Resolution of the Phosphorylated and Dephosphorylated cAMP-binding Proteins of Bovine Cardiac Muscle by Affinity Labeling and Two-dimensional Electrophoresis*

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The photoaffinity label 8-azido[32P]adenosine 3':5'-monophosphate (8-azido-cyclic [32P]AMP) was used to analyze both the cAMP-binding component of the purified cAMP-dependent protein kinase, and the cAMP-binding proteins present in crude tissue extracts of bovine cardiac muscle. 8-Azido-cyclic [32P]AMP reacted specifically and in stoichiometric amounts with the cAMP-binding proteins of bovine cardiac muscle. Upon phosphorylation, the purified cAMP-binding protein from bovine cardiac muscle changed its electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gels from an apparent molecular weight of 54,000 to an apparent molecular weight of 56,000. In tissue extracts of bovine cardiac muscle, most of the 8-azido-cyclic [32P]AMP was incorporated into a protein band with an apparent molecular weight of 56,000 which shifted to 54,000 upon treatment with a phosphoprotein phosphatase. Thus a substantial amount of the cAMP-binding protein appeared to be in the phosphorylated form. Autoradiograms following sodium dodecyl sulfate-polyacrylamide gel electrophoresis of both the pure and impure cAMP-binding proteins labeled with 8-azido-cyclic [32P]AMP revealed another binding component with a molecular weight of 52,000 which incorporated 32P from [γ-32P]ATP without changing its electrophoretic mobility. Limited proteolysis of the 56,000- and 52,000-dalton proteins labeled with 32P from either [γ-32P]ATP-Mg2+ or 8-azido-cyclic [32P]AMP showed patterns indicating homology. On the other hand, peptide maps of the major 8-azido-cyclic [32P]AMP-labeled proteins from tissue extracts of bovine cardiac muscle (Mr = 56,000) and rabbit skeletal muscle (Mr = 48,000) displayed completely different patterns as expected for the cAMP-binding components of types II and I protein kinases.

Both phospho- and dephospho-cAMP-binding components from the purified bovine cardiac muscle protein kinase were also resolved by isoelectric focusing on polyacrylamide slab gels containing 8 M urea. The phosphorylated forms labeled with 32P from either [γ-32P]ATP or 8-azido-cyclic [32P]AMP migrated as a doublet with a pi of 5.35. The 8-azido-cyclic [32P]AMP-labeled dephosphorylated form also migrated as a doublet with a pi of 5.40. The phosphorylated and dephos-

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Both phospho- and dephospho-cAMP-binding components from the purified bovine cardiac muscle protein kinase were also resolved by isoelectric focusing on polyacrylamide slab gels containing 8 M urea. The phosphorylated forms labeled with 32P from either [γ-32P]ATP or 8-azido-cyclic [32P]AMP migrated as a doublet with a pi of 5.35. The 8-azido-cyclic [32P]AMP-labeled dephosphorylated form also migrated as a doublet with a pi of 5.40. The phosphorylated and dephos-
protein kinase activity was immunoprecipitable with antisem directed against R and the remaining 5 to 10% was accounted for as nonimmunoreactive protein kinase I. 2) Hofmann et al. (18) extended these studies to soluble extracts of rabbit skeletal muscle, reporting that all of the CAMP-binding activity present in muscle extracts could be precipitated by the combined action of antisera specific for R and R. In soluble extracts of rabbit heart, liver, kidney, brain, and skeletal muscle, CAMP-binding protein activity was present in a 1:1 molar ratio with CAMP-dependent phosphotransferase activity; 3) Walter et al. (19) showed that the photoaffinity analog of CAMP, 8-azo-cyclic [32P]AMP (20, 21) was specifically and quantitatively incorporated into only two protein bands in extracts of several rat tissues. These proteins with subunit molecular weights of 47,000 and 54,000 were resolved by chromatography on DEAE-cellulose and shown to correspond to R and R, respectively.

The purified protein kinase II from bovine cardiac muscle incorporates 1 mol of phosphate from ATP into each of 2 seryl residues in its CAMP-binding protein dimer (22). This self-phosphorylation is characteristic of type II CAMP-dependent protein kinases (23, 24) and can occur by an intramolecular reaction (25). The dissociated phospho-R, but not the phospho-R residing in the holoenzyme, can be dephosphorylated by the action of phosphoprotein phosphatases (26) or by the reversal of the phosphotransferase reaction in the presence of ADP and Mg++. Although phospho- and dephospho-holoenzymes can be completely dissociated by CAMP (27) the dephospho-R reassociates with the catalytic subunit to regenerate the inactive holoenzyme, more readily than the phosphorylated R (27). Following complete dissociation of protein kinase by CAMP, the dephosphorylated enzyme achieves 90% reassociation at a concentration of CAMP 10-fold higher than the phosphorylated form (28). Previously, we predicted that the holoenzyme would exist in vivo primarily as a phosphoenzyme for the following reasons: 1) The self-phosphorylation of protein kinase occurs by an intramolecular reaction at concentrations of ATP (Km, 9.7 μM) several orders of magnitude below the physiological levels of ATP (8 to 10 mM [29-31]), and 2) phosphorylated holoenzyme, unlike the dissociated phospho-R, is not a good substrate for endogenous cardiac muscle phosphoprotein phosphatase activity (26). The objective of the present study was to develop methods to resolve the phosphorylated and dephosphorylated forms of the soluble CAMP-binding protein in bovine cardiac muscle in order to evaluate the state of phosphorylation of the CAMP-dependent protein kinase in vivo.

EXPERIMENTAL PROCEDURES

Materials

Sources—[γ-32P]ATP (10 Ci/mmol) was purchased from Amer- sham/Searle. Cyclic [3H]AMP (50 Ci/mmol) and Omnifluor were from New England Nuclear. 8-Azidoadenosine 3’-5’-monophosphate and other nucleotides were obtained from Sigma. 8-Azidoadenosine 3’-5’-[32P]monophosphate (30 Ci/mmol to 45 Ci/mmol), originally a generous gift of Drs. U. Walter and T. Greengard of Yale University, was purified at BDH Chemicals Ltd., Poole, England. Amphophos from LKB Instruments, Uppsala, Sweden was purchased from Sigma. Acrylamide and N.N’.methylenebisacylamide and x-ray film (SB-5) were from Eastman Kodak. Standardizing screens (Quanta II and Quanta III) were purchased from E. I. du Pont. Sodium laurel sulfate (specially pure, Product No. 30176) was purchased from BDH Chemicals Ltd., Poole, England. Polyethylene phosphoester (A grade, Catalogue No. 524528) was was from Calbiochem. Purified phosphoprotein phosphatase was prepared according to Chou et al. and was generously provided by Mr. C. K. Chou (26). Staphylococcus protease was obtained from Miles Laboratories. Chymotrypsin, thermolysin, subtilisin, and papain were from Sigma.

Methods

Protein Kinase Assay—Protein kinase was assayed as described (18). The reaction mixture (200 μl) contained 20 mM potassium phosphate buffer (pH 7.1), 10 mM MgSO4, 50 μM [γ-32P]ATP (30 to 50 cpm/pmol), 10 mM dithiothreitol, 0.25 mM of proteose peptone, 0.5 mg of bovine serum albumin, and, where indicated, 20 μM CAMP. Incubations were for 2 to 3 min at 37°C. One unit of enzyme activity is defined as that amount catalyzing the transfer of 1 pmol of [γ-32P]ATP to protamine per min.

Cyclic AMP-binding Assay—Cyclic [3H]AMP binding was assayed according to Gilmour (34) except that 50 mM potassium phosphate buffer, pH 7.1, was substituted for 0.05 M sodium acetate buffer, pH 4.4. Reaction was measured by the method of Lowry et al. (35) using bovine serum albumin as standard.

Preparation of Protein Kinase—Protein kinase, purified from bovine cardiac muscle (16), had a specific activity of 800 units/mg of protein and bound 2 mol of cyclic [3H]AMP/mol of holoenzyme. Preparations of purified protein kinase vary in their ability to accept phosphate from ATP (28) and the enzyme from R-250 in 10.0 to 1.9 mol of phosphorus incorporated/mol of holoenzyme (25, 27). When assayed for self-phosphorylation (27), the particular preparation used in these studies incorporated 1.2 mol of [32P] from [γ-32P]ATP/mol of holoenzyme. It was estimated to be at least 90% pure by polyacrylamide gel electrophoresis in the absence (36) or presence of sodium dodecyl sulfate (37). Calculations of the molar concentrations of protein kinase used in these experiments are based upon molecular weights of 174,000 for the holoenzyme, 98,000 for the CAMP-binding protein dimer, and 38,000 for the catalytic subunit (16, 17).

To prepare [32P]-labeled protein kinase, 10 μg of purified holoenzyme were incubated for 60 min at 4°C in a final volume of 50 μl containing 20 mM Tris-HCl (pH 7.5), 5 μM 2-mercaptoethanol, 10 mM MgCl2, and 50 μM [γ-32P]ATP (1000 to 2300 cpm/pmol). The reaction was terminated by applying 45 μl of the mixture to a column (5 x 40 mm) containing Sephadex G-25 fine, equilibrated with 50 mM 2-(N morpholino)ethanesulfonic acid buffer, pH 6.2. The enzyme was eluted with the same buffer in 100-μl fractions collected in plastic tubes. Fractions containing protein were identified by the presence of a concave meniscus and assayed for [32P] by dissolving a 2-μl aliquot in a mixture containing 0.5 ml of distilled water and 4.5 ml of Triton X-100:toluene (30:70) and Omnifluor (4 g/liter). [32P] bound to cyclic AMP-binding protein was estimated by pipetting a duplicate aliquot into 1 ml of cold 50 mM potassium phosphate buffer, pH 7.1, containing 1 ng/ml of bovine serum albumin. This was then poured onto chilled, wet Millipore filters, washed twice with 10 ml of the same buffer, dried, and assayed for [32P]. The column efficiently separated the [32P]-labeled enzyme from [γ-32P]ATP: 98% of the total radioactivity in the fractions containing protein kinase was protein-bound. This close agreement also reflected the quantitative retention of protein kinase by the filters. [32P]-labeled protein kinase was recovered from the column in 85 to 90% yield and was ready to be used.

Incorporation of 8-azo-cyclic [32P]AMP into Protein Kinase—Incorporation of 8-azo-cyclic [32P]AMP into protein kinase was performed as described by Walter et al. (19). The reaction mixture (50 μl) containing 50 mM sodium Mes buffer, pH 6.2, the indicated amounts of protein for each experiment (30 to 100 μg) and 1 mM 8-azo-cyclic [32P]AMP (50 to 5000 pmol) was prepared in 100 μl of MES buffer at 37°C. Reaction mixtures were cooled to 4°C for 30 min and then irradiated for 10 min at 4°C with a Minilab ultraviolet lamp (254 nm) at a distance of 10 cm. Covalent binding of the affinity label was assayed by applying the reaction mixture to Millipore filters. Filters were washed twice with 10 μl of 50 mM potassium phosphate buffer (pH 7.1), dried, and assayed for [32P]. Assay blanks were incubated with 10

Isocyclic Focusing Staining Solution—Staining Solution I was prepared according to Malik and Berrie (32), slightly modified as follows: 100 ml of a 2% aqueous solution of Coomassie brilliant blue R-250 was added to 100 ml of 2 N sulfuric acid. The mixture was left standing at room temperature for at least 2 hr. The precipitate formed was removed by filtration and the green filtrate was neutralized with 20 ml of 10 N KOH. Trichloroacetic acid (21 g) was then added to the dark blue solution, turning it dark green.

Staining Solution II was made according to Righetti and Drysdale (33) and contained 57 ml of glacial acetic acid, 75 ml of ethanol, 150 ml of distilled water, 320 mg of cupric sulfate, and 150 mg of Coomassie Brilliant Blue R-250 in a final volume of 327 ml. It was routinely prepared the day before use and was filtered through Whatman No. 54 filter paper.
Table I
Incorporation of 8-azido-cyclic [32P]AMP and binding of cyclic [3H]AMP to soluble cyclic AMP-binding proteins of bovine cardiac muscle

| Experiment | Additions | Cyclic nucleotide bound | S_{amb} | pm
<table>
<thead>
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<tr>
<td>1</td>
<td>Cyclic [3H]AMP</td>
<td>7.72</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8-Azido-cyclic [32P]AMP</td>
<td>7.19</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8-Azido-cyclic [32P]AMP + hv + cAMP</td>
<td>7.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8-Azido-cyclic [32P]AMP + cAMP + hv</td>
<td>0.18</td>
<td></td>
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![Fig. 1. A, sodium dodecyl sulfate-electrophoresis of the phospho and dephospho forms of purified protein kinase. Sodium dodecyl sulfate sample buffer (20 μl) containing 5 μg of purified protein kinase was added to each well and subjected to electrophoresis at a constant current of 20 mA on sodium dodecyl sulfate-polyacrylamide gels. The gels were then stained for protein with Coomassie brilliant blue R-250. Channel 1, untreated protein kinase; Channel 2, [32P]-labeled protein kinase incubated for 10 min at 22°C with 50 μM cyclic AMP and 0.1 μg of bovine cardiac muscle phosphoprotein phosphatase; Channel 3, [32P]-labeled protein kinase. B, densitometric tracings and [32P] content of the electrophoretograms presented in A. The stained gels depicted in A (Channels 2 and 3) were scanned with a microdensitometer as indicated under "Methods." The content of [32P] was measured by cutting the gel into 1-mm slivers and assaying for [32P] by liquid scintillation spectrometry.](http://www.jbc.org/doi/10.1074/jbc.251.1.39)
a flat comb the gel strip was then pushed down until it made contact with the stacking gel. The gel strip was maintained in this position by leaving the flat comb on top of it until polymerization was complete (10 min). The upper chamber of the gel apparatus was then filled with 0.5 liter of running buffer containing 30 μl of a 0.1% aqueous solution of bromophenol blue. The flat comb was withdrawn and electrophoresis was performed at a constant current of 20 mA (approximate time, 5.5 h). The slab gel was removed from the chamber and stained for 20 min with 0.1% Coomassie brilliant blue R-250 in 50% trichloroacetic acid and destained in 10% ethanol dissolved in 10% acetic acid.

**Tissue Extracts**—Bovine hearts from freshly killed animals were obtained from a slaughterhouse and after a maximum interval of 2 h in ice were homogenized in a Waring Blendor at 4°C in 40 mm potassium phosphate, pH 7.2, containing 4 mM 2-mercaptoethanol, 1 mm paramethyisulfonyl fluoride in 4 mM EDTA and 50 mM sodium fluoride (4:1, v/wet weight of tissue). Extracts were centrifuged at 30,000 × g for 15 min, filtered through cheesecloth, and used as indicated.

**RESULTS**

Following photoactivation, 8-azido-cyclic [32P]AMP binds covalently and in stoichiometric amounts to cardiac muscle protein kinase in crude tissue extracts (see Table I). With purified kinase, 2 mol of 8-azido-cyclic [32P]AMP bind/mol of holoenzyme. Cyclic AMP was unable to displace 8-azido-cyclic [32P]AMP when added following ultraviolet irradiation (Table I). At saturating concentration of analog, binding to protein kinase in purified preparations or in unpurified tissue extracts could be assayed by determining either the radioactivity retained on Millipore filters or in the individual cAMP-binding protein resolved after electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (19).

To analyze the cAMP-binding subunit of protein kinase in tissue extracts after labeling with 8-azido-cyclic [32P]AMP we studied the behavior of such proteins on the high resolution polyacrylamide gel system described by Laemmli (38). As shown in Fig. 1A (Gel 1), the purified, largely nonphosphorylated (22) cyclic AMP-binding subunit of bovine cardiac muscle protein kinase migrated as a doublet with apparent molecular weights of 54,000 (R) and 52,000 (R'), respectively (Fig. 1B). When the phosphorylated enzyme was examined, the cyclic AMP-binding subunit exhibited an apparent molecular weight of 56,000 (R-P, Fig. 1A, Gel 3) (10, 16). The Mr = 52,000 component did not change its mobility detectably. Treatment of the 32P-labeled protein with purified cardiac muscle phosphoprotein phosphatase (26) or with potato acid phosphatase (45) under conditions in which 90 to 95% of the 32P is removed restored the electrophoretic pattern observed with the unphosphorylated enzyme (Fig. 1, A (Gel 2) and B). The doublet was also observed in electrophoretograms of 8-azido-cyclic [32P]AMP-labeled protein kinase in crude extracts of bovine cardiac muscle (Fig. 2A), indicating that this finding was not an artifact of purified protein kinase. Since the major component labeled with the photoaffinity label under these conditions had an apparent molecular weight of 56,000 (Fig. 2A) and co-migrated with the pure phosphoenzyme (detected by protein stain, affinity labeling or 32P incorporation from [γ-32P]ATP), a significant portion of the cyclic AMP-binding

![Fig. 2. Incorporation of 8-azido-cyclic [32P]AMP into cytosolic cAMP-binding proteins of bovine cardiac muscle. A, sodium dodecyl sulfate buffer (50 μl) containing 100 μg of protein from bovine cardiac muscle cytosol and 0.75 pmol of 8-azido-cyclic [32P]AMP (9920 cpm/pmol) incorporated under standard conditions (see “Methods”) was applied to each sample well and subjected to electrophoresis as indicated in Fig. 1A. The gel was then stained for protein and dried and the individual channels were sliced into 1-mm slices and assayed for 32P. Top gel, Electrophoretogram of the purified bovine cardiac muscle kinase run in a parallel channel of the same slab gel and presented for comparison; middle panel, 32P content corresponding to protein pattern of bovine cardiac cytosol shown in bottom gel. B, bovine cardiac muscle cytosol (100 μg) was incubated with 1 μM [γ-32P]ATP (33,147 cpm/pmol) and 10 mM MgCl2 in a final volume of 10 μl of 50 mM potassium phosphate buffer, pH 7.1. After 10 min incubation at 4°C, the reaction was terminated by the addition of 30 μl of sodium dodecyl sulfate sample buffer, boiled for 2 min, and subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide slab gels. The slab gel was then stained and dried. Individual channels were sliced into 1-mm slices and assayed for 32P. The top gel is the same as that in A. The middle panel depicts the 32P content corresponding to the protein pattern of bovine cardiac cytosol shown in the bottom gel.
protein in the extract was in its phosphorylated form. This suggestion was strengthened by the incorporation of \(^3\)P from \([\gamma-^{32}\text{P}]\text{ATP}\) into the \(M_r = 56,000\) and 52,000 components (Fig. 2B), after incubation of bovine cardiac muscle cytosol with \([\gamma-^{32}\text{P}]\text{ATP}\) under conditions favoring self-phosphorylation: low concentrations of ATP (5 \(\mu\text{M}\)), low temperature (4°C), and no cyclic AMP (11, 25).

The remarkable specificity of the incorporation of \(^3\)P from \([\gamma-^{32}\text{P}]\text{ATP}\) into the electrophoretic doublet of the cyclic AMP-binding protein of bovine cardiac muscle cytosol probably reflects the intramolecular nature of the self-phosphorylation reaction (25). When similar experiments were conducted in the presence of cyclic AMP, there was considerably less incorporation of \(^3\)P into the cyclic AMP-binding protein and concomitant appearance of many other \(^3\)P-labeled proteins.

**Dephosphorylation of R-P in Bovine Cardiac Muscle Cytosol**—If the incorporation of 8-azido-cyclic \([^{32}\text{P}]\text{AMP}\) into a protein band of apparent molecular weight 56,000 (Fig. 2A) is due to the presence of a phosphorylated cyclic AMP-binding protein in bovine cardiac muscle cytosol then subsequent treatment of such extracts with a phosphatase should regenerate the 54,000-dalton protein band observed for the purified dephospho-cyclic AMP-binding protein (Fig. 1B). Fig. 3 shows that incubation of 8-azido-cyclic \([^{32}\text{P}]\text{AMP}\)-labeled cytosolic extract with phosphatase regenerated the 54,000-dalton form of the cyclic AMP-binding protein. This change in electrophoretic mobility is the result of dephosphorylation of the cyclic AMP-binding protein rather than another type of modification (i.e. limited proteolysis) since (a) a control incubation in the presence of phosphatase and 50 mM potassium orthophosphate (a potent inhibitor of the phosphatase (45)) did not show the shift in electrophoretic mobility (Fig. 3, Channel 2), (b) a control incubation in the presence of phosphatase and 50 mM potassium orthophosphate (a potent inhibitor of the phosphatase (45)) did not show the shift in electrophoretic mobility (Fig. 3, Channel 2).

**Resolution of \(R-P\) in Cyclic AMP-binding Proteins**—When the 8-azido-cyclic \([^{32}\text{P}]\text{AMP}\)-labeled phospho- or dephospho-cyclic AMP-binding proteins purified from bovine cardiac muscle were subjected to isoelectric focusing from pH 5.5 to pH 7 on polyacrylamide slab gels containing 8 M urea, the two forms of the cyclic AMP-binding protein were resolved (Fig. 6). The phosphorylated subunit migrated as a doublet with apparent pl values of 5.34 to 5.35 and a minor component with pl 5.30. After treatment of the phosphorylated binding proteins with phosphatase, the doublet shifted to pl values of 5.34 to 5.35, consistent with the observation that the phosphorylated form of the cyclic AMP-binding protein migrates at a higher pl than the dephosphorylated form (46). This suggests that the site for phosphorylation is present on both forms of the cyclic AMP-binding protein. Further, the phosphorylation of the cyclic AMP-binding protein is reversible, as evidenced by the reversion of the doublet to a single form upon incubation with cyclic AMP (47). The phosphorylation of the cyclic AMP-binding protein is also reversible upon incubation with cyclic AMP (47). The phosphorylation of the cyclic AMP-binding protein is also reversible upon incubation with cyclic AMP (47). The phosphorylation of the cyclic AMP-binding protein is also reversible upon incubation with cyclic AMP (47). The phosphorylation of the cyclic AMP-binding protein is also reversible upon incubation with cyclic AMP (47).
Resolution of cAMP-binding Proteins

Fig. 4. Peptide maps of the 56,000- and 52,000-dalton cAMP-binding proteins labeled with $[^{32}P]$ from either 8-azido-cyclic $[^{32}P]$AMP (A) or $[^{32}P]$ATP (B). A, peptide maps of the 56,000- and 52,000-dalton cAMP-binding proteins labeled with 8-azido-cyclic $[^{32}P]$AMP. Fifty micrograms of purified protein kinase from bovine cardiac muscle were incubated for 20 min at 4°C in a final volume of 50 μl of 50 mM Mes buffer, pH 7.0, containing 10 mM MgCl$_2$, 0.1 mM ATP, and 10 μM 8-azido-cyclic $[^{32}P]$AMP (10,000 cpm/pmol). After 10 min of UV irradiation, 100 μl of 0.065 M Tris-HCl buffer, pH 6.8, containing 2.3% sodium dodecyl sulfate and 5 mM 2-mercaptoethanol were added. Twenty microliters of this mixture were then applied to gels as indicated under “Methods.” The slab gel was then briefly stained and destained and the individual protein bands corresponding to the proteins of molecular weights 56,000 and 52,000 were excised and subjected to peptide mapping on a second sodium dodecyl sulfate-polyacrylamide (15%) slab gel following partial proteolysis (20 min, 23°C) with Staphylococcus aureus protease or $\alpha$-chymotrypsin. After electrophoresis, the gels were stained for protein, dried, and autoradiographed. Channel 1, autoradiograph of the untreated 56,000-dalton protein; Channel 2, the 56,000-dalton protein subjected to electrophoresis with 25 ng of S. aureus proteinase; Channel 3, the 52,000-dalton protein subjected to electrophoresis with 25 ng of S. aureus proteinase; Channel 4, the 56,000-dalton protein subjected to electrophoresis with 25 ng of α-chymotrypsin; Channel 5, the 52,000-dalton protein subjected to electrophoresis with 25 ng of α-chymotrypsin. B, peptide maps of the 56,000- and 52,000-dalton cAMP-binding proteins labeled with $^{35}S$ from either 8-azido-cyclic $[^{35}S]$AMP or $[^{35}S]$ATP. Fifty micrograms of purified protein kinase from bovine cardiac muscle were incubated with 10 μM 8-azido-cyclic $[^{35}S]$AMP as indicated in A or with 25 μM $[^{35}S]$ATP (11,000 cpm/pmol) in a final volume of 50 μl containing 50 mM Mes buffer (pH 7.0), 10 mM MgCl$_2$. After a 30-min incubation at 4°C the reactions were terminated by electrophoresis as indicated in A. Channels 1 to 4 were treated with 12 ng of S. aureus protease; Channels 5 to 8 were treated with 25 ng of this enzyme. The autoradiograms are presented. Channel 1, the 56,000-dalton protein labeled with 8-azido-cyclic $[^{35}S]$AMP; Channel 2, the 52,000-dalton protein labeled with 8-azido-cyclic $[^{35}S]$AMP; Channel 3, the 56,000-dalton protein labeled with $^{35}S$ from $[^{35}S]$ATP; Channel 4, the 52,000-dalton protein labeled with $^{35}S$ from $[^{35}S]$ATP; Channel 5, the 56,000-dalton protein 8-azido-cyclic $[^{35}S]$AMP; Channel 6, the 52,000-dalton protein 8-azido-cyclic $[^{35}S]$AMP; Channel 7, the 56,000-dalton protein labeled with $^{35}S$ from $[^{35}S]$ATP; Channel 8, the 52,000-dalton protein labeled with $^{35}S$ from $[^{35}S]$ATP. Similar results were obtained with subtilisin, thermolysin, and papain.
Resolution of cAMP-binding Proteins

To provide further evidence that the 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP-labeled protein bands seen in Fig. 6 were indeed the phosphorylated and dephosphorylated forms of the cAMP-binding component of protein kinase, an isoelectric focusing gel of the \( ^{32}P \)-phosphorylated binding protein before and after treatment with the phosphatase was performed. As shown on Fig. 8A, the \( ^{32}P \)-phosphorylated and the phosphatase-treated binding proteins showed an identical protein pattern when compared with their counterparts labeled with 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP (Fig. 6). For reasons that are not clear, when the \( ^{32}P \)-labeled binding protein was treated with phosphatase (Fig. 8B, Channel 2), a minor component of the putative dephosphorylated protein retained some radioactive phosphate. This radioactivity was also detectable in the \( ^{32}P \)-phosphorylated preparation (see Fig. 8B, Channel 1); it was not altered by extensive incubation with either \( [\gamma]^{32}P \)ATP-Mg\(^{2+}\) or phosphatase. The peptide map derived from limited proteolysis of this \( ^{32}P \)-phosphorylated band with \textit{Staphylococcus} protease was the same as that derived from the major phosphoprotein as were the 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP-labeled peptides derived from both proteins (data not shown).

**Extent of Phosphorylation of Cyclic AMP-binding Protein in Tissue Extracts**—Since it is possible to resolve the purified 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP-labeled phospho- and dephospho-cyclic AMP-binding protein components of protein kinase by isoelectric focusing with or without subsequent electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, we tested the applicability of this method to the 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP-labeled cyclic AMP-binding proteins in bovine cardiac muscle cytosol. As shown in Fig. 9A the pattern of labeling, \textit{i.e.} the cardiac muscle extract (Channel 1), was identical to the 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP-labeled purified phospho-binding protein (Channel 2) with the major bands having \( pI \) values of 5.30 and 5.34 to 5.35. This was different from the pattern observed for the purified enzyme following treatment with phosphatase (Channel 3). The appearance of both 56,000- and 52,000-dalton components in each of these bands on the

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**Fig. 6.** Isoelectric focusing in 8 M urea of purified phospho- and dephosphoprotein kinase labeled with 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP. Purified bovine heart protein kinase (122 µg) was incubated for 1 h at 4°C with 0.1 mM ATP and 10 mM MgCl\(_2\) in a volume of 50 µl of 50 mM potassium phosphate buffer, pH 7.1, containing 4 mM P-mercaptoethanol. 8-Azido-cyclic \( \left[ ^{32}P \right] \) AMP (6,000 cpm/pmol) was then added to a final concentration of 20 nM. Following incubation at 4°C for 15 min, the mixture was irradiated with UV light as described under "Methods." The mixture was then applied to a Sephadex G-25 (fine) column (5 x 40 mm) under the same conditions described under "Methods" for the preparation of \( \left[ ^{32}P \right] \) protein kinase. As indicated below, approximately 10 µg of phosphorylated 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP-labeled protein kinase was treated with 0.3 µg of potato acid phosphatase for 20 min at 22°C in the presence of 50 mM Mes buffer, pH 6.2, in a volume of 12 µl. The reaction was terminated by adding 7.6 µg of urea/10 µl of reaction mixture and loaded onto the sample wells of the isoelectric focusing polyacrylamide slab gel. After focusing, pH was measured along an empty channel parallel to one of the samples and the gel was stained for protein and autoradiographed. The photograph depicts an autoradiogram. Channel 1, 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP-labeled phospho-cAMP-binding protein; Channel 2, 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP-labeled phospho-cAMP binding protein treated with phosphatase.

**A**

(--) \( \leftarrow \) IEF \( \rightarrow \) (+)

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**B**

(--) \( \leftarrow \) IEF \( \rightarrow \) (+)

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**Fig. 7.** Two-dimensional electrophoresis of the purified phospho- and dephosphoprotein kinases-labeled with 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP. A, an identical sample to the one described in Fig. 6 (Channel 1) was subjected to isoelectrofocusing (IEF) on the same polyacrylamide slab gel and then subjected to electrophoresis in a second dimension on a sodium dodecyl sulfate (SDS)-polyacrylamide slab gel. The autoradiograph of the first dimension showed in Fig. 6 (Channel 1) is also presented for comparison. B, the same as described in A except that phosphatase-treated kinase labeled with 8-azido-cyclic \( \left[ ^{32}P \right] \) AMP was used (see Fig. 6, Channel 2).
second dimensional analysis indicated that both proteins were phosphorylated (Fig. 9B). This confirms the evidence derived from one-dimensional sodium dodecyl sulfate electrophoresis that the cAMP-binding component of protein kinase in extracts of bovine cardiac muscle is largely in the phosphorylated form.

**DISCUSSION**

A considerable amount is known about the biochemical properties of the purified type II cyclic AMP-dependent protein kinase from bovine cardiac muscle (23). This enzyme catalyzes the phosphorylation of its own cyclic AMP-binding protein component by an intramolecular reaction (25). Studies performed in vitro indicate (a) that the dephosphorylated cyclic AMP-binding protein regenerates the inactive protein kinase holoenzyme more readily than the phosphorylated protein (27, 28) and (b) dephosphorylation occurs principally if not exclusively when the phosphorylated binding protein is dissociated from the holoenzyme (26). Evaluation of the biological significance of the covalent modification of the cyclic AMP-binding protein will depend upon documentation that both phospho and dephospho forms occur in vivo and that these forms are functionally distinct under physiological conditions. To this end we have begun to develop methods for evaluating the phosphorylation state of cyclic AMP-binding proteins in fresh tissue extracts using the photoaffinity analog of cAMP, 8-azido-cyclic [32P]AMP which reacts specifically and in stoichiometric amounts with cAMP-binding components of protein kinases in tissue extracts (19, 21, 24).

The major phosphorylated form of the cAMP-binding subunit of purified bovine cardiac muscle protein kinase showed a different apparent molecular weight (56,000) on sodium dodecyl sulfate electrophoresis in Tris/glycine buffer than the unphosphorylated form (M, = 54,000). This change is attributed to a decrease in the electrophoretic mobility of the phosphorylated cAMP-binding protein since subsequent dephosphorylation with a phosphoprotein phosphatase restored the electrophoretic pattern observed in the untreated, predominantly unphosphorylated enzyme. Hofmann et al. (10) reported a similar shift in apparent molecular weight from 55,000 to 59,000 upon phosphorylation of the RII of bovine cardiac muscle. This change was apparent when electrophoresis was performed in Tris/glycine buffer but not in phosphate buffer.
of both phospho- and dephospho-cAMP-binding proteins on isoelectric focusing may be due to enzyme heterogeneity which becomes detectable with the narrow pH gradient used in these experiments. The two-dimensional electrophoreograms of the cAMP-binding proteins present in soluble extracts of bovine cardiac muscle demonstrated that this protein is predominantly in the phosphorylated form, in accordance with the results obtained with one-dimensional electrophoresis. It is interesting in this regard that Steinberg et al. (49) using affinity chromatography and two-dimensional electrophoresis (39) have reported that the type I protein kinase of S4 lymphoma cells is also phosphorylated in vivo.

The results of our analysis are schematically depicted in Fig. 10 and can be summarized as follows: 1) phospho and dephospho forms of RII can be resolved in crude tissue extracts by labeling with 8-azido-cyclic [32P]AMP followed by isoelectric focusing on polyacrylamide slab gels with or without a second dimension electrophoretic analysis; 2) in tissue extracts of bovine heart, protein kinase II is present predominantly in the phosphorylated form; 3) peptide maps of types I and II cyclic nucleotide binding proteins are clearly different from each other whereas maps of the 52,000- and 56,000-dalton cAMP-binding proteins are homologous.

REFERENCES

The 52,000-dalton cAMP-binding protein is also seen in extracts of other bovine and murine tissues (Rangel-Aldao, R., and Rosen, O. M., unpublished data).
252, 2855–2859
Resolution of the phosphorylated and dephosphorylated cAMP-binding proteins of bovine cardiac muscle by affinity labeling and two-dimensional electrophoresis.

Rafael Rangel-Aldao, James William Kupiec, and Ora Mendelsohn Rosen

Page 2507, Fig. 10

Due to a printer's error, Fig. 10 was printed incorrectly. The correct figure is printed below.

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