Novel protein kinase, AUT-PK 85, isolated from adrenocortical carcinoma: Purification and characterization
(cyclic AMP/photoaffinity/gel electrophoresis/hydrophobic chromatography/affinity chromatography)

GOURI SHANKER, HELGA AHRENS, AND RAMESHWAR K. SHARMA

Department of Biochemistry and Memphis Regional Cancer Center, University of Tennessee, Center for the Health Sciences, Memphis, Tennessee 38163

Communicated by Henry Lardy, September 11, 1978

ABSTRACT We describe the purification to apparent homogeneity of a protein kinase (designated AUT-PK 85) from adrenocortical carcinoma 494, as evidenced by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The enzyme binds cyclic AMP (cAMP) and autophosphorylates but does not use histone, casein, or polyornithine as substrates in the presence or absence of cAMP. Stoichiometry of phosphate incorporation was 0.71 mol/mol of enzyme. The enzyme was found to have a molecular weight of 85,000 based on gel filtration. The protein was composed of polypeptides having the same molecular weight, 42,000, and thus it appears to consist of two subunits of equal size. The enzyme bound two cAMP molecules, indicating that each subunit binds one molecule of cAMP. The homogenous enzyme did not inhibit the protein kinase activity of the free catalytic subunit of normal adrenal cAMP-dependent protein kinase under conditions such that recombination with the free regulatory subunit occurred. cAMP bound specifically to the enzyme with an apparent dissociation constant (cKd) of 1.2 X 10^{-4} M. Scatchard plot data indicated one type of binding sites for cAMP. The enzyme did not bind adenosine. This novel autophosphorylating, cAMP-binding, protein kinase may be a characteristic of certain adrenal neoplasms.

Studies with isolated adrenocortical carcinoma cells (1), cultured carcinoma cells (2), and tumor slices (3) have shown that, in contrast to what occurs in the normal adrenal cell, neither corticotropin (ACTH) nor cyclic AMP (cAMP) stimulates the synthesis of corticosterone. It has been further demonstrated that the route (14) pregnenolone \rightarrow progesterone \rightarrow deoxycorticosterone \rightarrow corticosterone is at least partly intact in the tumor cell (2, 4-6). Based on the premise that a cAMP-dependent protein kinase (7) is involved in cAMP-activated adrenal steroidogenesis, it was proposed (4-6) that the defect in the tumor may be due to a defect in the cAMP-dependent protein kinase system (for a review, see ref. 8). Recently we isolated a crude protein kinase that bound cAMP with a high degree of specificity but was unable to phosphorylate histone in the presence or absence of the nucleotide (9). These results were in contrast to the cAMP-dependent protein kinase found in the normal adrenal gland, which binds cAMP and catalyzes the phosphorylation of histones (10). This report deals with the purification to homogeneity and characterization of this enzyme, designated AUT-PK 85.

METHODS

cAMP binding activity was determined as described (11) except that the reaction was terminated with 2 ml of ice-cold 50% saturated ammonium sulfate. After 20 min the precipitate was collected on GF/C filters and washed twice with 2 ml of 15% saturated ammonium sulfate. The filters were dried and assayed for radioactivity. Protein kinase activity was assayed by the incorporation of 32P from [γ-32P]ATP into either histone as exogenous substrate or into the self-phosphorylating enzyme (12), using GF/C filters to collect the final precipitate.

All assays were performed in duplicate and the data were corrected for background radioactivity determined with the complete reaction mixture without enzyme.

Protein was determined by the method of Lowry et al. (13) or by the fluorescamine assay (14).

For electrophoresis in a non-denaturating system, polyacrylamide gels were prepared essentially as described (15, 16) with some modification. Denaturing polyacrylamide gels containing 0.1% sodium dodecyl sulfate were prepared by the method of Favre and Laemmli (17) as modified by Engbaek et al. (18).

RESULTS

Purification. All procedures were carried out at 0-4°C.

Step I: Homogenization and ammonium sulfate precipitation. One hundred grams of adrenal tumor (19) was minced, mixed with 200 ml of 20 mM Tris-HCl, pH 7.5/5 mM 2-mercaptoethanol/0.2 M EDTA (TMGE buffer), and homogenized in a Waring blender for 2-3 min. The homogenate was centrifuged for 45 min at 50,900 X g and the supernatant was brought to 50% saturation by the addition of solid ammonium sulfate. Approximately 85% of cAMP binding activity remained with the precipitate which was collected by centrifugation at 30,900 X g for 45 min and then dissolved in 10% (wt/vol) glycerol in TMGE buffer (TMGE buffer) and extensively dialyzed against TMGE buffer. Normal adrenal protein kinase was prepared in a similar fashion.

Step II: DEAE-cellulose chromatography. The dialyzed sample was fractionated on a DEAE-cellulose column with a linear NaCl gradient (0.04-0.25 M), and the fractions were assayed for binding and cAMP-dependent kinase activity (Fig. 1A). Two distinct cAMP binding peaks were obtained, one eluting at 0.10 M NaCl and the other eluting at 0.17 M NaCl. The latter was used for further purification. No cAMP-dependent kinase activity was detected in any of the column fractions. Earlier studies (9) showed that the cAMP-dependent protein kinase from bovine adrenal cortex elutes at 0.15 M NaCl on DEAE-cellulose chromatography. In this study the normal protein kinase was obtained by step elution of the DEAE-cellulose column with 0.25 M NaCl in TMGE.

Step III: Hydrophobic chromatography. The DEAE-cellulose cAMP-binding peak fractions eluting at 0.17 M NaCl were chromatographed on heyl-Sepharose (20), which removed most of the high molecular weight and hydrophilic proteins (Fig. 1B). The cAMP-binding peak fractions (50 mM NaCl) were pooled and dialyzed against TMGE buffer. cAMP-dependent kinase activity as assessed by the phosphorylation of

Abbreviations: cAMP, cyclic AMP; TME buffer, 20 mM Tris-HCl, pH 7.5/5 mM 2-mercaptoethanol/0.2 M EDTA; TMGE buffer, 10% (wt/vol) glycerol in TME buffer.
FIG. 1. Gradient elution profile of adenocortical carcinoma cAMP-binding protein kinases. (A) DEAE-Cellulose column. The ammonium sulfate precipitate was dissolved in TME buffer, dialyzed against TMGE buffer, and applied to a column (2.5 × 30 cm) of DEAE-cellulose previously equilibrated with TMGE. The column was washed at 70 ml/hr with 1 column vol (150 ml) of the equilibrium buffer and eluted with 500 ml of a linear gradient of NaCl (0.04–0.25 M) in the same buffer. Fractions (5 ml) were analyzed for cAMP-binding activity (■), cAMP-dependent kinase activity (▲); absorbance at 280 nm (○) was measured. (B) Hexyl-Sepharose. The cAMP-binding peak fractions eluting at 0.17 M NaCl on DEAE-cellulose (AUT-PK 85) were pooled, dialyzed against 5 mM 2-(N-morpholino)-ethanesulfonic acid, pH 6.5/15 mM 2-mercaptoethanol and fractionated on a hexyl-Sepharose column (1 × 25 cm) previously equilibrated with the same buffer. After washing at a flow rate of 15 ml/hr with 2 column vol (40 ml) of this buffer, the enzyme was eluted with a linear NaCl gradient (10–200 mM) in 2-(N-morpholino)-ethanesulfonic acid buffer. Fractions (2.5 ml) were collected and cAMP-binding activity (■) and absorbance at 280 nm (○) were determined.

histones was not detected in any of the cAMP-binding fractions.

Step IV: Affinity chromatography. Complete purification of the tumor enzyme was achieved when the dialyzed enzyme activity from the hydrophobic chromatography was adsorbed to a 10-ml N8-(2-aminomethyl)amino-cAMP-Sepharose column (21). Neither binding nor kinase activity could be detected in the column effluent.

To remove all impurities, the cAMP-Sepharose resin was washed extensively with TMGE buffer containing 0.1, 0.5, 1.0, and 2.0 M NaCl and the tumor protein kinase was recovered by incubating the resin with 50 ml of 20 mM cAMP in TMGE buffer at room temperature. The extracts were concentrated by adsorption to a 5-ml DEAE-cellulose column followed by a step-off elution with TMGE containing 0.5 M NaCl. The eluate was dialyzed extensively against TMGE buffer, and essentially all bound cAMP was removed when a dialysis bag containing charcoal suspended in TMGE was placed in the final dialysis buffer. Dialysis against storage buffer (TME/50% glycerol/0.1 M NaCl) resulted in a further 3-fold concentration and made it possible to store the enzyme for several months at −20°C without loss of activity. Table 1 summarizes the purification procedure. In only four steps the enzyme was purified almost 8000-fold with a 12% yield.

Molecular Weight and Purity. The AUT-PK 85 isolated by affinity chromatography yielded a single stained band of 42,000 daltons on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, indicating apparent homogeneity (Fig. 2B). In contrast, the normal adrenal enzyme migrated at 49,000 daltons. On a Bio-Gel P-150 gel filtration column, AUT-PK 85 eluted corresponding to 85,000 daltons whereas the normal adrenal regulatory subunit corresponded to 98,000 daltons (Fig. 3). This clearly showed that the tumor enzyme is different from the normal regulatory subunit and that both proteins are dimers in their native state.

Photoaffinity Labeling Studies. To ascertain that the stained band obtained after electrophoresis was the protein that binds

\[
\text{Table 1. Purification of AUT-PK 85}
\]

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein, mg</th>
<th>cAMP binding activity</th>
<th>Purification, fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>2500</td>
<td>7.50</td>
<td>0.003</td>
</tr>
<tr>
<td>Ammonium sulfate pellet</td>
<td>410</td>
<td>5.85</td>
<td>0.014</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>32.92</td>
<td>3.22</td>
<td>0.098</td>
</tr>
<tr>
<td>Hexyl-Sepharose</td>
<td>2.32</td>
<td>2.47</td>
<td>1.066</td>
</tr>
<tr>
<td>Affinity</td>
<td>0.040</td>
<td>0.93</td>
<td>23.200</td>
</tr>
</tbody>
</table>

FIG. 2. (A) Photoaffinity labeling of partially purified AUT-PK 85 obtained after hexyl-Sepharose chromatography. The enzyme preparation was preincubated with [3H]cAMP under standard conditions for 30 min and incubation was continued for 4 hr in the dark at 4°C in front of a Mineralite UVSL-25 hand lamp at a distance of 16 mm. The total incubation mixture was adjusted to pH 6.8 and was fractionated by sodium dodecyl sulfate gel electrophoresis. The protein band associated with radioactivity was determined by assaying gel slices dissolved in hydrogen peroxide. (B) Sodium dodecyl sulfate/polyacrylamide disc gel electrophoresis of purified AUT-PK 85 (lane 3) and molecular weight marker proteins (lanes 1 and 2). The gels were stained with Coomassie brilliant blue and destained in 7.5% acetic acid. The molecular weight markers were: RNA polymerase subunits (lane 1) and bovine serum albumin (lane 2). (C) Photoaffinity labeling of partially purified regulatory subunit of cAMP-dependent protein kinase from bovine adrenal cortex obtained after affinity chromatography. The experimental conditions were the same as in A.
cAMP, the \textsuperscript{3}H-labeled nucleotide was covalently incorporated by photolysis (22) and the pattern of radioactivity was analyzed (Fig. 2A). In the tumor extract, radioactivity was associated with a protein band at 42,000 daltons, confirming the molecular weight and establishing that the protein isolated by affinity chromatography was the cAMP-binding protein kinase. Analysis of the regulatory subunit from the normal adrenal cortex enzyme showed that the label was associated with a protein band at 49,000 daltons (Fig. 2C). This indicated that the tumor enzyme isolated was not the regulatory subunit of the cAMP-dependent protein kinase found in normal adrenal cortex. No 42,000-dalton cAMP-binding band was detected in the normal adrenal extract.

**cAMP Binding Characteristics.** Studies of the stoichiometry of cAMP binding with the homogeneous preparation of AUT-PK 85 showed that this enzyme bound 2 mol of cAMP per mol of the enzyme, indicating that each subunit binds cAMP in a 1:1 mol ratio.

Scatchard plot representation of cAMP binding yielded a straight line, indicating a single set of binding sites (Fig. 4). The apparent dissociation constant, \( K_d \), calculated from the slope was 1.15 \( \times \) 10\(^{-8} \) M. A similar \( K_d \), 1.3 \( \times \) 10\(^{-8} \) M, has been reported (10) for the regulatory subunit isolated from adrenal cortex, suggesting that the cAMP receptor of the tumor protein kinase is intact.

The receptor of AUT-PK 85 exhibited high specificity for cAMP. Only cyclic IMP competed to a small extent (18%) when the binding of radioactive cAMP was assayed in the presence of 500-fold excess of unlabeled cyclic CMP, cyclic IMP, cyclic UMP, or cyclic CMP. The binding of \[ ^{3}H \]adenosine with the enzyme was also evaluated but no affinity for the nucleoside was found. A study of the kinetics of cAMP binding showed that half of the binding sites became saturated within 3–4 min and complete saturation occurred within 25 min (data not shown).

**Phosphorylation of Histones.** It is well established that cAMP-dependent protein kinases from different sources phosphorylate histones (21, 23, 24). However, with AUT-PK 85, the amount of \textsuperscript{32}P incorporated in the presence or absence of histones, casein, or adrenal polyosomes was the same, indicating that these proteins did not serve as substrates. Furthermore, no effect of cAMP up to 10 mM was observed in this process.

**Autophosphorylation Studies.** It is established that the catalytic subunit of the protein kinase from bovine heart autophosphorylates its regulatory subunit (25). For investigation of a similar mechanism in normal adrenal kinase and AUT-PK 85, partially purified enzymes were preincubated with either \[ ^{3}H \]cAMP or \[ \gamma ^{32} \text{P} \]ATP and analyzed by nondenaturing electrophoresis. The tumor protein that bound cAMP comigrated with the phosphorylated protein, indicating that the enzyme underwent autophosphorylation (Fig. 5B). This was in contrast to the protein kinase from normal adrenal cortex with which neither the regulatory subunit alone was autophosphorylated (Fig. 5A) nor could its catalytic subunit phosphorylate the regulatory moiety (data not shown).

**Fig. 4.** Scatchard plot of the binding affinity of cAMP to AUT-PK 85. The amount of cAMP bound is plotted as a function of the ratio of the concentration of bound to free cyclic nucleotide. The concentration of free [\( ^{3}H \)]cAMP was obtained by subtracting the [\( ^{3}H \)]cAMP bound from the total [\( ^{3}H \)]cAMP concentration initially added to the incubation mixture. To evaluate \( K_d \), the molar concentration was determined from the total amount of ligand bound in 0.1 ml of incubation mixture.

The pH range for effective cAMP binding by AUT-PK 85 was between 5 and 8 in contrast to the normal protein kinase for which optimal binding is observed at pH 4–5.
Reconstitution Studies. To ascertain that the enzyme isolated from the adrenal tumor is not merely a regulatory subunit of a cAMP-dependent protein kinase, reconstitution with the catalytic subunit from the normal adrenal cortex enzyme was attempted. Incubation of the free catalytic subunit with the tumor kinase in the absence and presence of varying concentrations of cAMP had no effect on the amount of 32P incorporated into histones (Fig. 6B). This indicated that the tumor kinase cannot reconstitute with the catalytic subunit to an inactive holoenzyme. In contrast, the regulatory and catalytic subunits of the normal adrenal enzyme could be readily reconstituted to a cAMP-responsive holoenzyme.

DISCUSSION
To date, only one mode of action of cAMP has been thoroughly elucidated at the molecular level in eukaryotic cells—i.e., the cyclic nucleotide binds to the regulatory subunit of the protein kinase holoenzyme with the simultaneous release of the catalytic subunit (24, 25). In bacterial cells, the cAMP-binding receptor lacks kinase activity, binds to DNA, and promotes the transcription of mRNA of inducible enzymes (26, 27). On the other hand, various effects of cAMP on cellular and subcellular processes have been reported. These include: regulation of cell proliferation (28) and synthesis of protein (29) and RNA (30); chromosomal puffing (31); changes in membrane properties (31, 32); and mediation of the activity of various hormones (33).

This paper describes the presence of an autophosphorylating cAMP-binding protein kinase, AUT-PK 85, in adrenocortical carcinoma. This enzyme has been purified 8000-fold to apparent homogeneity as evidenced by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis. The enzyme appears to be dimeric with a molecular weight of 85,000. The stoichiometry of cAMP binding with the enzyme showed that it binds 2 mol of cAMP per mol of protein. This indicates that each receptor chain binds 1 mol of cAMP. The molecular weight of the regulatory subunit of the normal adrenal protein kinase was found to be 88,000, in broad agreement with the earlier reported value of 82,000 (24); it was found to be a dimer, each subunit having a molecular weight of 40,000.

As in the tumor enzyme, the normal receptor bound cAMP at a ratio of 1 mol/mol of receptor chain. The binding of the tumor enzyme was specific and exhibited the same dissociation constant as that of the normal enzyme. Both proteins, normal and tumor, exhibited one type of binding site as evidenced by the Scatchard plot.

In contrast to the normal adrenal protein kinase, the tumor enzyme lacked cAMP-dependent kinase activity in the phosphorylation of histones. The question was raised whether this enzyme might simply be a modified cAMP-binding protein that has lost the kinase subunit. This was not the case, however. For different tissues, the phosphotransferase activity of the free catalytic subunit has been shown to be inhibited by the addition of the regulatory subunit from the same enzyme (21, 34) (Fig. 6B). The tumor cAMP-binding protein did not inhibit the protein kinase activity of the free catalytic subunit of normal adrenal cAMP-dependent protein kinase under conditions such that recombination with the free regulatory subunit occurred. These results differentiate the tumor enzyme from the regulatory subunit of cAMP-dependent protein kinase.

Again, in contrast to the regulatory subunit of the normal adrenal protein kinase, the tumor enzyme was found to autophosphorylate. The stoichiometry of phosphate incorporation was 0.71 mol/mol of the enzyme. According to the terminology of Corbin et al. (35), this enzyme falls into the category of protein kinase type II, which has been shown to autophospho-
rylate (36-38) but the process of autophosphorylation, which is supposed to be an intramolecular rather than intermolecular process (39), requires the presence of intact enzyme (40). Furthermore, the molecular weights of the regulatory subunits of the autophosphorylating protein kinase isolated from rat liver and rat uterus have been found to be 54,000, which are quite different from the molecular weight of the tumor enzyme (41).

These studies indicate that the enzyme AUT-PK 85 reported here is distinct from the protein kinase enzyme present in the normal adrenal gland (10). Several investigators have reported the presence of cAMP receptors without protein kinase activity in eukaryotic cells (42-45). These proteins have at least one of the following characteristics. The dissociation constant for cAMP is about 10^{-7} M (43-45); adenine derivatives compete for the cAMP site (43-45); and specific adenosine binding sites have been described in enzymes from rat and mouse liver (46). The tumor enzyme described here, however, does not demonstrate any of these properties. Its dissociation constant for cAMP is at least 1 order of magnitude lower, and it does not possess any binding site for adenosine. Sy and Richter (47) have reported the isolation of another cAMP-binding protein from yeast; the molecular weight of this protein is 24,000, the Kd for cAMP is 5 × 10^{-9} M, and cGMP effectively competes with the cAMP-binding receptor. None of these properties is shared by the tumor enzyme. Therefore, AUT-PK 85 is different from the protein described by Sy and Richter (47).

At present, we cannot assign any physiological role to this protein kinase. It is tempting to speculate that the biochemical lesion responsible for the lack of cAMP-activated corticosterone synthesis in the adrenal tumor may be due to the presence of a variant protein kinase enzyme such as reported here.

This investigation was supported by Grant CA16091 from the National Cancer Institute.