Purification of a Ca\(^{2+}\)-activatable Cyclic Nucleotide Phosphodiesterase from Bovine Heart by Specific Interaction with Its Ca\(^{2+}\)-dependent Modulator Protein*  

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A Ca\(^{2+}\)-activatable cyclic nucleotide phosphodiesterase from bovine heart can be eluted from a DEAE-cellulose column either in the free form by buffers containing 0.1 mM ethylene glycol bis(\(\beta\)-aminoethyl ether)N,N',N"-tetraacetic acid (EGTA) or as a complex of the enzyme with its protein modulator by buffers containing 0.01 mM CaCl\(_2\). A purification procedure based primarily on the significantly different affinity of the two forms of the enzyme for DEAE-cellulose was developed for the purification of the enzyme from bovine heart. The procedure involves ammonium sulfate fractionation, three chromatographic steps on DEAE-cellulose, and gel filtration on Sephadex G-200 with a 5000-fold purification over the crude extract. The purified enzyme has a specific activity of 120 pmol of cAMP/mg/min, can be activated 5-fold by Ca\(^{2+}\), but is only 80% pure as judged by analytical disc gel electrophoresis. The purified enzyme is unstable but can be stabilized by addition of Ca\(^{2+}\) and the protein modulator; this is in contrast to the less pure preparations of Ca\(^{2+}\)-activatable phosphodiesterase which are destabilized by the protein modulator in the presence of Ca\(^{2+}\).

Cheung (1, 2) has demonstrated the existence of a protein activator of cyclic nucleotide phosphodiesterase in many mammalian tissues. Independently, Kakiuchi and co-workers (3, 4) discovered a protein modulator in rat brain which could enhance the activation of a Ca\(^{2+}\)-activatable cyclic nucleotide phosphodiesterase. Later work from several laboratories (5-7) established that the modulator and the activator are identical proteins. Using a homogeneous preparation from bovine heart (8), we showed that the modulator is a Ca\(^{2+}\)-binding protein (5) and, subsequently, protein modulators from other sources were also shown to bind Ca\(^{2+}\) (6, 7, 9).

The mechanism of activation of cyclic nucleotide phosphodiesterase by Ca\(^{2+}\) and the protein modulator has been studied by several investigators. The enzyme activation depends on the simultaneous presence of both Ca\(^{2+}\) and the protein modulator (5-8). In addition to being necessary for enzyme activation, calcium is also required for the association between the enzyme and its protein modulator, which has been shown to undergo changes in physical properties and stability as a result of Ca\(^{2+}\) binding (10-14). The following scheme has been proposed to describe the activation of the enzyme by its modulator (10):

\[
\begin{align*}
C_{A}^{2+} + \text{Modulator} & \rightarrow \text{[Modulator-Ca\(^{2+}\)] (Low activity)} \\
\text{[Modulator-Ca\(^{2+}\)]} & \rightarrow \text{[Modulator-Ca\(^{2+}\)] (Activated)} \\
\end{align*}
\]

\[
\begin{align*}
\text{[Modulator-Ca\(^{2+}\)]} + \text{E} & \rightarrow \text{[Modulator-Ca\(^{2+}\)-E]} (Low activity) \\
\text{[Modulator-Ca\(^{2+}\)-E]} & \rightarrow \text{[Modulator-Ca\(^{2+}\)-E] (Activated)} \\
\end{align*}
\]

The equations are only intended to indicate the sequence of events since determinations of the strength and stoichiometry of interaction require more exact measurements than possible with impure enzyme preparations.

The Ca\(^{2+}\)-activatable cyclic nucleotide phosphodiesterase is one of the multiple forms of the mammalian enzyme (15-18). Partially purified preparations of mammalian cyclic nucleotide phosphodiesterase have been obtained from bovine heart (19, 20) and bovine brain (21), but these purified preparations are either refractory to the protein modulator (20, 21) or activated by the modulator to a very small extent (19); i.e. 2-fold as compared to the 6- to 10-fold observed with the impure enzyme (2, 8). Specific activities of the purified preparations range from 2.5 to 10 pmol of cAMP/min/mg (19, 21). These values are considerably lower than those reported for homogeneous preparations of the enzyme from yeast (22) and frog rod outer segment disc membranes (23).

In this report, we describe a procedure for the purification of Ca\(^{2+}\)-activatable bovine heart cyclic nucleotide phosphodiesterase. The procedure is based on the Ca\(^{2+}\)-induced specific interaction between the enzyme and its protein modulator and enzyme preparations with specific activities of better than 100 pmol of cAMP/min/mg and which can be activated 4- to 6-fold by Ca\(^{2+}\) have been obtained.

MATERIALS AND METHODS

Fresh bovine hearts were obtained from a local slaughterhouse. The hearts were cut into small pieces and stored at -20° for 2 to 5 days prior to use. Cyclic nucleotides, 5'-nucleotidase, and bovine serum albumin were from Sigma Chemical Co. Cyclic [\(^{3}H\)JAMP and cyclic [\(^{3}H\)JGMP were from Schwarz/Mann. DEAE-cellulose (Cellex...
The modulator-deficient phosphodiesterase was purified by the procedure of Tec et al. (8) with modifications to scale up the purification. The reactions were prepared by the method described previously (9). The modulator-deficient phosphodiesterase was mixed with 0.2 M NaCl and the sample was then applied to a DEAE-cellulose column (2.5 × 60 cm) previously equilibrated with 20 mM imidazole buffer, pH 6.5, containing 1 mM MgCl2 and 0.2 mM NaCl. The column was first eluted with 600 ml of the buffer containing 0.2 M NaCl and then with a linear gradient composed of 600 ml each of buffer containing 0.2 M and 0.6 M NaCl, respectively. Fractions containing the modulator activity were pooled and concentrated by precipitation with 60% (NH4)2SO4, at pH 4.0. The precipitate could be dissolved in 20 to 30 ml of Tris buffer (20 mM Tris, pH 7.5, 1 mM MgCl2, 10 mM CaCl2). Aliquots (6 to 10 ml) of the sample were subjected to gel filtration on a column (2.5 × 85 cm) of Sephadex G-100 previously equilibrated with the same buffer; fractions containing the modulator were pooled and stored frozen at -20°C and these preparations were 90 to 95% pure as judged by polyacrylamide disc gel electrophoresis on 15% gel. One preparation could yield 20 to 25 mg of the protein modulator.

The modulator-deficient phosphodiesterase needed for the assay of the modulator was prepared as previously described (10). The preparation was obtained in small aliquots at -30°C until used.

The (NH4)2SO4 pellet used in the experiment of Fig. 1 was obtained at an intermediate step in the preparation of the modulator-deficient phosphodiesterase. Prior to its use, the pellet was dissolved in and dialyzed overnight against the column equilibrating buffer (see legend for Fig. 1) and then clarified by centrifugation at 100,000 × g. The sample contained saturating amounts of the protein modulator; its phosphodiesterase activity was not enhanced by the addition of exogenous protein modulator.

In the case of experiments for which it was necessary to carefully control the Ca2+ concentration (Fig. 5), reagents and protein samples were purified using Chelox 100 resin as previously described (5). The purified reagents and samples were always monitored by atomic absorption spectrometry (Perkin-Elmer model 303) and stored in plastic containers.

The activity of cyclic nucleotide phosphodiesterase was measured as previously described (8, 24). The procedure involved coupling of the phosphodiesterase reaction to a 5'-nucleotidase reaction which was followed by analyzing the resulting phosphate. The 5'-nucleotidase reaction was carried out concurrently with one stage assay or after the phosphodiesterase reaction (two stage assay). The enzyme activity was routinely measured at 30°C, pH 7.5, in a reaction mixture containing 25 mM Tris, 25 mM imidazole, 3 mM magnesium acetate, 1.2 mM cyclic nucleotide, 0.4 unit of 5'-nucleotidase, and either 0.01 mM CaCl2 and 50 units of protein modulator (activated activity) or 0.1 mM EGTA (nonactivated activity). One unit of enzyme was defined as the amount of enzyme hydrolyzing 1 μmol of cAMP/min.

For enzyme assays at low concentrations of the nucleotide substrates, a method using 3H-labeled cyclic nucleotides based on the procedure of Thomsen and Appelman (25) was used. The method has been described previously (18).

The protein modulator was assayed on the basis of its ability to activate cyclic nucleotide phosphodiesterase under conditions where the modulator is limiting as previously described. One unit is defined as the amount of modulator which gives rise to half-maximal activation of 0.012 unit of enzyme (9).

Protein concentrations were determined by methods of Lowry et al. (26) or a modification thereof (27). Bovine serum albumin was used as the standard and standard curves were constructed with the albumin in buffer solutions identical with those of the sample. Analytical disc gel electrophoresis was carried out as described by Davis (28). The protein was stained with Amido black. To locate the enzyme activity on the gel, an unstained gel was sliced into 2-mm slices and each slice was extracted with 100 μl of buffer (20 mM Tris, pH 7.5, 1 mM MgCl2, 10 mM CaCl2) containing 50 units of protein modulator. The extracts were then analyzed for phosphodiesterase activity.
pellet described under "Materials and Methods") is affected by the presence of protein modulator in the preparation and the Ca²⁺ concentration of the eluting buffers. If the elution buffers contained 0.1 mM EGTA the phosphodiesterase was eluted from the DEAE-cellulose column in two activity peaks (PI and PI₁) at NaCl concentrations of 0.11 and 0.21 M, respectively (Fig. 1A); the PI phosphodiesterase could be activated 5-fold by added protein modulator and calcium and this activation was completely eliminated by 1 mM EGTA, whereas the PI enzyme was neither activated by the protein modulator nor inhibited by EGTA. From the results in Fig. 1A it can be seen that the Ca²⁺-activatable (PI) and the Ca²⁺-independent form of cyclic nucleotide phosphodiesterase can be separated on a column of DEAE-cellulose using EGTA-containing buffers; the Ca²⁺-activatable phosphodiesterase so obtained is free of its protein modulator. In contrast, as shown in Fig. 1B, the two forms of phosphodiesterase together with saturating amounts of protein modulator were eluted from the column as a single activity peak at a NaCl concentration of 0.2 M when the eluting buffers were made 10 μM in Ca²⁺. One possible explanation for this observation is that the PI phosphodiesterase associates with the endogenous acidic protein modulator in the presence of Ca²⁺ and is, therefore, retarded on the column so that it elutes together with the Ca²⁺-independent form. This suggestion is supported by the data in Fig. IC which show that a preparation of Ca²⁺-activatable phosphodiesterase which is free of endogenous modulator (see "Materials and Methods") was eluted from the column at a NaCl concentration of 0.115 M effect if the buffers contained Ca²⁺. In this study we have exploited this effect of Ca²⁺ on the behavior of the enzyme on DEAE-cellulose as a step in the purification of the enzyme.

Purification of Ca²⁺-activatable Phosphodiesterase

Extraction and Ammonium Sulfate Fractionation — All purification steps were carried out in the cold room. Frozen bovine heart tissue (3 kg) was ground in a meat grinder and then homogenized in batches (5-quart Waring Blender, 10 s) in a total volume of 6 liters of cold, 0.1 M Tris/HCl buffer, pH 7.5. The homogenate was centrifuged at 3,000 x g for 20 min and the supernatant adjusted to pH 8.5 with 5 M NaOH and made 1 mM in EDTA using a 0.15 M neutralized EDTA solution. Solid ammonium sulfate was then added to obtain 55% saturation. The solution was gently stirred for 30 min and then centrifuged at 11,000 x g for 20 min. The resultant pellet was dispersed in 150 to 300 ml of Buffer A (20 mM Tris/HCl, pH 7.0, buffer containing 1 mM imidazole, 1 mM magnesium acetate, and 15 mM 2-mercaptoethanol). The resulting turbid solution was dialyzed for 40 h against two changes of 10 liters each of Buffer A containing 0.1 mM EGTA. The dialyzed sample, still turbid, was centrifuged at 120,000 x g for 1 h. The resulting supernatant (1.2 liters) usually had an ionic strength of less than 0.1.

First and Second DEAE Cellulose Columns — The supernatant obtained as described above was applied to a DEAE-cellulose column (5 x 85 cm) previously equilibrated with Buffer A containing 0.1 mM EGTA. After application, the column was washed with 2 liters of the same buffer containing 0.05 M NaCl and then eluted with a linear salt gradient made up of 2.5 liters each of the same buffer containing 0.05 and 0.4 M NaCl, respectively. The flow rate was 250 to 300 ml/h and the Ca²⁺-activatable phosphodiesterase eluted in a broad peak with its highest activity appearing at approximately 0.11 M NaCl. Fractions with the highest activity were pooled and dialyzed overnight against 8 liters of Buffer A containing 0.1 mM EGTA. The dialyzed sample (800 ml) was rechromatographed on a second DEAE-cellulose column (2.5 x 85 cm) previously equilibrated with the same buffer. The flow rate was 150 ml/h and the column was eluted with 600 ml of the equilibrating buffer containing 0.05 M NaCl, followed by 1.8 liters of a linear NaCl gradient from 0.05 to 0.35 M. Fig. 2A shows that phosphodiesterase activity elutes as a single activity peak with its highest activity at 0.105 M NaCl. Fractions containing the highest activity were pooled (approximately 300 ml).

Third DEAE-Cellulose Column — Purified bovine heart protein modulator (4 mg) and CaCl₂ (1.5 ml of a 0.1 M solution) were added to the pooled fractions obtained as described above. The preparation was dialyzed overnight against 4 liters of Buffer A containing 10 μM CaCl₂. The dialyzed sample was applied to a DEAE-cellulose column (2.5 x 40 cm) previously equilibrated with Buffer A containing 10 mM CaCl₂ and 10 mM EGTA. The enzyme was neither activated by the protein modulator nor inhibited by EGTA. From the results in Fig. 1A it can be seen that the Ca²⁺-activatable phosphodiesterase so obtained is free of the endogenous modulator in the presence of Ca²⁺ and is, therefore, retarded on the column so that it elutes together with the Ca²⁺-independent form. This suggestion is supported by the data in Fig. IC which show that a preparation of Ca²⁺-activatable phosphodiesterase which is free of endogenous modulator (see "Materials and Methods") was eluted from the column at a NaCl concentration of 0.115 M effect if the buffers contained Ca²⁺. In this study we have exploited this effect of Ca²⁺ on the behavior of the enzyme on DEAE-cellulose as a step in the purification of the enzyme.

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Third DEAE-Cellulose Column — Purified bovine heart protein modulator (4 mg) and CaCl₂ (1.5 ml of a 0.1 M solution) were added to the pooled fractions obtained as described above. The preparation was dialyzed overnight against 4 liters of Buffer A containing 10 μM CaCl₂. The dialyzed sample was applied to a DEAE-cellulose column (2.5 x 40 cm) previously equilibrated with Buffer A containing 10 μM CaCl₂ and 10% EGTA. The enzyme activity was eluted in the NaCl concentration range of approximately 0.19 to 0.22 M NaCl, with the peak at 0.21 M NaCl, whereas most of the protein material appeared as a peak in the NaCl concentration range of 0.09 to 0.15 M (Fig. 2B).

Gel Filtration on Sephadex G-200 — Fractions from the third DEAE-cellulose column containing enzyme activity (approximately 120 ml) were pooled and concentrated to 3 ml by diaflo ultrafiltration using an Amicon UM 10 membrane. The concentrated sample was applied to a column (2.5 x 85 cm) of Sephadex G-200 previously equilibrated and also eluted with

![Fig. 2. Chromatography of phosphodiesterase on the second and third DEAE-cellulose columns. Elution profile for the second (A) and third DEAE-cellulose column chromatographies (B). Details are described under "Results." Phosphodiesterase activity was measured in the presence of 0.1 mM Ca²⁺ and 10 units of the protein modulator.](http://www.jbc.org/)
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Buffer A containing 10 \textmu M CaCl\textsubscript{2}, 0.1 M NaCl, and 10\% sucrose. The phosphodiesterase activity appeared in a sharp peak which corresponded approximately with one of the protein peaks. Fractions under the peak region of the enzyme activity were pooled separately from the side fractions as indicated in Fig. 3. The two pooled preparations were concentrated to 2 to 3 ml each by ultrafiltration on an Amicon UM-10 membrane and stored in small aliquots at -20\(^\circ\) until use.

Summary of Purification - Table I summarizes the results for a typical purification of the Ca\textsuperscript{2+}-activatable phosphodiesterase. If only the fractions under the peak activity region of the G-200 Sephadex elution profile are considered, the purification factor and recovery were 5000-fold and 3.5\%, respectively, and the specific activity achieved was over 120 units/mg. The purified enzyme could usually be activated 4- to 5-fold by Ca\textsuperscript{2+}.

The most effective step in the purification procedure is the third DEAE-cellulose column: an enrichment of over 75-fold results from this single step. As shown in Fig. 2A, the phosphodiesterase activity was eluted from the second DEAE-cellulose column at NaCl concentrations between 0.08 and 0.14 M and, therefore, was contaminated by proteins eluted at the salt concentration. Under the conditions of the third DEAE-cellulose column the enzyme was eluted as the phosphodiesterase-modulator complex at much higher NaCl concentrations and thus was freed from most of the proteins contaminating it after the second DEAE-cellulose column; these proteins still eluted at NaCl concentrations between 0.09 and 0.15 M (Fig. 2B).

Disc Gel Electrophoresis - The enzyme preparation obtained after the Sephadex G-200 filtration step was not homogeneous. Fig. 4A shows the disc gel electrophoresis patterns of samples at several stages of purification. It is clear that the third DEAE-cellulose column results in a very significant improvement in enzyme purity. The fraction representing the activity peak from the Sephadex G-200 step shows only two distinct bands and a minor diffuse staining region. A similar gel pattern was observed for the side fraction of the activity peak after Sephadex G-200 except that there is relatively more of the lower protein band (result not shown). Densitometric tracing of the sample from the G-200 Sephadex peak fraction suggests that the predominant band represents approximately 80\% of the total protein; this band corresponds to the enzyme activity as determined from a sliced unstained gel (Fig. 4B).

Attempts to purify this enzyme preparation further were unsuccessful. In a few trials, the enzyme was rechromatographed on a Sephadex G-200 column using buffers containing 1 mM EGTA. In other trials, the enzyme was rechromatographed on two successive small DEAE-cellulose columns eluted with buffers containing 0.01 mM Ca\textsuperscript{2+} and 0.1 mM EGTA, respectively. These attempts all resulted in the loss of approximately 90\% of the total enzyme activity and a drastic decrease in specific enzyme activity to approximately 20 to 30 units/mg.

Table I

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Protein</th>
<th>Enzyme activity</th>
<th>Specific activity</th>
<th>Activation by Ca\textsuperscript{2+}</th>
<th>Yield</th>
<th>Purification</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>Activated(^	ext{a})</td>
<td>Nonactivated(^	ext{a})</td>
<td>Activated</td>
<td>Nonactivated</td>
<td>%</td>
</tr>
<tr>
<td>Crude extract</td>
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<td>0.08</td>
<td>71</td>
<td>1</td>
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<tr>
<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitate</td>
<td>14,400</td>
<td>1,167</td>
<td>0.29</td>
<td>0.032</td>
<td>900</td>
<td>32</td>
</tr>
<tr>
<td>1st DEAE-cellulose(^	ext{c})</td>
<td>1,800</td>
<td>527</td>
<td>0.29</td>
<td>0.032</td>
<td>900</td>
<td>32</td>
</tr>
<tr>
<td>2nd DEAE-cellulose(^	ext{d})</td>
<td>1,280</td>
<td>510</td>
<td>0.40</td>
<td>0.045</td>
<td>900</td>
<td>31</td>
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<tr>
<td>3rd DEAE-cellulose(^	ext{e})</td>
<td>8.0</td>
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<td>4.63</td>
<td>680</td>
<td>15.3</td>
</tr>
<tr>
<td>Sephadex G-200(^	ext{f})</td>
<td></td>
<td></td>
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<tr>
<td>Peak</td>
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<td>11</td>
<td>129</td>
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<td>510</td>
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<tr>
<td>Sides</td>
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<td>16.4</td>
<td>470</td>
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<tr>
<td>Total</td>
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<td>110</td>
<td>22.5</td>
<td>97</td>
<td>19.9</td>
<td>490</td>
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</table>

\(^	ext{a}\) Enzyme activity was measured in the presence of saturating concentrations of the protein modulator and Ca\textsuperscript{2+}.

\(^	ext{b}\) Enzyme activity was measured in the presence of 0.1 mM EGTA.

\(^	ext{c}\) Calculation was based on the activated activity of the enzyme.

\(^	ext{d}\) The Ca\textsuperscript{2+}-independent form of phosphodiesterase was removed at this step.

\(^	ext{e}\) Enzyme preparations showed absolute dependence of added protein modulator for Ca\textsuperscript{2+} activation.

\(^	ext{f}\) Enzyme preparations contained saturating amounts of the protein modulator.
we have found that the protein modulator and Ca$^{2+}$ exerted different effects on the thermal stability of the enzyme at different stages of purification.

As shown in Fig. 5A the enzyme, as obtained after the second DEAE-cellulose column, was relatively stable at 55°C, pH 7.5, even in the presence of either Ca$^{2+}$ or protein modulator, but lost activity rapidly when both Ca$^{2+}$ and protein modulator were present simultaneously. It thus appears that destabilization of the enzyme at this stage of purification requires both Ca$^{2+}$ and the modulator, just as both are required for enzyme activation. The thermal inactivation of phosphodiesterase appeared to follow first order kinetics either in the absence or presence of saturating amounts of both effectors (Fig. 5A). In experiments where inactivation of the enzyme took place in the presence of varying amounts of the modulator or Ca$^{2+}$, all of the rates were found to conform to first order kinetics. The dependence of the apparent rate constant of inactivation upon modulator or Ca$^{2+}$ concentration resembled a ligand saturation curve with apparent dissociation constants of 0.65 unit of modulator and 2 μM Ca$^{2+}$, respectively (Fig. 5B). These values are remarkably close to the kinetic parameters obtained by steady state kinetic analysis of enzyme activation, which gave dissociation constants of 1 unit of modulator and 2.3 μM Ca$^{2+}$, respectively (5).

In contrast to the above results, Fig. 6A shows that enzyme obtained after gel filtration on Sephadex G-200 was inacti-
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The enzyme preparation used was saturated with the protein modulator so that further addition of the protein modulator was not needed for enzyme stabilization. In addition to Ca\textsuperscript{2+} and the protein modulator, bovine serum albumin also could stabilize the purified phosphodiesterase against heat inactivation; in the presence of albumin the stability of the enzyme was only slightly affected by Ca\textsuperscript{2+} (Fig. 6A).

In contrast to the less pure phosphodiesterase preparations which are stable during storage in refrigeration, the purified enzyme was rapidly inactivated by incubation at 0\(^\circ\) in buffers containing EDTA. This inactivation could be significantly retarded by addition of bovine serum albumin to the incubation mixture (Fig. 6B). Removal of the protein modulator from the purified enzyme preparations by DEAE-cellulose chromatography also resulted in a rapid loss of enzyme activity.

Other Properties — The purified Ca\textsuperscript{2+}-activatable cyclic nucleotide phosphodiesterase has been characterized in terms of its catalytic properties. Under routine assay conditions (see "Materials and Methods"), linear progress was observed for either the activated or the nonactivated enzyme reaction. Inclusion of 0.2 mg/ml of bovine serum albumin in the reaction mixture resulted in approximately 20% increase in enzyme activity. Presumably, serum albumin could protect the enzyme against inactivation during the reaction. For the kinetic characterization of the purified phosphodiesterase, serum albumin was always included in the reaction mixture. The initial reaction rate exhibited a linear dependence on the enzyme concentration in the range of 0.1 to 2 \mu l/ml. The purified enzyme was found to catalyze the hydrolysis of cAMP or cGMP with a maximal velocity for cAMP approximately 3-fold that for cGMP. The enzyme exhibited a higher affinity for cGMP than for cAMP; the apparent \(K_m\)'s values of the activated enzyme for cGMP and cAMP were 0.22 and 0.009 \mu M, respectively. At pH 7.5 the Ca\textsuperscript{2+}-activated enzyme could be further stimulated 2- to 3-fold by 20 \mu M imidazole, whereas the nonactivated (basal) enzyme was not significantly affected by imidazole. These kinetic properties are essentially the same as those described for a less pure preparation of the enzyme (18).

The molecular weight of the Ca\textsuperscript{2+}-activatable cyclic nucleotide phosphodiesterase was estimated on a calibrated Sephadex G-200 column. Similar to the brain phosphodiesterase (13, 14), the enzyme from bovine heart could be chromatographed on the gel filtration column either in its free state or as an enzyme-modulator complex. Fig. 7 shows that molecular weights of the free enzyme and the enzyme-modulator complex are 155,000 and 230,000, respectively. These values are similar to those reported for the Ca\textsuperscript{2+}-activatable phosphodiesterase from mammalian brain (13, 14).

**DISCUSSION**

The bovine heart Ca\textsuperscript{2+}-activatable cyclic nucleotide phosphodiesterase has been purified and the purest enzyme preparation has a specific activity of 120 \mu mol of cAMP/min/mg; this value is at least an order of magnitude greater than those previously reported for a mammalian cyclic nucleotide phosphodiesterase. Since the preparation appeared approximately 80% pure as judged by gel electrophoresis, a homogeneous preparation of the Ca\textsuperscript{2+}-activatable phosphodiesterase would
probably have a specific activity of approximately 160 μmol of cAMP/min/mg. Cyclic nucleotide phosphodiesterase preparations with high specific activity have been obtained by other investigators from Baker's yeast (22) and frog rod outer fragments membrane (23). Specific activities for the purified yeast and frog phosphodiesterases are 265 and 165 μmol of cAMP/min/mg, respectively.

It has been reported that the Ca2+-activatable cyclic nucleotide phosphodiesterase often becomes unresponsive to Ca2+ and its protein modulator during purification or upon storage (18, 21, 27). The purified enzyme obtained in this study still retained significant Ca2+ sensitivity; it could usually be activated 4- to 6-fold. However, relatively impure bovine heart enzyme could often be activated up to 10-fold by Ca2+. Thus, some desensitization of the enzyme did occur during the enzyme purification. The reason for the decline in Ca2+ activation during the enzyme purification is not clear. It is possible that the nonactivatable enzyme is a degradative product of the activatable enzyme, since it has been shown that limited proteolytic digestion results in the desensitization of the Ca2+-activatable phosphodiesterase (2). However, a systematic investigation of this possibility has not been carried out.

To a large extent, the present purification procedure utilizes the Ca2+-dependent reversible association between the enzyme and its protein modulator. Teshima and Kakiuchi (13), and independently Lin et al. (14), showed that brain phosphodiesterase is separated from its protein modulator on a Sephadex G-200 column in the absence of free Ca2+, but eluted as an enzyme·modulator complex if Ca2+ is present. They concluded that Ca2+ promotes the association of the enzyme and the protein modulator. Similar results have been obtained in the present study using the enzyme and the modulator from bovine heart (Fig. 7). In addition, the bovine heart enzyme can be eluted from a DEAE-cellulose column either in its free form by buffers containing EGTA or as the enzyme modulator complex by buffers containing Ca2+. Since the protein modulator is a highly acidic protein with an isoelectric point of 4.0 (29), the complexed form of the enzyme is bound more strongly to the anion exchange column and thus eluted at considerably higher salt concentration than is the free enzyme. Thus, the chromatographic properties of the enzyme on DEAE-cellulose can be specifically altered by changing Ca2+ concentration of the eluting buffers. This step in our purification procedure is reminiscent of the specific substrate or modifier elution method described by Pogell and Sarngadharan (30) for the purification of fructose-1,6-diphosphatase. However, their method is based on the ligand-induced conformational change of the enzyme rather than on protein-protein interaction.

It has been recently shown by Brostrom et al. (31) and confirmed by Cheung and co-workers (32) that the Ca2+-regulated protein modulator also activates a detergent-solubilized mammalian brain adenylyl cyclase. Furthermore, the mechanism of activation of adenylyl cyclase by the modulator also has been shown to involve a Ca2+-dependent association of the modulator and the soluble enzyme (33). Thus, it is not inconceivable that the purification procedure described here can be adapted for the purification of brain adenylyl cyclase. The present procedure for the purification of the modulator-regulated enzymes requires pure protein modulator. One large scale preparation of the bovine heart protein modulator (see "Materials and Methods") is usually sufficient for five to six phosphodiesterase preparations. Since bovine brain is a very rich source for the protein modulator, large quantities of the modulator can be more readily obtained from the brain tissue than from bovine heart. Recently, Watterson et al. (34) have developed a purification procedure which yields 70 mg of the protein modulator from each kilogram of the brain tissue.

Although the purified phosphodiesterase is similar to a less pure preparation of the enzyme (18) in catalytic properties, it exhibits different stability properties. While the protein modulator and Ca2+ facilitate the heat inactivation of less pure enzyme preparations, the stability of the most purified preparation is actually enhanced by these effectors. Based on the observed effect of Ca2+ and the modulator on less pure enzyme preparations, we have suggested previously that enzyme activation by the Ca2+-protein modulator complex is accompanied by a conformational change in the enzyme (10). The present observation that the enzyme changes its stability properties during the course of purification does not negate the previous suggestion. Presumably, the change in enzyme conformation induced by Ca2+ and the protein modulator may either stabilize or destabilize the enzyme depending on the presence of other components in the solutions.

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Purification of a Ca2+-activatable cyclic nucleotide phosphodiesterase from bovine heart by specific interaction with its Ca2+-dependent modulator protein.
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