8. There is an apparent zero order release of P$_i$ both in the presence and absence of 5 μM cAMP, as can be measured by observing ADP or P$_i$ formation. The release of P$_i$ is quite slow, but it is stimulated by 5 μM cyclic AMP to an extent (8-fold) comparable to that for the stimulation of histone phosphorylation. A cyclic AMP-stimulated ATPase has not been reported. Our results on the cyclic AMP stimulation suggest that the ATPase activity seen by us is reasonably attributed to protein kinase I.

These experiments also permitted the detection of a phosphorylated enzyme under relatively mild conditions. Erlichman et al. (17) have reported the incorporation of 2 mol of phosphate from ATP into the cyclic AMP-binding component of the bovine cardiac muscle protein kinase of molecular weight 174,000. The occurrence of autophosphorylation in a bovine brain cyclic AMP-dependent protein kinase preparation has been reported (18) but the results obtained were expressed in arbitrary units and not converted to picomoles. The results shown in Fig. 8 indicate that a maximum of 12% (9 pmol compared with 75 pmol total in 200 μl of reaction volume) of the protein kinase I ($M_r = 162,000$) present was phosphorylated within 5 min. The same result (corrected for recovery) was found with the trichloroacetic acid precipitation method. Insufficient phosphorylenzyme was obtained to permit a test of the hypothesis that this species might be an intermediate in the catalytic phosphorylation of histone.

The results obtained on adding 0.2 μg/μl of histone to the same incubation mixture both in the presence and absence of 5 μM cyclic AMP are illustrated in Fig. 9. Fig. 10 shows that the rate of decrease in the ATP concentration fits a reasonable first order rate law in both cases. In the absence of cyclic AMP the rate of release of P$_i$ is roughly equal to the rate of histone phosphorylation and of the decrease in the ATP concentration. In the presence of cyclic AMP, the phosphorylation of histone appeared to be complete within 2 min. Within experimental error both the rate of decrease in the ATP concentration and that of the release of P$_i$ fit first order kinetics based on concentrations at infinite time equal to the difference between the initial concentration of ATP and that incorporated into histone (Fig. 10b). The observation of first order kinetics is
TABLE II
Survey of substrates for protein kinase I

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Type</th>
<th>% of absorbance at 230 nm</th>
<th>Units*</th>
<th>Corrected units*</th>
<th>cAMP Stim.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histones</td>
<td>II</td>
<td>100</td>
<td>8.2</td>
<td>112</td>
<td>8.2 112</td>
</tr>
<tr>
<td>50 μg by weight</td>
<td>IV</td>
<td>61</td>
<td>5.3</td>
<td>68</td>
<td>8.7 111</td>
</tr>
<tr>
<td>(0.25 μg/μl)</td>
<td>V</td>
<td>67</td>
<td>8.6</td>
<td>75</td>
<td>13 112</td>
</tr>
<tr>
<td>Protamine sulfate (salmine)</td>
<td>VI</td>
<td>39</td>
<td>3.9</td>
<td>44</td>
<td>19 112</td>
</tr>
<tr>
<td>Casein hydrolyzate, salt free</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat liver ribosomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>40 μg by A 260 nm</td>
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<tr>
<td>(0.2 μg/μl)</td>
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<tr>
<td>Bovine serum albumin</td>
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<td></td>
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<tr>
<td>(0.26 μg/μl)</td>
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</table>

* One unit is the number of picomoles of 32P incorporated by 1 mg of protein kinase into recovered protein in the standard assay system (200 μl total volume) at 30° for 5 min.

This correction is based on the assumption that the histone species have equal extinction coefficients at 230 nm and that there is a direct relationship between rate and the histone concentration.

**cAMP, cyclic AMP.**

TABLE III
Histone stimulation of catalytic phosphorylation by protein kinase I

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Histone</th>
<th>Cyclic AMP</th>
<th>Activity units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40 μg</td>
<td>(5 μM)</td>
<td>10</td>
</tr>
<tr>
<td>+</td>
<td>100</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>40 μg</td>
<td>+</td>
<td>101</td>
</tr>
<tr>
<td>+</td>
<td>100</td>
<td>+</td>
<td>173</td>
</tr>
</tbody>
</table>

Anticipated for the enzymatic hydrolysis of ATP observed here (at ATP concentrations which are less than K_m, as will be shown later) because a significant amount of ATP is being consumed, in contrast to the negligible amount which was hydrolyzed in the reactions illustrated by Fig. 8. Although this technique did not resolve the course of the reaction prior to 2 min, the rate of the first order release of Pi seen after 2 min is considerably less than the rate of transfer of phosphate to histone; but it is considerably faster than the rate of release of Pi observed in the absence of histone. The good fit of the data to a first order plot does not suggest that there is a lag period for Pi release prior to 2 min. As seen in Fig. 10c, when the enzyme concentration was increased 2-fold, the rate of decrease in the ATP concentration still fits apparent first order kinetics. Calculation of a second order rate constant for reaction by dividing the observed first order constant by the enzyme concentration gave a value about the same as that obtained from the data in Fig. 10b. Also, even when the protein kinase I concentration was increased 3-fold the maximal amount of phosphate incorporated into histone under the conditions employed remained about 520 pmol (100% recovery). These results indicate the limit of histone phosphorylation and show again that, except for possible transient phosphoryl-enzyme formation which has not been excluded, an insignificant degree of enzyme autophosphorylation occurs during the phosphorylation of histone catalyzed by protein kinase I.

An amino acid analysis of the histone substrate indicated that the 520 pmol of phosphate introduced under our experimental conditions corresponded to only 4% of the available threonine or serine residues. An analysis by the method of Traugh and Traut (19) of the phosphorylated histone we obtained indicated the presence of only phosphoserine and phosphothreonine. It must be pointed out, however, that the conditions employed in this often-used procedure are such that these phosphorylated residues would be expected to be the most stable ones. The small extent of incorporation of phos-
Phosphorylation by Bovine Brain Protein Kinase

Fig. 7. Analysis of thin layer assays. [γ-32P]ATP graph: Δ, *P1 formation in the absence of cyclic AMP; O, *P1-protein formation in the presence of 5 μM cyclic AMP; †, *P1-protein formation in the presence of 5 μM cyclic AMP. The arrow indicates level at which 14% of total ATP has decomposed to form P1 or P-protein. [U-14C]ATP graph: Δ, [U-14C]ADP formation in the absence of cyclic AMP; O, [U-14C]ADP formation in the presence of 5 μM cyclic AMP. The arrow indicates level at which 18% of total ATP has decomposed.

Fig. 8. ATP decomposition catalyzed by protein kinase I in the absence of histone. γ-32P]ATP graph: 0, *P1 formation in the presence of 5 μM cyclic AMP; Δ, *P1-protein formation in the presence of 5 μM cyclic AMP; O, *P1 formation in the absence of cyclic AMP. *P1-protein formation in the presence of cyclic AMP. [U-14C]ATP graph: 0, [U-14C]ADP formation in the presence of 5 μM cyclic AMP.

Fig. 9. Analysis of assay of protein kinase I with both histone and γ-32P]ATP present. Results with 5 μM cyclic AMP present: Δ, γ-32P]ATP; Δ, *P1-protein; O, *P1. Results of assays performed in the absence of cyclic AMP: . . .

Fig. 10. Semilog plots of assays in the presence of histone and high concentrations of protein kinase I. a, 75 pmol of protein kinase I (200 μl total volume) in the absence of cyclic AMP. b, 75 pmol of protein kinase I assayed in the presence of 5 mM cyclic AMP. c, 150 pmol of protein kinase I assayed in the presence of 5 μM cyclic AMP. Δ, γ-32P]ATP; O, *P1, Δ, *P1-protein.

Encouraged by these results we decided to carry out more detailed experiments for the determination of the kinetic parameters of histone and ATP. In this work the assay system included 6 × 10^-4 M protein kinase I in a total volume of 200 μl of 40 mM sodium acetate, pH 6.0, containing 10 mM magnesium acetate with ATP concentrations varying from 0.28 to 3.0 μM, while histone varied from 0.125 to 0.5 μg/ml. The conditions were such that the initial concentrations of ATP and histone could be used in the kinetic analysis, and complete association of ATP with Mg^2+ could be assumed.

As illustrated in Fig. 11, the data of 12 initial rate measurements were plotted according to Equation 1, the steady state expression for a double displacement mechanism. Employing the statistical method described by Cleland (20) for kinetic data, the computerized analysis converged within 10 iterations and yielded

\[ K_{m,ATP} = 13 \mu M \pm 2 \mu M, K_{m,histone} = 1.1 \mu M \pm 0.2 \mu M, \]

and \[ k_{cat} = 220 \text{ min}^{-1} \pm 30 \text{ min}^{-1}. \]

\[ v = \frac{k_{cat} E_s \text{(Mg-ATP)} \text{(histone)}}{K_{m,histone} \text{(Mg-ATP)} + K_{m,ATP} \text{(histone)} + \text{(Mg-ATP)} \text{(histone)}}. \]
FIG. 11. Double reciprocal plots of initial rate data fitted to Equation 1.

**DISCUSSION**

A detailed study of the phosphorylation of histone catalyzed by a cyclic AMP-stimulated bovine brain protein kinase, including kinetic measurements using a new assay technique, has been presented here. The protein kinase (protein kinase I) employed had a similar specific activity to the brain enzymes obtained by Miyamoto et al. (3) and appeared to be homogeneous by gel electrophoresis and by analytical ultracentrifugation. A value of about $3 \times 10^{-8}$ M for the half-maximal stimulation of the catalytic activity of protein kinase I by cyclic AMP was found. Cyclic GMP was about 3 times less effective than cyclic AMP in the stimulation of protein kinase I activity. Protein kinase I did not have significant cyclic AMP phosphodiesterase or histone phosphatase activity. Little autophosphorylation of protein kinase I was observed during assays performed in the present work. Also, only a 2-fold stimulation of protein kinase I by histone was seen in the absence of cyclic AMP.

The PEI-cellulose method for following the phosphorylation of histone catalyzed by protein kinase I, as described here, gives good agreement with results obtained by the trichloroacetic acid method, and the former technique permits a more detailed analysis of the course of the reaction with respect to all the substrates and products involved in it. A significant amount of error was introduced by the presence of $^{32}$P, at the solvent front in the PEI-cellulose assays involving $^{32}$P, but the analysis of assays performed with [U-$^{14}$C]ATP was straightforward. To the best of our knowledge this is the first use of [U-$^{14}$C]ATP to assay protein kinases. It should prove to be a useful and general assay due to the longer half-life of $^{14}$C as compared to that of $^{32}$P and because of the relative simplicity of the PEI-cellulose technique. To increase the efficiency of this technique, we suggest the use of a thin layer chromatogram radioactivity scanning system or of measurements with 5-μl samples of $10^{-8}$ M cold ATP and ADP to locate the labeled spots on the thin layer sheets rapidly.

An initial cleavage of ATP in the active site of protein kinase I prior to histone phosphorylation is supported by the observation that protein kinase I is able to catalyze the hydrolysis of ATP in the absence of histone and by the observed release of P$_i$ even in the presence of histone. Our observation on the release of P$_i$ also suggests that there is a competition between water and histone for the $\gamma$-phosphate of ATP. It is interesting to make a rough comparison of the rates of P$_i$ release obtained under the conditions used for the experiments illustrated in Fig. 7 where pseudo-zero order kinetics are observed and in Fig. 10 where pseudo-first order kinetics have been seen. The results shown in Fig. 7 were measured in the presence of 5 μM cyclic AMP and 0.2 μg/μl of histone with 2 pmol of protein kinase I in the assay mixture. By dividing the observed zero order rate constant by the enzyme concentration, a first order rate constant of 1.1 min$^{-1}$ can be calculated for P$_i$ production. A similar calculation gives a value of 15 min$^{-1}$ for the first order rate constant for [U-$^{14}$C]ADP release. Computation of a first order rate constant for P$_i$ release from the data of Fig. 10 by dividing the observed rate constant by the enzyme concentration and multiplying by the initial ATP concentration gave a value of 4.0 min$^{-1}$. Thus, the rate constants estimated for P$_i$ formation from the experiments illustrated in Figs. 7 and 10 agree within a factor of 4 over a range in the enzyme concentrations from $10^{-8}$ M to $4 \times 10^{-7}$ M. In view of the crude nature of the comparison, these findings suggest that the rate of P$_i$ release has a direct dependence on the enzyme concentration in this range. The rate constants just calculated for P$_i$ and [U-$^{14}$C]ADP release in the presence of 0.2 μg/μl of histone and 5 μM cyclic AMP can be compared to those obtained from the data of Fig. 9 where assays were performed in the absence of histone but in the presence of 5 μM cyclic AMP. From the measurements illustrated in Fig. 9 first order rate constants for P$_i$ release of 0.14 min$^{-1}$ and [U-$^{14}$C]ADP formation of 0.16 min$^{-1}$ can be obtained. Taking the average of the rate constants calculated for P$_i$ production from the measurements of Fig. 7 and 10, it appears that the rate of P$_i$ release is increased approximately 20-fold by the presence of 0.2 μg/μl of histone. Similarly, the rate of [U-$^{14}$C]ADP formation is estimated to be increased roughly 100-fold. Since the rates of production of ADP and histone-P seen in our experiments seem to be comparable, the release of these products is probably not rate-limiting.

Nothing definite can be said concerning the changes in covalent bonds occurring during the transfer of the phosphate moiety to histone. It does not form a long lived intermediate with the enzyme under the conditions employed here since no lag period for P$_i$ release has been seen and the rates of release of ADP and P$_i$ are similar in the absence of histone. In experiments to be reported elsewhere we have shown that phosphate buffers do not competitively inhibit the phosphorylation of histone so it does not seem likely that P$_i$ is transferred directly to histone.

These facts are consistent with a double displacement mechanism such as the scheme shown in Equation 2 where Mg$^{2+}$-ATP is cleaved prior to the phosphorylation of histone. In this scheme either water or histone can act as phosphate receptor. Equation 2 represents only a first approximation to the mechanism of action of protein kinase I. The rate expression obtained by a steady state treatment of this scheme under initial rate conditions (product concentrations negligible relative to substrate concentrations) is illustrated in Equation 3,
and it can be easily seen how this corresponds to the relationship of Equation 1

\[
E \frac{k_1(Mg\cdotATP)}{k_{-1}} (E\cdotMg\cdotATP) \frac{k_1}{k_{-1}} (E\cdotP) + Mg\cdotADP
\]

II

\[
\frac{k_4(acceptor)}{k_{-4}} (E\cdotacceptor-P) \frac{k_4}{k_{-4}} E + acceptor-P
\]

where

\[
K_{m,MgATP} = \frac{h_4(k_{-1} + k_2)}{k_4(k_2 + k_4)}
\]

\[
K_{m,histone} = \frac{h_4(k_{-1} + k_2)}{k_4(k_1 + k_2)}
\]

The equivalence of the mathematical form of the theoretical Equation 3 and the experimentally fitted relationship (Equation 1) shows that the kinetic behavior of the enzymatic phosphorylation of histone is consistent with the postulated double displacement mechanistic scheme. The values obtained for the constants in equation 1 are only first estimates in view of the complications introduced by the use of an inhomogeneous histone substrate with a high tendency to aggregate. A further complication arises from the lack of information concerning the Mg\cdotATP complex under the conditions employed (to be discussed in a future publication). In light of these experimental difficulties, it is not expedient at this time to carry out a complete kinetic analysis of the system in order to establish the strict adherence of the reaction kinetics to the double displacement mechanistic scheme.

The study of histone phosphorylation catalyzed by protein kinase I which has been described here has been carried out primarily in the presence of 5 µM cyclic AMP (fully stimulating). As described elsewhere, the apparent \( K_m \) for histone is not greatly influenced by the addition of cyclic AMP (12), while the \( K_m \) for ATP is considerably decreased by the presence of cyclic AMP (2, 12). Further studies on the mechanism of action of protein kinase I are being pursued in our laboratory.

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