A Guanosine 3'':5''-Monophosphate-dependent Protein Kinase from Bovine Heart Muscle

PURIFICATION AND PHOSPHORYLATION OF HISTONE I AND IIb*

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Guanosine 3'':5''-monophosphate-dependent protein kinase from bovine heart muscle was purified to apparent homogeneity using affinity chromatography. The kinase activity was purified at least 16,400-fold with an overall recovery of 6%. Sodium dodecyl sulfate-gels of purified kinase showed one stained band corresponding to a molecular weight of 82,000. When histone I was used as substrate protein, half-maximal stimulation of kinase activity by cGMP and cAMP was observed at concentrations of 0.025 and 6.8 μM, respectively.

Of various histone fractions tested highest phosphorylation rates were observed with histone IIb. Optimal rates were obtained in the presence of 60 mM magnesium. With histone I as substrate biphasic curves for phosphate incorporation were observed when the concentration of histone I was varied in the presence of low (5 mM) and high (60 mM) concentrations of magnesium. Sodium dodecyl sulfate-gel electrophoresis of histone I phosphorylated under different conditions revealed that phosphate co-migrated with the histone I band when low concentrations of histone I (0.2 mg/ml) and magnesium (2 mM) were used. At high concentrations of histone I (4.0 mg/ml) and magnesium (60 mM), almost all of the bound radioactivity migrated at a position which showed no stained band but which corresponded to that of histone IIb. After addition of low concentrations of histone IIb (0.02 mg/ml) to tubes containing low concentrations of histone I (0.2 mg/ml) and magnesium (2 mM), almost all of the protein-bound phosphate co-migrated with the band of histone IIb. It is suggested that millimolar concentrations of magnesium are required for the phosphorylation of histone I and IIb and that high magnesium concentrations may facilitate the dissociation of histone IIb from the enzyme.

Guanosine 3'':5''-monophosphate-dependent protein kinase has partially been purified from several tissues of mammalian and nonmammalian origin (1-6). More recently, purification of cGMP-dependent protein kinase from bovine lung to homogeneity has been reported (7-8). Purified cGMP and cAMP-dependent protein kinases are able to phosphorylate several identical substrates (9). cGMP-dependent protein kinase, however, differs from the cAMP-dependent enzyme and is composed of two presumably identical subunits (7-8).

Takai et al. (5) reported that the activity of partially purified cGMP-dependent protein kinase is dependent on the presence of high concentrations of magnesium. This observation has been confirmed for cGMP dependent protein kinases partially purified from other tissues and has also been reported for the enzyme purified from bovine lung (4-8, 10). In general, cGMP-dependent protein kinase activity has been determined by using histones as protein substrates. Since histones are basic proteins and since partially purified cGMP-dependent protein kinase has an isoelectric point of pH 5.7 (5, 6), the possibility has been considered that the observed magnesium dependency was the result of nonspecific interaction between the histones and the kinase and not a specific property of the kinase itself. To test this possibility the effect of magnesium on the phosphorylation of two histone subfractions was studied.

**Materials and Methods**

ATP, cyclic AMP, cyclic GMP, histone IIa and IIb, bovine serum albumin, phosphorylase α, lysozyme, phosvitin, protamine sulfate, and 2-(N-morpholino)ethanesulfonic acid were obtained from Sigma. Histone HLY, arginine-rich histone HF, and HA, slightly lysine-rich histone Hf2a, and lysine-rich histone Hf1, were purchased from Worthington. Cyclic (PH)GMP (specific activity, 20 Ci/mmol) was from Amersham/Buchler. DEAE-cellulose (Whatman DE52) was obtained from Beeve Angel, London. Horseradish peroxidase, catalase, phenylmethyisulfonfyl fluoride and sodium dodecyl sulfate were obtained from Serva, Heidelberg.

Assays—Catalytic activity of cGMP-dependent protein kinase was determined at pH 7.4 in a total volume of 0.1 ml containing 5 μM of Tris/HCl, 40 nmol of ethylene glycol bis(β-aminoethyl ether) N',N'-tetraacetic acid, 10 μmol of °°P-ATP, 100 μmol of magnesium acetate, 20 μg of histone HLY, ±50 μmol of cGMP, and diluted enzyme to start the reaction. Incubations were carried out for 5 min at 30°C. Reactions were terminated and the amount of °°P incorporated into protein substrate was determined as described (Method B) by Reimann et al. (11). The 10 and 5% trichloroacetic acid washes were replaced by 20% trichloroacetic acid washes when histone I was used as protein substrate. cGMP binding capacities were determined at pH 8.0 in the presence of a 10-fold excess of unlabeled cAMP over tritiated cGMP (12).

Polyacrylamide gel electrophoresis (7.5% acrylamide) in the pres-
Purification of cGMP-dependent Protein Kinase - Fresh bovine heart muscles were obtained from a local slaughter house and freed of all visible fat. If not stated otherwise, further steps were carried out at 4°C, and buffers contained 10 mM potassium phosphate, pH 7.0, and 10 μM phenylmethylsulfonyl fluoride (Buffer A). Five kilograms of bovine heart muscle were ground and homogenized in 4 volumes of Buffer A containing 6 mM EDTA and centrifuged at 9,000 × g for 25 min. The supernatant was filtered through glass wool, and 2-mercaptoethanol was added to a final concentration of 0.2 mM. Two and one-half liters of settled DEAE-cellulose (Whatman DE52) equilibrated in the Buffer A containing 2 mM EDTA and 10 mM 2-mercaptoethanol were added, and the suspension was stirred slowly for 30 min. The resin was collected on a Buchner funnel, washed with 2 volumes of the equilibration buffer and finally poured into a column (8 × 80 cm). The column was eluted with 4 volumes of the equilibration buffer using a linear gradient from 0 to 550 mM potassium chloride. Fractions were tested for cGMP-dependent protein kinase activity and cGMP binding capacities, which co-eluted at a conductivity of about 2.8 mSī (Fig. 1). Active fractions were pooled and dialyzed extensively, and solid ammonium sulfate (0.291 g/ml) was added. After stirring for 30 min precipitated protein was collected by centrifugation at 9,000 × g for 30 min. The precipitated protein was dissolved in 60 ml of the Buffer A containing 0.2 mM EDTA, 150 mM KC1, and 15 mM 2-mercaptoethanol. Dissolved protein was immediately applied to an 8-ml 8-NH(CH₂)₂S-cGMP/Sepharose 4B affinity column (14) equilibrated with the same buffer. The column was then washed overnight with about 400 ml of the above buffer with the exception that the concentration of KC1 was raised to 1 M. Elution of the affinity column was carried out at 25°C with a linear gradient order with 3 volumes of Buffer A containing 0.2 mM EDTA, 15 mM 2-mercaptoethanol and the following additions: 1) 150 mM KC1, 2) 150 mM KC1 and 10 mM CAMP, 3) 150 mM KC1, and 4) 150 mM KC1 and 10 mM cGMP. Protein kinase activity eluting in the presence of cGMP was pooled and dialyzed overnight against the above buffer containing 0.2 mM EDTA and 15 mM 2-mercaptoethanol. The dialyzed fractions were next applied to a 1-ml DE52 cellulose (Whatman DE52) column. The column was washed extensively with Buffer A containing 0.2 mM EDTA, 50 mM 2-mercaptoethanol, and 20 mM KC1 until the adsorbance of the eluting buffer at 280 and 260 nm approached zero. The column was then washed at 22°C with 40 ml of the above buffer. The column was then washed again at 4°C with 80 ml of the above buffer containing no CAMP, and the kinase was eluted with 200 mM KC1. Fractions were pooled by their absorbance at 280 nm and dialyzed overnight against a 2 mM cGMP concentration in the presence of 2 mM 2-mercaptoethanol and 15 mM 2-mercaptoethanol. The cGMP-dependent kinase activity and the molecular weight as judged by SDS-polyacrylamide gel electrophoresis remained constant for at least 4 weeks when the enzyme was stored at 4°C.

RESULTS AND DISCUSSION

Comments on Purification - The results of a typical purification starting with 5 kg of bovine heart muscle are shown in Table I. Apparent homogeneity was obtained after an at least 16,400-fold purification. About 6% of the original enzyme activity were recovered. Both values are only estimates since accurate determination of kinase activity in the 9,000 × g supernatant was difficult due to high background phosphorylation. In addition, the concentration of magnesium required for optimal enzyme activity changed during purification when histone H1Y was used as protein substrate. Magnesium concentrations of 10 to 20 and 60 to 100 mM were required for optimal kinase activity, when the 9,000 × g supernatant and purified kinase were used, respectively. From values for cGMP binding capacities (not shown) a 40,000-fold purification of the enzyme was calculated.

Elution of the cGMP affinity column with CAMP at low concentrations was found to remove a protein (M₀ ~ 53,000), which otherwise co-eluted with the cGMP-dependent protein kinase during the final elution step. The enzyme activity obtained is stimulated under optimal conditions at least 10-fold by cGMP without using a charcoal absorption step (8). This indicates that cGMP used for elution was reduced to concentrations appreciable below the apparent activation constant for cGMP. This conclusion is further supported by the finding that 0.92 ± 0.02 mol of cGMP were bound/82,000 g of protein.

Purification of cGMP-dependent protein kinase from bovine heart muscle resulted in an essentially pure enzyme preparation as judged by SDS-polyacrylamide gel electrophoresis (Fig. 1a). Scans of stained gels indicated a purity of over 97%. The molecular weight of the stained band was found 82,000, which value is identical to that reported for the subunit of cGMP-dependent protein kinase purified from bovine lung (8). An apparent molecular weight of 150,000 was estimated from gel filtration of the purified enzyme on Sepharose 6B columns. The same value was obtained for the enzyme present after the ammonium sulfate precipitation step. This would indicate that the molecular weight of the enzyme did not change during the affinity column step.

Phosphorylation of Histone I and IIb - The ability of several histone fractions, including histone mixtures, HI, HIIa, HIIb, and III, to serve as protein substrates in the kinase reaction was studied at a fixed concentration of 10 mM magnesium. All histone fractions were phosphorylated, although the amount of phosphate incorporated varied not only from fraction to fraction but also to some extent with the source of substrates. As shown previously for the purified bovine lung enzyme (7) higher rates of phosphate incorporation were obtained when HIIb was used as substrate. Phosphorylation of the various fractions was stimulated by cGMP between 1.6- and 4.7-fold. When HI (0.2 mg/ml) in the presence of 2 mM magnesium was used as substrate, half-maximal stimulation of phosphorylation was found at concentrations of 0.025 and 6.8 μM, respectively. Slightly lower apparent activation constants for cGMP and CAMP being 0.012 and 2.0 μM, respectively, were obtained when HIIb (0.2 mg/ml) was used as protein substrate in the presence of 60 mM magnesium.

Since the amount of phosphate incorporated varied considerably when different histone mixtures were used, further studies were carried out by using HI and HIIb purified by the method of Johns (15). SDS-polyacrylamide gels of HIIb showed one stained band and that of HI showed two major bands, corresponding to H1 (17), and one minor band which presumably was a contaminating protein amounting to 5 to 10% of the total protein (see also Fig. 6a). The amount of phosphate incorporated in the presence of CAMP into HIIb increased up to a concentration of 60 mM magnesium and decreased at higher concentrations of magnesium (Fig. 3). Since phosphorylation in the absence of cGMP decreased at lower concentrations of magnesium than that in the presence of cGMP activity ratios (-cGMP/+cGMP) decreased with increasing concentrations of magnesium. On SDS-polyacrylamide gels of...
cGMP-dependent Protein Kinase from Bovine Heart Muscle

FIG. 1 (left). DEAE-cellulose column profile of soluble bovine heart muscle protein kinase activities. Chromatography of bovine heart muscle extract on DEAE-cellulose was carried out as described in the text. Volume of fractions was 29 ml. Aliquots of 10 and 40 μl were used for determination of cGMP-dependent protein kinase in the absence (●—●) and presence (○—○) of cGMP (cG) and cGMP binding capacities (■—■), respectively. Aliquots of 5 μl were used for determination of cAMP-dependent protein kinase activity (△—△) in the presence of cAMP (cA). —, absorbance at 280 nm; ×—×, conductivity.

FIG. 2 (right). SDS-polyacrylamide gel electrophoresis of cGMP-dependent protein kinase purified from bovine heart muscle. Two and one-half micrograms of purified cGMP-dependent protein kinase were heated with 2% SDS and 5% 2-mercaptoethanol for 5 min at 100°C. Gel was run from top to bottom and stained with Coomassie blue.

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Total activity (nmol/min/volume)</th>
<th>Specific activity (nmol/min/mg)</th>
<th>Recovery (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant, 9,000 × g</td>
<td>129,000</td>
<td>1290&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0098&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>1,890</td>
<td>480</td>
<td>0.26</td>
<td>39.1</td>
<td>27</td>
</tr>
<tr>
<td>50% (NH₄)SO₄</td>
<td>960</td>
<td>380</td>
<td>0.42</td>
<td>30.6</td>
<td>43</td>
</tr>
<tr>
<td>cGMP/Sepharose&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5</td>
<td>82</td>
<td>164</td>
<td>6.5</td>
<td>16,400</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activity measured in the absence of cGMP (1958 nmol/min/volume) has been subtracted. The other values were determined in the presence of cGMP, and values obtained in the absence of cGMP have not been subtracted.

<sup>b</sup> Values have been obtained after concentration of the enzyme on the 1-ml DE52 cellulose column.

HIib phosphorylated in the presence of cGMP and 60 mM magnesium, radioactive phosphate co-migrated with the HIib band, suggesting that under this condition phosphate was incorporated into the protein substrate. Identical results were obtained when saturating concentrations of HIib (1 mg/ml) were used. In addition, the dependency on high magnesium concentrations for optimal phosphorylation rates was not changed when higher concentrations of ATP (0.5 mM) were used.

The influence of magnesium on the phosphorylation of HI was studied at two concentrations of the protein substrate. At low concentrations of HI (0.2 mg/ml), phosphate incorporation in the presence of cGMP increased up to a concentration of 5 mM magnesium and decreased when higher concentrations of magnesium were added. In contrast, when the same experiment was carried out at a concentration of 4.0 mg/ml of HI, the decrease in phosphate incorporation was observed at magnesium concentrations above 30 mM. Under both conditions phosphorylation was stimulated by cGMP. Variation of the concentration of HI in the presence of two concentrations of magnesium (5 and 60 mM) resulted in biphasic curves (Fig. 4).

The biphasic phosphorylation curves suggested that they could be the result of the presence of two substrates which require different concentrations of magnesium for optimal phosphorylation. This possibility was further investigated by

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Fig. 3. Phosphorylation of histone Iib at different concentrations of magnesium. Phosphorylation of histone Iib (0.2 mg/ml) was carried out in the absence (●) and presence (○) of cGMP (cG) at the indicated concentrations of magnesium. Reactions were started by the addition of 0.01 μg of purified enzyme. The top panel shows the activity ratio (−cGMP)/+cGMP).
using SDS-polyacrylamide gel electrophoresis of HI phosphorylated under different conditions. From Fig. 5 it is evident that radioactive phosphate co-migrated with HI, when phosphorylation was carried out in the presence of 0.2 mg/ml of HI and 2 mM magnesium. However, when phosphorylation was carried out in the presence of 4.0 mg/ml of HI and 60 mM magnesium, almost all of the radioactivity migrated at a position where no distinct stained band was found. Under each condition phosphate incorporation was stimulated by cGMP (Table II), and recovery of protein-bound phosphate was found to be 70 ± 5% of that amount layered on the gels. The principal pattern as described in Fig. 5 and Table II was not changed when HI purchased from Worthington (code: HL) was used or when HI was dialyzed extensively against 2 mM EDTA followed by dialysis against distilled water prior to use. In addition, identical results were obtained when phosphorylated HI was precipitated by trichloroacetic acid prior to incubation of the sample with SDS and 2-mercaptoethanol. The phosphate incorporated into HI or HIIb under the conditions described above had the characteristics of an acid-stable, alkali-labile phosphate bond.

The position of bound phosphate incorporated in the presence of high concentrations of HI and magnesium corresponded to that of HIIb, suggesting that the bound radioactivity was eventually incorporated into HIIb present as a contamination in the HI preparation. This possibility was further evaluated by phosphorylating HI at low concentrations (0.2 mg/ml) in the presence of HIIb (0.02 mg/ml) and 2 mM magnesium. After SDS-polyacrylamide gel electrophoresis, almost all of the bound radioactivity co-migrated with the HIIb band (Table III).

Possible self-phosphorylation of cGMP-dependent protein kinase (18) was examined in experiments in which the purified enzyme was added at a concentration of 2 mg/tube. These experiments were conducted since it was possible that such a reaction may have been influenced under the different conditions used for phosphorylation of histone I and IIb. After SDS-polyacrylamide gel electrophoresis, $^{32}$P was found to co-migrate with the band of cGMP-dependent protein kinase amounting to 0.1 ± 0.05 mol of phosphate/82,000 g of protein. However, this amount of incorporated phosphate did not change significantly under any of the conditions used which included incubation of enzyme in the absence and presence of HI or HIIb. Addition of cGMP or cAMP to these tubes did not change the amount of phosphate incorporated.

cGMP-dependent protein kinase from bovine heart muscle appears to be quite similar in several respects to the enzyme

![Fig. 5. SDS-polyacrylamide gel electrophoresis of histone I phosphorylated at different conditions. Histone I at concentrations of 0.2 mg/ml (○) and 4.0 mg/ml (□) was phosphorylated in the presence of 2 mM and 60 mM magnesium, respectively. After incubation for 5 min at 30°C, reactions were terminated by the addition of 30 μl of a solution containing 5% SDS and 2% 2-mercaptoethanol. Tubes were immediately placed in a boiling water bath for 5 min. Aliquots containing 6.2 μg of histone I were then layered on gels. Gels were stained with Commassie blue, destained by diffusion and scanned at 546 nm. Thereafter gels were sliced into 1-mm slices starting from the top. Individual slices were dried on filter paper squared at 80°C, and radioactivity was then counted in a toluene based scintillator. The upper panel shows a representative scan of phosphorylated histone I. The lower panel gives the distribution of $^{32}$P radioactivity. In each case phosphorylation was carried out in the presence of cGMP and 0.01 μg of purified enzyme. The radioactivity observed per slice has been corrected for the aliquots layered on individual gels. Specific activity of γ-$^{32}$P-ATP was 3000 cpm/pmol. BPB, bromphenol blue.](http://www.jbc.org/content/272/28/3398/F5)

### TABLE II

<table>
<thead>
<tr>
<th>Localization of radioactivity on SDS-polyacrylamide gels of histone I phosphorylated under different conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental procedures were as given in legend to Fig. 5 with the exception that gels were sliced into larger segments as indicated.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Histone I</th>
<th>MgCl$_2$</th>
<th>cGMP</th>
<th>Phosphate bound in gel slice ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/ml</td>
<td>mM</td>
<td></td>
<td>pmol/slice</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>2</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>0.2</td>
<td>2</td>
<td>+</td>
<td>25.8</td>
</tr>
<tr>
<td>4.0</td>
<td>60</td>
<td>-</td>
<td>1.7</td>
</tr>
<tr>
<td>4.0</td>
<td>60</td>
<td>+</td>
<td>9.3</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>2</td>
<td>+</td>
<td>23.2</td>
</tr>
<tr>
<td>4.0</td>
<td>60</td>
<td>+</td>
<td>8.4</td>
</tr>
</tbody>
</table>

* In millimeters from top of gel.

![Fig. 4. Effect of variation of histone I concentration on phosphate incorporation at two concentrations of magnesium. Histone I at the indicated final concentrations was added to tubes containing 60 mM (A) or 3 mM (B) magnesium. For further details see legend to Fig. 3.](http://www.jbc.org/content/272/28/3398/F4)
purified from bovine heart lung (7, 8). It shares with this enzyme a dependency on high magnesium concentrations for optimal phosphorylation rates when several histone fractions are used (7, 8). From this study it is evident that different magnesium concentrations are required for optimal phosphorylation rates of HI and HIIb. Although the reason for this difference is not clear, it may suggest that the different magnesium dependency was entirely due to the protein substrate used.

As shown in Table III rather low concentration of HIIb competed effectively with the phosphorylation of HI, suggesting that HIIb has a much higher affinity for the enzyme than HI. In this experiment low concentrations of magnesium were used. In addition, HIIb was also phosphorylated at low concentrations of magnesium in the absence of HI as shown in Fig. 3. Therefore, one may speculate that the high concentrations of magnesium necessary for optimal phosphorylation rates of HIIb are not important with respect to the phosphate transfer reaction but rather for dissociation of phosphorylated HIIb from the enzyme. This suggestion does not exclude a contaminating factor which may be present in the HIIb preparation and may be responsible for the apparent magnesium dependency. So far, the apparent dependency of the enzyme on high concentrations of magnesium for optimal rates appears to be the result of the in vitro conditions used and of no physiological significance.

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A guanosine 3’:5’-monophosphate-dependent protein kinase from bovine heart muscle. Purification and phosphorylation of histone I and IIb.

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