Adenosine 3':5'-Monophosphate-dependent Protein Kinase from Yeast

Yoshimi Takai, Hirohei Yamamura, and Yasutomi Nishizuka

From the Department of Biochemistry, Kobe University School of Medicine, Kobe, Japan

SUMMARY

Adenosine 3':5'-monophosphate (cyclic AMP)-dependent protein kinase which catalyzes the phosphorylation of histone and protamine is purified about 100-fold from the soluble fraction of bakers' yeast by streptomycin treatment, ammonium sulfate fractionation, followed by DEAE-cellulose column chromatography and isoelectricfocusing electrophoresis. A divalent cation, Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$, is needed for the enzyme activity. The apparent $K_m$ value for cyclic AMP is about $2 \times 10^{-6}$ M. The mode of action of the cyclic nucleotide is essentially similar to that described for mammalian enzymes. Comparative studies have revealed that yeast and rat liver cyclic AMP-dependent protein kinases exhibit closely similar but not exactly identical kinetic and catalytic properties. Nevertheless, the catalytic and regulatory units from yeast and liver enzymes are crosswise reactive, and recombination of these units of the heterologous sources produces a "hybrid" cyclic AMP-dependent protein kinase.

The protein kinase is separable from casein kinase described by Rabinowitz and Lipmann (14), and the mode of action of cyclic AMP is shown to be essentially similar to that described for mammalian protein kinases. Available evidence suggests that in yeast cyclic AMP acts as a regulator in the protein phosphorylation reactions rather than in the catalytic machinery as has been described for E. coli. Comparison will also be made of the kinetic and catalytic properties of the yeast and rat liver cyclic AMP-dependent protein kinases.

EXPERIMENTAL PROCEDURE

Materials—Fresh bakers' yeast (Saccharomyces cerevisiae) in pressed cake (Oriental Yeast Co., Ltd., Kobe), which was grown aerobically at 30°C in a 5% sucrose medium supplemented with 0.15% urea and 0.015% dibasic ammonium phosphate and harvested at late logarithmic phase, was employed for the present studies. Rat liver cyclic AMP-dependent protein kinase and its catalytic and regulatory units were prepared as described previously (15, 16), and the enzyme preparations employed here were practically free of endogenous phosphate acceptor under the conditions of the standard assay. Calf thymus histone was prepared by the method of Johns (17). Salmon sperm protamine (Lot 26B 8060), trypsin (type III), bovine serum albumin, and horse heart cytochrome c (type III) were obtained from Sigma. Bovine casein (Hammarsten) was purchased from Merek AG-Darmstadt. Egg yolk phosphitin was obtained from Mann. Human γ-globulin (Fraction II) and ovalbumin (twice recrystallized) were obtained from Nutritional Biochemicals. [γ-32P]ATP was prepared by the method of Glim and Chappel (18). Cyclic [3H]-AMP (4.47 Ci per mmole) was a product of New England Nuclear, and the radiochemical purity was examined by thin layer chromatography before use. Other chemicals were obtained from commercial sources.

Assay of Protein Kinases and Cyclic AMP-binding Protein—Protein kinase activity was assayed by measuring the radioactivity of [γ-32P]ATP incorporated into an acid-precipitable material as described previously (19). The standard reaction mixture (0.25 ml) contained 2.5 μmoles of Tris-Cl at pH 7.5, 1.25 μmoles of magnesium acetate, 2.5 nmoles of [γ-32P]ATP (2 to 8 $\times 10^4$ cpm per n mole), 100 μg of either histone, protamine, or casein as phosphate acceptor, and an enzyme preparation to be assayed. Cyclic AMP (0.4 μM) was added where indicated. Cyclic AMP-binding protein was assayed by measuring the binding of cyclic [3H]-AMP using a Millipore filter as described previously (19). The mode of action of the cyclic nucleotide is essentially similar to that described for mammalian proteins. Available evidence suggests that in yeast cyclic AMP acts as a regulator in the protein phosphorylation reactions rather than in the catalytic machinery as has been described for E. coli. Comparison will also be made of the kinetic and catalytic properties of the yeast and rat liver cyclic AMP-dependent protein kinases.

Cyclic AMP, adenosine 3':5'-monophosphate.
mined using a Nuclear-Chicago Geiger Muller gas flow counter, model 4388, and that of $^3$H samples was determined using a Packard Tri-Carb liquid scintillation spectrometer, model 3320, with Bray's solution (20). Protein was determined by the method of Lowry et al. (21) with bovine serum albumin as a standard. Molecular weight of the proteins was estimated by gel filtration on a Sephadex G-75 column (93 × 2.5 cm) by the method of Andrews (22). The proteins used as standards were human γ-globulin, bovine serum albumin, ovalbumin, and horse heart cytochrome c. Detailed experimental conditions for gel filtration were described in a preceding paper (16).

**Other Procedures—** Isoelectrofocusing electrophoresis was performed by the method of Svensson (23) using a 110-tol column (LKB Instruments, Inc.) maintained at 0°. The detailed experimental conditions were described in a preceding paper (24).

Tryptic digestion and acid hydrolysis of enzymically phosphorylated radioactive histone preparations were carried out under the conditions specified previously (16). Ascending paper chromatography was carried out with Whatman No. 3MM filter paper with 1-butanol-pyridine-acetic acid-water (15:10:3:12) as a solvent at room temperature. High voltage paper electrophoresis was carried out under the conditions described previously (16). Ascending thin layer chromatography was carried out with an Eastman cellulose sheet, No. 6064, and 1 m ammonium acetate-ethanol (30:70) as a solvent (25). For autoradiography an x-ray film, type IX, Fuji Photo Film Company, was exposed to a filter paper under test.

**RESULTS**

**Purification of Cyclic AMP-dependent Protein Kinase—** All manipulations were carried out at 0–4°. Bakers' yeast (40 g wet weight) was suspended in 5 volumes of 50 mM Tris-Cl at pH 7.5 containing 1 mM EDTA and 6 mM 2-mercaptoethanol, and the cells were crushed twice in a French press cell at 20,000 p.s.i. The homogenate was centrifuged for 20 min at 20,000 × g. To the supernatant solution (200 ml), 10.5 ml of 20% streptomycin sulfate were added with continuous stirring, and the resulting precipitate was removed by centrifugation. The supernatant solution (190 ml) was brought to 50% saturation with solid ammonium sulfate (60 g) and centrifuged for 20 min at 20,000 × g. The precipitate was dissolved in about 30 ml of Buffer A (10 mM Tris-Cl, pH 7.5, and 6 mM 2-mercaptoethanol) and dialyzed for more than 12 hours against a large volume of Buffer A. The dialyzed solution (660 mg of protein) was applied to a DEAE-cellulose (DE52) column (15 × 3 cm) equilibrated with Buffer A. After the column was washed with 200 ml of the buffer, elution was carried out with a 1,200-ml linear concentration gradient of NaCl (0 to 0.4 M) in Buffer A. Fractions of 12 ml each were collected. When each fraction was assayed for protein kinase with histone as substrate, three peaks of activity appeared as shown in Fig. 1. The first peak was stimulated greatly by cyclic AMP, whereas the second and third peaks were not stimulated or inhibited by the cyclic nucleotide. The latter two protein kinases reacted with protamine more than 10 times faster than with histone and, therefore, the enzymes are tentatively referred to hereafter as protamine kinases. Each protamine kinase was distinguishable from the catalytic unit of cyclic AMP-dependent protein kinase (first peak) in kinetic and catalytic properties, and available evidence indicates that both protamine kinases belong to entirely different entities. Fig. 1 also shows two peaks of cyclic AMP-binding protein; one was

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*Detailed properties of protamine kinases will be described elsewhere.*

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**Fig. 1.** DEAE-cellulose (DE52) column chromatography of protein kinase and cyclic AMP-binding protein from yeast. Detailed experimental conditions were described in the text. ○—○ and □—□, protein kinase with histone as substrate in the presence and absence of cyclic AMP (0.4 μM), respectively; ■—■ and □—□, protein kinase with casein as substrate in the presence and absence of cyclic AMP (0.4 μM), respectively. X---X, radioactivity of cyclic [γ-32P]AMP bound.

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Detected in the fraction of cyclic AMP-dependent protein kinase, and the other did not coincide with any of the protein kinases and was identified as free regulatory unit (see below). When each fraction was assayed for protein kinase with casein as substrate, again two peaks appeared as also shown in Fig. 1. The major peak appeared in the fraction of cyclic AMP-dependent protein kinase, but the enzyme was completely separated from the latter enzyme by the subsequent manipulation described below. This protein kinase was independent of cyclic AMP and reacted with casein as well as phosphatase but not with histone and protamine; the enzyme is probably identical with casein kinase which has been described by Rabinowitz and Lipmann (14) and recently purified by Sy and Richter (13).

The cyclic AMP-dependent protein kinase (Fractions 16 through 30 in Fig. 1) was precipitated by the addition of solid ammonium sulfate (60% saturation), centrifuged for 20 min at 20,000 × g, and dissolved in 10 ml of Buffer A. The enzyme solution was dialyzed for several hours against the same buffer and then subjected to isoelectrofocusing electrophoresis. As shown in Fig. 2 the cyclic AMP-dependent protein kinase appeared as a single peak at pH 7.7, and two peaks of cyclic AMP-binding protein were detected; one which appeared at pH 7.7 coincided exactly with protein kinase activity, and the other binding protein which appeared at pH 9.1 was identified as free regulatory unit as described below. Under these conditions casein kinase was found at pH 8.9 and clearly separated from the cyclic AMP-dependent protein kinase. Table I summarizes a typical result of these purification procedures. The cyclic AMP-dependent protein kinase was purified at least 100-fold with an over-all recovery of approximately 40%. This preparation was free of endogenous phosphate acceptor under the conditions for the standard assay and was separated from protamine kinases as well as from casein kinase.

**Phosphatase Acceptors**—The purified preparation of yeast cyclic AMP-dependent protein kinase phosphorylated histone and protamine. Table II shows the relative effectiveness of sub-
protein kinases. The radioactive histone preparations were sub-
phosphorylated each specific seryl and threonyl residue of sub-
strate proteins, histone was phosphorylated separately with these
threonine was about 22% of that of phosphoserine. Under the
same conditions rat liver protein kinase produced phosphothreo-
dase followed by paper electrophoresis, a major radioactive
spot was associated with phosphoserine; the amount of phospha-
tine in the amount of about 3% of that of phosphoserine.

Calf thymus histone was fully phosphorylated, and then subjected to acid-hy-
drolysis followed by paper electrophoresis, a major radioactive
protein became insoluble and was associated with the spot. The radioactive histone preparations were sub-
jected to trypsin digestion and, subsequently, to paper chroma-
tography followed by paper electrophoresis. As illustrated in
Fig. 3, the radioactive peptides obtained in this manner were
closely similar but not exactly identical. Under these conditions
both histone preparations were equally digested to produce
identical sets of more than 38 spots visualized by the ninhydrin
reaction.

Effect of Cyclic AMP—The maximum stimulation by cyclic
AMP was obtained at 8 × 10^{-4} M, and the concentration neces-
sary for the half maximum stimulation was 2 × 10^{-5} M. Among
several nucleoside 3':5'-monophosphates, cyclic AMP was far
more effective than any other nucleotide, and inosine 3':5'-
monophosphate was about 37% as active as cyclic AMP at
1 × 10^{-5} M. 3':5'-Monophosphates of guanosine, cytidine, and
uridine were all inactive at this concentration.

Other Properties—The enzyme preparation showed an absolute
requirement for a divalent metal ion; either Mg^{2+}, Co^{2+}, or Mn^{2+}
supported protein kinase activity and Mg^{2+} was most active as
shown in Fig. 4. At higher concentrations these ions were
inhibitory. The enzyme was specific for ATP with the K_m value
of 1.2 × 10^{-4} M, and the maximum enzyme activity was obtained
at pH 7.5 in Tris-Cl buffer. The cyclic AMP-dependent protein
kinase was relatively unstable, and approximately 50% of the
activity was lost when stored at 4° for 2 weeks or when heated
at 50° for 2 min. The catalytic unit of the protein kinase was
very labile and the activity was completely lost at 4° for several
days. Table III summarizes the properties of yeast cyclic AMP-
dependent protein kinase in comparison with rat liver enzyme.

TABLE II

Table II summarizes the properties of yeast and liver cyclic AMP-dependent
protein kinases.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Yeast protein kinase</th>
<th>Liver protein kinase</th>
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<tbody>
<tr>
<td></td>
<td>Without cyclic AMP</td>
<td>With cyclic AMP</td>
</tr>
<tr>
<td>Calf thymus histone</td>
<td>10.9</td>
<td>43.2</td>
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<tr>
<td>Salmon sperm protamine</td>
<td>43.3</td>
<td>101.2</td>
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<td>Bovine casein</td>
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<td>Egg yolk phosvitin</td>
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<td>0</td>
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<td>Bovine serum albumin</td>
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<td>0</td>
</tr>
<tr>
<td>Human γ-globulin</td>
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Substrate specificity of yeast and liver cyclic AMP-dependent
protein kinases

Enzyme activity was assayed under the standard conditions
except that 100 μg each of the acceptor protein indicated and
either yeast (9 μg) or rat liver (15 μg) cyclic AMP-dependent
protein kinase were added. The column numbers and those in paren-
theses indicate picomoles of phosphate incorporated and percent-
age activities with calf thymus whole histone as 100, respectively.

Site of Phosphorylation—Yeast cyclic AMP-dependent protein
kinase phosphorylated mainly seryl and some threonyl residues of
phosphate acceptor proteins. When calf thymus whole his-
tone was fully phosphorylated, and then subjected to acid-hy-
drolysis followed by paper electrophoresis, a major radioactive
spot was associated with phosphoserine; the amount of phospha-
threonine was about 22% of that of phosphoserine. Under the
same conditions rat liver protein kinase produced phosphothreo-
dine in the amount of about 3% of that of phosphoserine.

In order to ascertain whether the yeast and rat liver enzymes
phosphorylated each specific seryl and threonyl residue of sub-
strate proteins, histone was phosphorylated separately with these
protein kinases. The radioactive histone preparations were sub-

Table I

Summary of purification of cyclic AMP-dependent protein kinase from yeast

Detailed experimental conditions are described in the text. One unit is defined as that amount of enzyme which incorporates
1 nmole of phosphate from [γ-32P]ATP into histone per min under
the standard assay conditions.

<table>
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<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
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<tr>
<td>Crude supernatant</td>
<td>mL</td>
<td>mg</td>
<td>units</td>
<td>units/mg</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>190</td>
<td>2,660</td>
<td>26.6</td>
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</tr>
<tr>
<td>Ammonium sulfate</td>
<td>30</td>
<td>660</td>
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<td>0.04</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>10</td>
<td>48</td>
<td>47.0</td>
<td>0.08</td>
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<tr>
<td>Isoelectrofocusing electrophoresis</td>
<td>20</td>
<td>3.6</td>
<td>15.0</td>
<td>4.17</td>
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Table II

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Dissociation of Protein Kinase into Subunits by Cyclic AMP—
When the purified preparation of yeast cyclic AMP-dependent
protein kinase was subjected once again to isoelectrofocusing
electrophoresis under the same conditions given in Fig. 2, the
enzyme was recovered at pH 7.7 as shown in Fig. 5A. The cyclic
AMP-binding protein coincided well with kinase activity. If, how-
however, the same enzyme preparation was mixed with cyclic
AMP (0.2 μM) at 0°C prior to the electrophoresis, the protein kinase was found to shift partly and was recovered at pH 6.9 as shown in Fig. 5B. The protein kinase newly produced did not bind cyclic AMP nor was it stimulated by the cyclic nucleotide. This protein kinase, therefore, seemed to be the catalytic unit of the protein kinase. A cyclic AMP-binding protein found at pH 9.1 appeared to be the regulatory unit dissociated from the original cyclic AMP-dependent protein kinase. Under these conditions the dissociation of the protein kinase was sometimes incomplete, presumably due to reassociation of the catalytic and regulatory unit during the electrophoresis, since free cyclic AMP rapidly migrated to the anode, while these units showed relatively close isoelectric points as illustrated in this figure. The significance of a small shoulder of kinase activity appearing in this figure was unknown; the kinetic and catalytic properties of the shoulder were indistinguishable from those of the main peak.

Recombination of Catalytic and Regulatory Units—Gill and Garren (26) reported that the protein kinase isolated from bovine adrenal cortex was inhibited by a cyclic AMP-binding protein obtained from the same tissue. A preceding report from this laboratory (16) showed that a cyclic AMP-dependent protein kinase obtained from rat liver was reconstituted from its catalytic and regulatory units. In addition, a catalytic unit from one tissue and a regulatory unit from another were shown to be crosswise reactive and a “hybrid” cyclic AMP-dependent protein kinase was produced (27). Experiments diagrammed in Fig. 6 showed that the catalytic and regulatory units obtained from yeast and rat liver were also crosswise reactive, and the activity of either one of these catalytic units was inhibited progressively by the addition of increasing amounts of a regulatory unit from the homologous as well as from the heterologous source. This inhibition was completely overcome by the cyclic nucleotide which resulted in the full restoration of the original activity. The yeast regulatory unit employed for the present experiments was the free cyclic AMP-binding protein found at pH 9.1 upon isoelectrofocusing electrophoresis and was essentially free of protein kinase activity with histone as substrate.

Molecular Weight—Yeast cyclic AMP-dependent protein kinase showed a symmetrical peak upon gel filtration on a Sephadex G-75 column, and the molecular weight was estimated to be

![Fig. 3. Autoradiography of tryptic digests of radioactive histone preparations phosphorylated by yeast and rat liver cyclic AMP-dependent protein kinases. Detailed experimental conditions were described in the text. Paper chromatography (Direction a) and subsequent paper electrophoresis at pH 3.5 (Direction b) were carried out under the conditions specified under “Experimental Procedure.” A, yeast protein kinase; B, rat liver protein kinase.](image)
about 58,000. The catalytic and regulatory units dissociated in the presence of cyclic AMP were isolated by isoelectrofocusing electrophoresis under the conditions given in Fig. 5B. These units were then subjected separately to gel filtration analysis as described under “Experimental Procedure,” and the molecular weight was estimated to be approximately 30,000 for the catalytic unit and 28,000 for the regulatory unit (Table III). During the gel filtration of the regulatory unit, a small quantity of cyclic AMP-binding protein with the molecular weight of about 14,000 was produced in addition to the 28,000 molecular weight regulatory unit. This small cyclic AMP-binding protein appeared to be subunits of the regulatory unit since, when the regulatory unit was subjected repeatedly to gel filtration in the presence of cyclic AMP, 2 mM mercaptoethanol (6 mM), and 10% glycerol in 10 mM Tris-Cl at pH 7.5, a small amount of the 14,000 molecular weight cyclic AMP-binding protein was consistently produced. The precise subunit structure of the regulatory unit is under investigation.

**DISCUSSION**

The present studies clearly demonstrate a cyclic AMP-dependent protein kinase in the soluble fraction of bakers’ yeast which is capable of phosphorylating histone and protamine. Cyclic AMP dissociates the protein kinase into catalytic and regulatory units in a manner similar to that described for mammalian enzymes (26, 28–31). A preceding report from this laboratory has shown that cyclic AMP-dependent protein kinases obtained from various mammalian tissues of different species exhibit closely similar kinetic and catalytic properties and phosphorylate the same specific sites of histone and protamine (10). However, the yeast cyclic AMP-dependent protein kinase presented in this paper apparently resembles but is distinguished from mammalian enzymes in several respects, including $K_m$ value for ATP, optimum pH, optimum Mg$^{2+}$ ion, isoelectric point, and heat stability (Table III). The seryl and threonyl residues of histone phosphorylated by the yeast and rat liver enzymes appear to be overlapping but not exactly identical as judged by the fingerprint procedure (Fig. 3); this slight difference is not simply due to endogenous phosphate acceptor which may contaminate the enzyme preparations, although the possibility of contamination of another unknown protein kinase may not be definitely excluded at the present time. Nevertheless, the catalytic and regulatory units obtained from yeast and rat liver are crosswise reactive, and recombination of these units results in the formation of a “hybrid” cyclic AMP-dependent protein kinase.

The present paper also describes at least three classes of protein kinases in yeast. One is the cyclic AMP-dependent protein kinase described in this paper. The second is a protein kinase which phosphorylates preferentially casein and phosvitin but not histone and protamine. This protein kinase is independent of cyclic AMP and seems to be casein kinase described by Rabinowitch and Lipmann (14). The third is a class of enzymes which greatly favor protamine rather than histone as substrate and do not respond to the cyclic nucleotide (Fig. 1); the enzymes show isoelectric points of pH 5.5 to 5.6 and are clearly distinguishable from the catalytic unit of cyclic AMP-dependent protein kinase.
Recently, Sy and Richter (13) have isolated a cyclic AMP-binding protein from yeast which shows the molecular weight of 24,000. Since casein kinase present in yeast is not stimulated by cyclic AMP nor is inhibited by the cyclic AMP-binding protein, these authors have thought that the binding protein might play a role in the catabolite gene activation as reported for prokaryotic organisms (1). The cyclic AMP-binding protein presented in this paper is shown to be associated with cyclic AMP-dependent protein kinase, and available evidence indicates that in yeast cyclic AMP may act as a regulator in the protein phosphorylation reactions as reported for various mammalian tissues. The biological role as well as the naturally occurring phosphate acceptors of yeast cyclic AMP-dependent protein kinase will be explored by further investigations.

Acknowledgments—The authors are grateful to Mr. Hideki Katagami, Miss Mieko Nakashima, Mrs. Sachiko Nishiyama, and Miss Miwako Kuroda for their skillful technical and secretarial assistance.

REFERENCES
