Cyclic GMP-dependent protein kinase in extracts of bovine aortic tissue eluted from DEAE-cellulose ion-exchange resins as two distinct peaks of activity. This elution pattern was preserved when the peaks were combined, precipitated with ammonium sulfate, dialyzed, and rechromatographed. Proteolysis did not appear to account for the two forms of kinase because (i) aging of the extract did not cause interconversion of the two forms, and (ii) both forms retained GMP sensitivity unlike the proteolytically formed monomer. In addition, treatment with saturating concentrations of GMP (10 μM) did not cause interconversion of the two forms. The first peak of GMP-dependent protein kinase eluting from DEAE-cellulose (form 1) had a slightly greater mobility on gradient sodium dodecyl sulfate-polyacrylamide gels than the second peak (form 2). On native, non-denaturing gradient polyacrylamide gels, however, form 2 displayed the greater electrophoretic mobility. Furthermore, form 1, when bound to CAMP-agarose, appeared to exchange more rapidly with GMP than form 2 when subjected to affinity chromatography. Peptide maps generated from the two forms by protease treatment were very similar, although trypsin produced a unique peptide in form 1 and Streptomyces griseus protease gave rise to unique peptides in forms 1 and 2. Phosphorylation did not appear to account for the physical differences because both enzymes could be phosphorylated to similar extents and dephosphorylation by alkaline phosphatase did not result in the conversion of one form to the other. These results suggest that either differences in primary structure or post-translational modification, other than phosphorylation, are responsible for the presence of two forms of GMP-dependent protein kinase in aortic tissue.

Furthermore, it is usually found at lower concentrations than the cAMP-dependent enzyme. Nevertheless, the GMP-dependent protein kinase has been purified and characterized from lung (7, 8) and heart (9), and the sequence of the lung enzyme has been determined (10).

The elucidation of the role of GMP-dependent protein kinase has not been successful. In smooth muscle, and particularly vascular smooth muscle, GMP has been suggested to mediate the relaxation of nitric oxide containing vasodilators (e.g. nitroprusside and nitroglycerine), atrial natriuretic factor, endothelial-derived relaxing factor, and certain wavelengths of ultraviolet radiation (see Ref. 11 for a review). The major receptor and only measurable binding protein for GMP in vascular smooth muscle is the GMP-dependent protein kinase. Activation of the GMP-dependent protein kinase in response to atrial natriuretic factor, endothelial-derived relaxing factor, and nitroprusside has been described in rat aorta and analogs of GMP have been shown to cause endogenous protein phosphorylation (12). Recently, Francis et al. (13) have shown that cyclic nucleotide analogs that relax smooth muscle have high affinity for cGMP-dependent protein kinase but have little activity toward the cAMP-dependent protein kinase.

During the course of our investigations on the role of GMP-dependent protein kinase in vascular tissue, we observed that the GMP-dependent protein kinase from bovine aorta eluted as two peaks of activity from DEAE-cellulose columns. Wolfe et al. (14) have also observed this phenomenon for GMP-dependent protein kinase from extracts of several tissues including aorta. They attributed this chromatographic behavior to the binding of GMP to some molecules of GMP-dependent protein kinase, thereby altering its elution from DEAE-cellulose. A similar effect was observed by these investigators for the cAMP-dependent protein kinase (15). In this report, we present evidence for two forms of GMP-dependent protein kinase from aorta that are most likely due to different structural properties of the two forms of the kinase. These differences appear to be related to either covalent modification of the enzyme or, more likely, to differences in the primary sequences of the enzymes.

**EXPERIMENTAL PROCEDURES**

Materials—Bovine aortas were either obtained from a local abattoir or were purchased from Pel-Freez, Rogers, AR. DEAE-cellulose (DE52) was from Whatman, and adenosine 3':5'-monophosphate (C-8-aminohexylaminom)-agarose was purchased from Sigma. γ-32P-ATP and 72I-Protein A were from Du Pont-New England Nuclear. Histone H2b was purchased from Worthington. Polyacrylamide gel electrophoresis supplies and equipment were purchased from Pharmacia LKB Biotechnology Inc. (Phast System). Trypsin (type I from bovine pancreas), protease (type VI from Streptomyces griseus), and protease V8 (type XVII from Staphylococcus aureus) were from Sigma; Pronase and S-chymotrypsin were from Miles Laboratories Ltd., South Africa. Alkaline phosphatase (type I and II) was purchased from Boehringer Mannheim. The enzyme was first identified in arthropod tissues (4) and was subsequently shown to be present in several mammalian tissues, although, unlike the cAMP-dependent protein kinase, the GMP-dependent enzyme does not appear to be ubiquitous (5, 6).

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‡ Established Investigator of the American Heart Association.

§ Predoctoral Fellow for the Pharmaceutical Manufacturer's Association.
from calf intestine) was from Sigma.

Purification of Bovine Aortic cGMP-dependent Protein Kinase—

500 to 1000 g of frozen tissue (stored at −70 °C) was ground to a fine powder using a meat grinder cooled to the temperature of liquid N₂. The powder could either be stored at −70 °C or could be used immediately. Powdered tissue was suspended in 2 volumes of 20 mM potassium phosphate, pH 6.8, 2 mM EDTA, 15 mM 2-mercaptoethanol (PEM buffer) at 4 °C until thawed and homogenized in a 4-liter capacity Waring blender at low, medium, and high speed for 30 s each. The homogenate was centrifuged at 10,000 × g in a Sorvall GS-3 rotor for 30 min, and the supernatant fraction was filtered into a 2-liter flask through glass wool. The extract was diluted with an equal volume of buffer and applied to a DEAE-cellulose column (5 × 35 cm) equilibrated in PEM. After washing the column with 4 liters of PEM containing 50 mM NaCl, the column was eluted with a linear gradient of NaCl from 50 to 350 mM (total volume 4 liters). Fractions (approximately 20 ml each) were collected and assayed for cGMP-dependent protein kinase activity, protein, and conductivity. Active fractions were pooled and treated with solid ammonium sulfate (40 g/100 ml) for 1 h with occasional stirring. The precipitate was collected by centrifugation (10,000 × g for 30 min) and suspended in a small volume of PEM. The suspended protein was dialyzed against 20 volumes of PEM overnight (>15 h), centrifuged to remove insoluble material, and applied to a 2-ml cAMP-agarose affinity column (50 ml/h). This step was accomplished in less than 3 h. Once the protein had been applied, the column was washed with 5 volumes of PEM followed by 10 volumes of PEM containing 1 M NaCl. This latter fraction was collected separately, dialyzed against PEM to remove salt, and concentrated on a 0.5-ml DEAE-cellulose column by elution with 1.5 ml of 0.3 M NaCl. This fraction will be referred to as NaCl-cGMP kinase. The majority of cGMP-dependent protein kinase, which was still bound to the cAMP affinity column, was eluted as follows: 2 volumes of PEM (to remove NaCl) was added to the column followed by 0.5 ml of 0.1 mM cGMP in PEM. This latter buffer was allowed to flow into the column. The column was then removed from the 4 °C chamber and allowed to set at room temperature for 10 min. The column was then eluted with PEM + 0.1 mM cGMP at room temperature and fractions of 2 ml were collected on ice. Twenty affinity column fractions were assayed for kinase activity and protein, and aliquots were subjected to SDS-polyacrylamide electrophoresis on 10–15% gradient gels. In some instances, fractions were subjected to electrophoresis on either 8–25 or 10–15% polyacrylamide gels at 4 °C under nondenaturing (i.e. native) conditions.

Peptide Mapping of cGMP-dependent Protein Kinase—Peptide maps of cGMP-dependent protein kinase were performed according to the Cleveland procedure (16), as modified for use with the Phast System. Twenty µl of cGMP-dependent protein kinase (0.12 mg/ml) were incubated with 2 µl of 12 µg/ml specific protease for various periods of time ranging from 1 to 12 h at 37 °C. The reactions were terminated by the addition of 2 µl of an electrophoresis stop mix (3.7 M mercaptoethanol + 10% SDS) and heated at 85 °C for 3 min. Samples (0.3–1 µl) were applied to 8–25% polyacrylamide gels and subjected to electrophoresis for 65 V/h. Gels were removed from the bonding material using a cheese slicer, and peptides were localized by silver staining using the procedure of Wray et al. (17).

Other Procedures—Autophosphorylation of the cGMP-dependent protein kinase was performed in a total volume of 0.1 ml in a mixture containing 50 mM Tris-Cl, pH 7.5, 0.05 mM ATP (1000 cpm/pmol), 5 mM MgCl₂, 0.1 mM EGTA, and cyclic nucleotides at different concentrations. Reactions were conducted at 30 °C at various times and were terminated by the addition of 10 µl of electrophoresis stop mix as described above. 10–15% SDS-PAGE was performed using the Phast System to 60 V/h, or 8% SDS-PAGE was performed according to the method of Laemmli (18). Phosphorylated protein was visualized by autoradiography. Cyclic GMP-dependent protein kinase activity was determined as described previously (6). Protein was determined by the method of Bradford (19), and cGMP was determined by radioimmunoassay (20).

RESULTS

Chromatographic Behavior of Bovine Aortic cGMP-dependent Protein Kinase—Cyclic GMP-dependent protein kinase from bovine aorta eluted as two distinct peaks from DEAE-cellulose columns (Fig. 1). Form 1 eluted at approximately 20 mmho while form 2 eluted at approximately 26 mmho. In addition, a shoulder of cGMP-dependent protein kinase was usually observed after the peak of both forms of the enzyme, especially form 2. This might represent those molecules of cGMP-dependent protein kinase that contain bound cGMP and thus elute at the slightly higher ionic strength compared with the cGMP-free enzyme (14). Interconversion between the two forms of cGMP-dependent protein kinase was not observed under the following conditions: (i) the addition of cGMP (10⁻⁴ M) to cGMP-dependent protein kinase form 1 eluted by 1 M NaCl from cAMP-agarose (NaCl-cGMP kinase) prior to rechromatography on DEAE-cellulose (Fig. 2); (ii) aging of the extract (6–24 h at 4 °C); (iii) the use of fresh aorta as opposed to frozen tissue; (iv) phosphorylation of the purified enzymes in the presence of Mg²⁺ and ATP; (v) treatment of form 2 with 1:10 (w/w) of chymotrypsin. These results suggest that neither form of the enzyme is derived from the other due to tissue processing, purification procedures, proteolysis, or other modifications. In addition, the ratio of cGMP-dependent protein kinase forms was fairly consistently 3:2, form 1:form 2, following DEAE-cellulose. In order to examine further the relationship between the elution profiles of cGMP-dependent protein kinase, both forms were pooled together and treated with ammonium sulfate (40 g/100 ml) to precipitate protein. The precipitate was collected by centrifugation, dialyzed for 24 h against 100 volumes of PEM buffer which was changed three times, and rechromatographed on DEAE-cellulose. Again, two forms of cGMP-dependent protein kinase eluted from DEAE-cellulose in similar positions to those observed when the crude extract was chromatographed (not shown). These results suggested that the chromatographic behavior of cGMP-dependent protein kinase from bovine aorta was not influenced by loosely bound diffusible molecules that might influence retardation of some or all of the cGMP-dependent protein kinase when eluted from DEAE-cellulose with NaCl.

Purification and Electrophoretic Properties of the Two Forms of cGMP-dependent Protein Kinase from Bovine Aorta—Table I illustrates the purification data obtained from approximately 600 g of frozen bovine aorta. The specific

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**Fig. 1.** DEAE-cellulose chromatography of bovine aortic extract. Approximately 750 g of frozen aortic tissue was chromatographed as described under "Experimental Procedures." One unit is the amount of enzyme required to transfer 1 pmol of phosphate from ATP to histone H2b in 1 min.
activity of each enzyme was similar (approximately \(3.0 \times 10^5\) units/mg of protein). Each enzyme probably required a 1,500-fold purification from the crude extract for apparent homogeneity, although this was difficult to assess because of inaccuracies in determining the specific activity of cGMP-dependent protein kinase in the crude extract. Only one major band was observed migrating at a position corresponding to \(M_\text{r}\) 79,000, using nongradient SDS-PAGE (not shown). However, when cGMP-dependent protein kinase was subjected to SDS-PAGE on 10–15% gradient gels, clear mobility differences were observed between the two forms of cGMP-dependent protein kinase. In Fig. 3, cGMP-dependent protein kinase form 1 was separated from form 2 by DEAE-cellulose ion-exchange chromatography and was applied to a cAMP affinity column. Form 2, however, was only partially separated from form 1 on DEAE-cellulose. This pool was applied to a separate cAMP affinity column. Each column was eluted with 0.1 mM cGMP and fractions of 2 ml were collected. Aliquots of each fraction were subjected to gradient SDS-PAGE and protein was detected by silver staining. As shown in the figure, most of the cGMP-dependent protein kinase form 1 was eluted in the first two fractions from the affinity column, and a single protein band with an \(M_\text{r}\) of 75,000 was observed. However, cGMP-dependent protein kinase form 2 was heavily contaminated with form 1, and two bands were eluted. The first fraction contained mainly the band that corresponded to form 1 with respect to mobility, while the second fraction had a mixture of form 1 and a lower mobility substance which was identified as form 2. Continued elution of the column with 0.1 mM cGMP yielded mostly form 2 (fractions 3 and 4). In lanes 9 and 10, the pooled form 1 and form 2 are compared. These data illustrate two points: first, form 1 and form 2 have different electrophoretic mobilities on SDS-PAGE suggesting different sizes, shapes, or SDS binding capacities; second, form 1 elutes more rapidly from cAMP-agarose than form 2, suggesting that either the rate of exchange between immobilized cAMP and cGMP is slower for form 2 or that form 2 interacts more readily with the cAMP-agarose matrix than does form 1. In either case, the results indicate that some structural differences exist between forms 1 and 2.

Electrophoretic properties of cGMP-dependent protein kinase on 8–25% nondenaturing (native) gels are shown in Fig. 4. On native gels, the more rapidly eluting form 1 had a lower mobility than form 2. This was not surprising in that form 2 is apparently more negatively charged than form 1 based on its chromatographic behavior on DEAE-cellulose. Because the gel used for this experiment contained an 8–25% gradient of acrylamide solution, proteins were separated according to size as well as charge. Thus, these results indicated that any molecular weight differences between forms 1 and 2 were trivial, since the mobilities were quite similar. This seemed to rule out the possibility that form 1 was a monomeric form of cGMP-dependent protein kinase.

In order to examine further whether or not form 1 was the proteolytically derived active monomeric form of cGMP-dependent protein kinase, the extent of activation of both forms of enzyme by cGMP was examined. Because small amounts of cGMP-dependent protein kinase (0.1% of total) can be eluted from cAMP-agarose columns by 1 M NaCl in the absence of cyclic nucleotide, the extent of activation of cyclic nucleotide-free kinase by cGMP can be determined. Both forms 1 and 2 NaCl-cGMP kinase activities were stimulated by cGMP between 7.5- and 10-fold (activity ratio of form 1 = 0.10; activity ratio of form 2 = 0.15). Cyclic AMP was approximately 10-fold less active in stimulating both forms of enzyme using histone as the substrate. These results suggested

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**Table 1**

<table>
<thead>
<tr>
<th>Purification of cGMP-dependent protein kinase from bovine aorta</th>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Units</th>
<th>Units/mg</th>
<th>Fold</th>
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<td>2.019</td>
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<td></td>
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<td>2.6</td>
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<td></td>
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<td>0.57</td>
<td>0.3</td>
<td>526,316*</td>
<td>144</td>
</tr>
</tbody>
</table>

* cGMP-dependent protein kinase from form 1 was pooled in a manner to prevent contamination with form 2. Thus, the amount is an underestimation of the total amount of cGMP-dependent protein kinase present as form 1.

The total units of protein kinase activity in the crude extract equaled \(20.6 \times 10^3\), which exceeded the total amounts of form 1 and form 2 added together by about 5-fold. The reasons for this are not well understood but could be due to the presence of other protein kinases as well as the presence of activators and substrates present in the extract itself.

* Some loss in activity under the elution conditions had probably occurred as a result of low amounts of protein on the column, proteolysis, or other factors. These problems may account for the low specific activity and yield compared with those seen for the bovine lung enzyme (8).

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**Fig. 2. Rechromatography of forms 1 and 2 cGMP-dependent protein kinase on DEAE-cellulose.** A, an aliquot of form 1 cGMP-dependent protein kinase eluted from the cAMP-agarose affinity column was dialyzed and applied to a DEAE-cellulose column (0.9 × 4 cm) and eluted with a linear gradient of NaCl (0–300 mM) in PEM buffer. Fractions were collected (3 ml) and assayed for cGMP-dependent protein kinase on DEAE-cellulose. Because the elution conditions (see "Experimental Procedures") were designed to determine the specificity of the enzyme, the cGMP-dependent protein kinase was incubated with 10 μM cGMP for 30 min prior to rechromatography on DEAE-cellulose as described in A.
Cyclic GMP-dependent Protein Kinase

that both forms 1 and 2 were dimeric forms of the enzyme and that neither had sufficient cGMP bound to it to produce activation of the enzyme.

Peptide Maps of GMP-dependent Protein Kinase—The chromatographic and electrophoretic properties of the two forms of cGMP-dependent protein kinase suggested that the enzymes differed structurally, perhaps in primary sequence. Therefore, we examined the electrophoretic profiles of peptides derived from forms 1 and 2 using the Cleveland mapping procedure as adapted by us for use with the Phast System. Cyclic GMP-dependent protein kinases forms 1 and 2 were subjected to proteolysis using five proteases (trypsin, chymotrypsin, protease from S. griseus, Pronase, and S. aureus V8). As shown in Fig. 5, digestion with the various proteases for 12 h produced peptide maps of the two enzymes which were very similar. Two differences were observed: trypsin produced a peptide in form 1 that appeared to be completely lacking in form 2, and protease from S. griseus produced a peptide in form 2 that appeared to be absent in form 1 (arrows in Fig. 5). Also, form 2 appeared to be somewhat less susceptible to proteolysis than form 1, as seen with S. aureus protease especially when digestion was performed for limited periods of time. It was necessary, then, to determine that complete proteolysis of cGMP-dependent protein kinase did indeed occur. In order to examine the extent of proteolysis, a time course was performed using S. griseus protease on both forms 1 and 2. As shown in Fig. 6, no further digestion of kinase occurred after 3 or 4 h using either kinase when protease was added after each hour. Similar results were found when trypsin was used instead of S. griseus protease (not shown).

Autophosphorylation of GMP-dependent Protein Kinase—The data described above could be explained either by differences in primary sequence between the two forms of cGMP-dependent protein kinase or by a covalent modification of one form of the enzyme that might be absent in the other form. One possibility is that form 2 of the cGMP-dependent is autophosphorylated to a greater extent than form 1, thus altering its elution profile from DEAE-cellulose and altering its susceptibility to proteolysis. As shown in Fig. 7, both forms

FIG. 3. SDS-PAGE of affinity purified cGMP-dependent protein kinase forms 1 and 2. Forms 1 and 2 were separately pooled from the initial DEAE-cellulose column, concentrated with ammonium sulfate, and dialyzed against PEM. The two concentrates were applied to separate CAMP-agarose columns as described under "Experimental Procedures" and eluted with 0.1 mM cGMP in PEM. Fractions (2 ml) were collected from each affinity column and prepared for SDS-PAGE on 10-15% gels. Proteins were silver-stained. Lanes 9 and 10 represent the pooled forms 1 and 2 that were concentrated following elution from the affinity column. PK, protein kinase.

FIG. 4. Native polyacrylamide gel electrophoresis of cGMP-eluted fractions of forms 1 and 2 cGMP-dependent protein kinase (PK) from cAMP-agarose affinity columns. Fractions were collected as described in the legend to Fig. 4 and subjected to electrophoresis at 4 °C on 8-25% nondenaturing polyacrylamide gels. Proteins were silver-stained.

FIG. 5. One-dimensional peptide maps of cGMP-dependent protein kinase. Forms 1 and 2 cGMP-dependent protein kinase (PK) were treated with 1:10 (w/w) of the specified proteases for 12 h at 37 °C as described under "Experimental Procedures." Reactions were terminated and aliquots were subjected to 8-25% SDS-PAGE. Gels were silver-stained as described. Gel A, forms 1 and 2 were treated with Trypsin (T), chymotrypsin (C), and type VI S. griseus protease (Pt). Gel B, forms 1 and 2 were treated with Pronase (Pn) and type XVII S. aureus protease (V8).
were treated with 1:10 (w/w) of S. griseus protease each hour for 4 h at 37 °C. At the end of each hour, aliquots of reaction mixture were removed and frozen in dry ice and a new aliquot of protease was added. Samples and gels were treated as described in the legend to Fig. 5.

Fig. 5. One-dimensional peptide maps of cGMP-dependent protein kinase. Forms 1 and 2 cGMP-dependent protein kinase were treated with 1:10 (w/w) of S. griseus protease each hour for 4 h at 37 °C. At the end of each hour, aliquots of reaction mixture were removed and frozen in dry ice and a new aliquot of protease was added. Samples and gels were treated as described in the legend to Fig. 5.

Fig. 7. Autoradiography of forms 1 and 2 cGMP-dependent protein kinase (PK) eluted from cAMP-agarose columns with 0.1 mM cGMP. The enzymes were concentrated on small (<0.5 ml) DEAE-cellulose columns and dialyzed exhaustively to remove free cGMP and some bound nucleotide. Equal concentrations of enzymes were diluted 1:10 in PEM before being added to the phosphorylation assay (10 μl of enzyme in a total volume of 0.1 ml). Phosphorylation was carried out for 5 min at 30 °C in the absence or presence of 0.1 mM cGMP or 1.0 μM cAMP. The phosphorylated 79,000-dalton subunit was visualized by autoradiography.

1 and 2 undergo autophosphorylation in the presence of either cAMP or cGMP. There were no differences in the extent of autophosphorylation when using cAMP to stimulate maximal phosphorylation, suggesting that both enzymes contained the same amount of endogenous phosphate. Stoichiometry was estimated to be ~1 mol/mol monomer for each form. This was further examined by using alkaline phosphatase to remove endogenous phosphate from both enzymes. Treatment with phosphatase did not alter the mobility of either form of cGMP-dependent protein kinase when subjected to SDS-PAGE (Fig. 8). When cGMP-dependent protein kinases were treated with phosphatase and then rephosphorylated, neither enzyme showed an increase in phosphate incorporation in the presence of either cAMP or cGMP (Fig. 8B). These results suggest that the differences in either electrophoretic mobility or DEAE-cellulose elution were not due to differences in endogenous phosphorylation of the enzymes.

Activation of cGMP-dependent Protein Kinase by Cyclic Nucleotides—The results described in Figs. 3 and 4 suggested that form 1 of cGMP-dependent protein kinase may have a lower affinity for cAMP than form 2. However, the Kₐ values for cAMP and cGMP for activating forms 1 and 2 cGMP-dependent protein kinase pooled from DEAE-cellulose columns were very similar if not identical (Kₐ cGMP = 0.1 μM; Kₐ cAMP = 1.0 μM). If form 2 were a phosphorylated form of the enzyme, it might have been expected to have a higher affinity for cAMP (21, 22).

**DISCUSSION**

The results described in this manuscript are the first to provide evidence for two forms of cGMP-dependent protein kinase that differ in structure due to a covalent modification other than phosphorylation, primary sequence, or a combination of the two. Wolfe et al. (14) observed that different eluting forms of cGMP-dependent protein kinase could be interconverted by cGMP binding. Although they found no evidence for a covalent difference between the two forms of cGMP-dependent protein kinase in their studies, our results suggest that in addition to changes in chromatographic behavior due to cGMP binding, there are differences based on permanent structural alterations in one or both forms of the enzyme. For example, the differences might be due to differences in the primary amino acid sequences between the two forms of cGMP-dependent protein kinase, post-translational modifications other than phosphorylation, or a combination of the two. The evidence for such differences is compelling: first, the differences in DEAE-cellulose elution are observed following rechromatography of the enzymes, precipitation with ammonium sulfate, and aging of the extracts. Second, gradient SDS-PAGE reveals differences in mobility even after SDS denaturation under reducing conditions. Native PAGE demonstrates that the two forms are not simply differences in the number of protomers that make up the holoenzyme, since form 1 has a slightly lower mobility on native gels than form 2 even though its mobility is slightly greater than form 2 on SDS-PAGE. Third, peptide maps produced by prolonged proteolysis reveal minor differences in structure, even though the most striking aspect of this experiment is the similarity in structure. That phosphorylation is not responsible for these structural differences is suggested by an examination of their properties following treatment with alkaline phosphatase. Alkaline phosphatase does not convert one form into the other when examined using SDS-PAGE. It is possible, how-
ever, that alkaline phosphatase does not completely dephosphorylate cGMP-dependent protein kinase so these results should be interpreted with caution. A more convincing experiment to suggest that differences in phosphorylation do not account for the structural differences is that both enzymes autophosphorylate to apparently the same extent. There also does not appear to be a greater sensitivity of form 2 to cAMP activation of protein kinase using histone H2b as the substrate. Since phosphorylated cGMP-dependent protein kinase is known to have a greater sensitivity to cAMP than unphosphorylated enzyme, our findings add further support to the contention that forms 1 and 2 do not simply represent phospho and dephospho forms of the cGMP-dependent protein kinase.

The possibility that form 1, which has a slightly greater mobility on SDS-PAGE, is derived from form 2 by proteolysis was also considered. However, this is not likely for several reasons. First, proteolytic cleavage of cGMP-dependent protein kinase is known to occur in the "hinge" region of the enzyme producing catalytically active monomers that are unable to undergo autophosphorylation (23, 24). This is not observed since form 1 is activated 5- to 10-fold by cGMP, is fully phosphorylated, and because form 1 has a mobility on gradient native PAGE close to that of form 2. We have further confirmed the size of form 1 as the dimeric form of cGMP-dependent protein kinase (M, = 150,000) on Sephacryl S-300 (not shown). Second, aging of the extract does not result in greater amounts of form 1 being formed relative to form 2. We have observed, however, that both forms 1 and 2 are reduced with aging of the extract and a third peak of kinase activity emerges from DEAE-cellulose columns before elution of form 1. This third peak most likely represents the monomeric form of cGMP-dependent protein kinase since it is fully active in the absence of cGMP and can be generated by treatment of either form 1 or form 2 with chymotrypsin. It seems reasonable to conclude that form 1 is not a proteolytic fragment of form 2. The possibility that form 1 may be a "slightly" proteolytically modified form of form 2 cannot be ruled out. If form 1 does represent a slightly proteolytically altered form of cGMP-dependent protein kinase, then this result is unprecedented.

The existence of two forms of cGMP-dependent protein kinase is not entirely unexpected, even though we and others (8, 9) have not found this situation to exist in tissues such as lung and heart. It is well known that cAMP-dependent protein kinase isoforms exist, namely type I and type II (25), and now it is clear that subtypes for the catalytic subunit (26) and possibly the regulatory subunit exist as well. These subunits appear to be different gene products in each case. More recently, it has been shown that protein kinase C is also a heterogeneous family of enzymes each of which appear to be different gene products (27). The fact that the cGMP-dependent protein kinase has been less extensively studied may account for the lack of information on different forms of this enzyme. However, as interest grows on the role of the enzyme in smooth muscle function, investigators may wish to examine it in more detail at both the structural and genetic level. Thus, a knowledge of the existence of multiple forms of cGMP-dependent protein kinase will undoubtedly be useful for these studies.

Of some interest is the possibility that the two forms of cGMP-dependent protein kinase may have different regulatory properties. Whether or not these may reflect a regulatory mechanism for the mediation of the biological actions of cAMP through activation of cGMP-dependent protein kinase is not known. To date, no information is yet available on the ability of cAMP to activate the cGMP-dependent protein kinase in intact tissues. However, a recent report by Francis et al. (13) demonstrates that cAMP analogs which selectively activate cGMP-dependent protein kinase are potent relaxing agents in smooth muscle. They make the interesting suggestion that cAMP relaxes smooth muscle in at least some tissues by activating the cGMP-dependent protein kinase. Whether or not this is true in aortic tissue is not known, but it is clear that analogs with higher affinity for the cGMP-dependent protein kinase are better relaxants than those that are selective for the cAMP-dependent protein kinase.

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