Purification and Properties of the Protein Activator of Bovine Heart Cyclic Adenosine 3',5'-Monophosphate Phosphodiesterase

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SUMMARY

A protein activator for cyclic adenosine 3',5'-monophosphate (cAMP) phosphodiesterase has been purified from bovine heart by a simple procedure which involves ammonium sulfate and acid precipitations, heat treatment, DEAE-cellulose and Sephadex G-100 column chromatography. The purified activator appears to be homogeneous by ultracentrifugal and disc gel electrophoretic criteria. The specific activity of the pure activator is approximately 8,000-fold that of the crude tissue extract. For optimal recovery of the activator, the buffer solutions used during purification should contain 1 mM Mg^{2+}. The activator is relatively stable in low concentrations of Mg^{2+}, 1 mM or lower. At higher concentrations of this metal ion, 10 mM or higher, the activator rapidly loses its activity during storage at 4°. The activator is also inactivated by low concentrations of EDTA. This inactivation is not reversed by the addition of Mg^{2+}.

Kinetic studies indicate that the activator activates cAMP phosphodiesterase by enhancing the V_{max} and decreasing the K_{m} for cAMP. The cyclic nucleotide, in turn, enhances the affinity of the enzyme toward the activator. The dependence of the enzyme activity on the protein concentration of a mixture of the enzyme and the activator is nonlinear, showing upward curvature. These results suggest that the activator and the enzyme may exist in equilibrium among their respective free forms and the active protein complex and that this equilibrium may be modulated by cAMP. The molecular weight of the activator has been determined by sedimentation-diffusion and gel filtration techniques to be 19,200 and 27,000, respectively.

Since cyclic nucleotides play important regulatory roles in many biological functions (1, 2), it is reasonable to assume that their intracellular concentrations are delicately controlled. Based mainly on such considerations, several groups of researchers (3-8) have investigated regulatory properties of cyclic nucleotide phosphodiesterase, the enzyme responsible for the hydrolytic destruction of these nucleotides. This enzyme was first demonstrated and partially purified from bovine heart muscle by Sutherland and Rall (9) and Butcher and Sutherland (10). The activity of the enzyme may be controlled in several different manners which include allosteric modulation (4, 5, 11) isoenzymes (7), enzyme localization (11), and protein-protein interaction (3-5). Regarding the last mechanism of regulation for this enzyme, Cheung (3) has demonstrated that cyclic nucleotide phosphodiesterase from bovine brain may become inactivated during purification and that the partially purified enzyme can be reactivated by a specific activator existing in the crude tissue extract. Since this activator is nondialyzable and inactivated upon incubation with trypsin, Cheung (3) has suggested that the activator is a protein molecule and that the active enzyme results from association of the protein activator with the enzyme unit. Other workers (4, 5) have also demonstrated the existence of such a protein activator in mammalian tissues. Although the mechanism of the enzyme regulation by this protein activator is not clear, the significance of its regulatory role is supported by the observation that the interaction between the enzyme and the protein activator may be modulated by cyclic adenosine 3',5'-monophosphate (8).

In the present communication, a simple procedure for the purification of the protein activator to apparent homogeneity is reported. Results on the stability and the structural and activating properties of this activator are also presented.

MATERIALS AND METHODS

Materials—Beef hearts were obtained fresh from a local slaughterhouse, Burns Food Ltd. of Winnipeg. The fresh hearts were cut into small pieces (approximately 1 inch^3) and stored frozen at -20° for 1 to 4 weeks prior to use. DEAE-cellulose was obtained from Bio-Rad. Ammonium sulfate and ammonium molybdate, both certified A.C.S. grade, were obtained from Fisher Scientific Co. Sephadex G-100, G-75, and G-25 were the products of Pharmacia. Cyclic AMP,1 5'-nucleotide (Sigma grade II) from venom of Croctalus adamanteus, 1-amino-2-napthol-4-sulfonic acid (Sigma grade II), and RNAse were all obtained from Sigma. Sperm whale myoglobin, ovalbumin, and beef pancreas chymotrypsinogen were obtained from Mann Research Laboratories. Ultracentrifugation membranes UM-10 and UM-2 were obtained from Amicon Corporation. Reagents used...

1 The abbreviation used is: cyclic AMP or cAMP, cyclic adenosine 3',5'-monophosphate.
for disc gel electrophoresis were from Canal Industrial Corporation.

**Assay of cAMP Phosphodiesterase**—The enzyme activity was measured by the procedure of Butcher and Sutherland (10) with slight modification (8). This procedure involved the conversion of 5'-AMP to adenosine by *C. atrox* venom, and the phosphate released was measured by the method of Fiske and SubbaRow (12). The purified 5'-nucleotidase was used in the present study instead of *C. atrox* venom. The purified nucleotidase was free of 5'-AMP to adenosine by *C. atrox* venom, and the phosphate released was measured by the method of Fiske and SubbaRow.

The catalytic activity of the enzyme was increased in a nonlinear manner. With saturating concentration of the activator, the enzyme is stimulated approximately 6-fold. One unit of activator is arbitrarily defined as the amount which is required to give 50% stimulation of this standard amount of cAMP phosphodiesterase.

To avoid possible variations in properties of the activator-deficient enzyme, a single preparation of the enzyme was used throughout the present work. The enzyme stored frozen at −20° was stable and gave reproducible standard curves. To measure the activator activity, the complex was heated for 3 min at 100° to destroy the phosphodiesterase enzyme activity. The catalytic activity was then measured at three different concentrations of the activator, each in triplicates.

**Preparation of Activator-deficient Phosphodiesterase**—Frozen beef heart (1.5 kg) was thawed, put through a meat mincer and homogenized with 2 volumes of 0.1 M Tris-HCl (pH 7.5) containing 1 mM imidazole and 1 mM magnesium acetate. The homogenate was centrifuged at 10,000 × g for 20 min. The supernatant was taken to 60% saturation with (NH₄)₂SO₄ and allowed to stand at 4° for 3 hours. It was then centrifuged at 10,000 × g at 4° for 15 min. The pellet was dissolved in minimal volume of 0.02 M Tris-HCl (pH 7.5) containing 0.08 M NaCl, 1 mM imidazole, and 1 mM magnesium acetate and dialyzed overnight against the same buffer. The solution was then applied to a DEAE-cellulose column (6 cm × 30 cm) and washed stepwise with Tris buffer (pH 7.5) containing successively 0.08 M, 0.2 M, and 0.5 M NaCl. The major part of the enzyme was eluted with buffer containing 0.2 M NaCl. This enzyme peak was then pooled and dialyzed against 0.02 M Tris-HCl containing 0.08 M NaCl, 1 mM imidazole, and 1 mM magnesium acetate. The solution was then applied to a second DEAE-cellulose column (2.5 cm × 44 cm) and eluted with a salt gradient starting from 0.08 M NaCl and ending with 0.25 M NaCl. The enzyme peak was pooled and dialyzed against 0.02 M Tris-HCl (pH 7.5) containing 1 mM imidazole and 1 mM magnesium acetate. The enzyme solution was stored frozen at −20° in 3-ml aliquots. The specific activity of the enzyme was 0.29 unit per mg of protein; the enzyme was purified 16-fold over the crude homogenate. The enzyme preparation still contained activator but its amount was difficult to determine. As the amount of the sample used for the activator assay was increased, a decrease in specific activator activity was observed. This might be due to the presence of inhibitors in the enzyme sample.

**Analytical Disc Gel Electrophoresis**—Analytical disc gel electrophoresis was performed by the method of Davis (14), using 7.5% polyacrylamide gels. Protein samples (10 to 30 μg) were applied to each gel. Amido-black was used for staining proteins. Carbohydrates were stained with periodic acid-Schiff stain by the method of Kapitany and Zebrowski (15). To locate the activator activity on the gel, an unstained polyacrylamide gel was cut into thin slices (0.1 cm thick). The slices were washed by a glass rod in 0.15 ml of 0.02 M Tris-HCl, 1 mM MgAc₂, and 1 mM imidazole buffer (pH 7.5) and centrifuged. The supernatant was used to measure the activator activity.

**Determination of Molecular Weight by Gel Filtration of Sephadex G-75 Column**—A column (3.0 cm × 90 cm) of Sephadex G-75 equilibrated with 0.02 M Tris-HCl (pH 7.5) containing 1 mM imidazole, 1 mM magnesium acetate, and 0.1 M KCl was used to determine the molecular weight of the activator. The flow rate used was 5 ml per hour. Fractions (1 ml) were collected. Dextran Blue was used to mark the void volume, and adenosine was used to mark the total bed volume. Molecular weight...
markers used were sperm whale myoglobin, ovalbumin, beef pancreas chymotrypsinogen, and RNase A.

**Ultracentrifugation**—A Beckman model E analytical ultracentrifuge was used. In the determination of diffusion constant the synthetic boundary cell was used, and the centrifugation was operated at low rotor speed of 6,000 rpm at 20°C. The diffusion constant was determined from the spreading of the boundary as a function of time. In the determination of sedimentation constant, the samples were centrifuged at 60,000 rpm at 20°C. Both diffusion and sedimentation constants were corrected to the standard conditions, i.e. 20°C in water.

**Density and Viscosity of Solvent**—The density of the buffer used in the ultracentrifugation studies was determined by the use of a pycnometer. The viscosity of the buffer was determined using an Oswald viscometer (Cannon Fenske, series 30).

**Amino Acid Composition**—A Beckman model 120C amino acid analyzer was used for amino acid analysis. Lyophilized pure activator (800 μg) was hydrolyzed with 6 N HCl at 105°C for 24 and 72 hours.

**RESULTS**

**Purification of the Protein Activator**

**Extraction and Ammonium Sulfate Fractionation**—For a typical preparation, 1.5 kg of the frozen bovine heart was ground in a meat grinder and homogenized in 3 liters of 0.1 M Tris-HCl buffer (pH 7.5) in the cold room. The homogenate was centrifuged at 3,000 × g for 20 min. The supernatant was titrated to pH 8.5 with 1 N KOH; 0.15 M neutralized EDTA solution was then added to the supernatant to make the final concentration of the chelating agent 1 mM. Cold (NH₄)₂SO₄ was added to the solution to bring the salt concentration to 60% saturation. The solution was then centrifuged at 10,000 × g for 20 min. The pellet from the centrifugation was dissolved in 500 ml of 0.02 M Tris-HCl (pH 7.5) containing 1 mM magnesium acetate and 1 mM imidazole and dialyzed against the same buffer. This solution contained 75 to 90% of the total enzyme activity and may be used for the purification of the activator-deficient enzyme (see "Materials and Methods").

**Acid Precipitation and Heat Treatment**—To the supernatant of the (NH₄)₂SO₄ fraction, 1 M MgAc₂ solution was added to make the final Mg²⁺ concentration 0.01 M, and the pH of the solution was adjusted to pH 4 with 1 N HCl. The acidified solution was left standing on ice for 1 hour and then centrifuged at 3,000 × g for 20 min. The pellet (henceforth referred to as pH 4 pellet) was dissolved in 400 ml of 0.03 M Tris-HCl (pH 7.5) containing 1 mM magnesium acetate and 1 mM imidazole and dialyzed against the same buffer. This solution contained 75 to 90% of the total enzyme activity and may be used for the purification of the activator-deficient enzyme (see "Materials and Methods").

**DEAE-cellulose Column Chromatography**—A DEAE-cellulose column (6 cm × 40 cm) was equilibrated with 0.03 M imidazole buffer (pH 6.5) containing 1 mM magnesium acetate and 0.2 M NaCl. The dialyzed sample from the heat treatment step was applied to the column, and the column was then eluted with 1.5 liters of the same buffer. Approximately 80% of the total protein of the original sample was contained in this initial elution, whereas all of the activator was retained on the column. After this initial elution, the column was eluted with a linear gradient generated from 1.7 liters of the buffer containing 0.2 M NaCl and 17 liters of the buffer containing 0.7 M NaCl. A profile of the elution of the DEAE-cellulose column chromatography is depicted in Fig. 2. The activator activity was eluted between 0.42 and 0.53 M NaCl, with a peak at a NaCl concentration of 0.46 M. The activator peak is not associated with any of the major protein peaks. In most experiments, the trailing edge of the activity peak overlapped a protein peak, thus the trailing part of the activity peak was usually discarded. For the experiment depicted in Fig. 2, fractions 510 to 550 were collected and concentrated to 2 ml by ultrafiltration through an Amicon UM-2 membrane.

**Sephadex G-100 Gel filtration**—The concentrated activator from the DEAE-cellulose column was applied to a Sephadex G-100 column (2.5 cm × 90 cm) which was pre-equilibrated with 0.02 M Tris-HCl buffer (pH 7.5) containing 1 mM magnesium acetate and 1 mM imidazole. The column was developed with the same buffer. An elution profile for the gel filtration is shown in Fig. 3. Most of the protein material was eluted prior to the activity peak of the activator. The fractions between 138 and 155 were collected and concentrated by ultrafiltration.

**RESULTS**

| FRACTION NUMBER | 100 | 200 | 300 | 400 | 500 | 600 |
|-----------------|-----|-----|-----|-----|-----|-----|
| LONP:STRAIN:GI  | 1.0 | 1.2 | 1.4 | 1.0 | 1.2 | 1.4 |

**Fig. 2.** DEAE-cellulose chromatography of the protein activator. The heat treated pH 4 pellet was dialyzed against 0.03 M imidazole buffer (pH 6.5) containing 1 mM magnesium acetate and 0.2 M NaCl. The sample was then applied to a DEAE-cellulose column (6 × 40 cm) which was pre-equilibrated with the same buffer and then eluted with a linear gradient obtained from 1.7 liters of buffer containing 0.2 M NaCl and 1.7 liters of buffer containing 0.7 M NaCl. Fractions (18 ml) were collected. The symbols used were: , activator activity, ionic strength, ; fractions pooled for further purification, solid bar under the activity peak.
TABLE I

| Fraction       | Protein | Total activity | Recovery | Specific activity |
|---------------|---------|----------------|----------|------------------|
| Homogenate    | 65,000  | 151            | 100      | 2.3              |
| pH 4 pellet after heat treatment | 4,400   | 119            | 79       | 27               |
| DEAE-cellulose | 35      | 60             | 39       | 1,720            |
| Sephadex G-100 | 1.2     | 21             | 14       | 18,100           |

Properties of the Activator

Stability—Cheung (3) showed that bovine brain extract after being boiled in neutral or acidic aqueous solution for a few minutes could still activate CAMP phosphodiesterase. To investigate further the thermal stability of the activator, we examined the effect of boiling on the activity of the activator from bovine heart at various stages of its purification. In order to measure accurately the activator activity prior to the boiling treatment, all samples were first heated at 80° for 3 min to eliminate the CAMP phosphodiesterase activity. With pure activator which did not exhibit phosphodiesterase activity, it was found that heating at 80° for 3 min had no effect on the activator activity. Our results indicated that the bovine heart protein activator was stable to boiling at pH 7.5 for 5 min at all stages of its purification. Partial loss in the activator activity (15 to 40%) did, however, result from the boiling. The activator samples in the presence or absence of 1 mM Mg²⁺ showed essentially identical thermal stability.

Although the activator possesses remarkable thermal stability, we have observed significant loss in total activator activity during its purification. In the Sephadex G-100 chromatography step, total activities recovered from the column were 60 to 70% of those applied. In two experiments when Mg²⁺ was omitted from the buffer solutions, the activator activities recovered from the Sephadex column were 1.5 and 5%. The requirement of Mg²⁺ in the buffer to provide good recovery of the activator activity suggests that Mg²⁺ stabilizes the protein activator.

To test further the effect of Mg²⁺ on the activity and stability of the activator, a purified sample of the activator in 0.02 M Tris-HCl buffer (pH 7.5) containing 1 mM imidazole was diluted into the same buffer containing various concentrations of Mg²⁺ and then incubated at 4°. At various times after the incubation, the activator activity in the diluted samples was determined. As shown in Fig. 6, samples incubated in high concentrations of Mg²⁺ (10 mM or greater) have much lower activator activities. More than 50% of the activity was lost after incubation for 1
Fig. 6. Stability of the protein activator at different concentrations of Mg**+. The protein activator fraction from the Sephadex G-100 column was rendered free of Mg**+ by a further gel filtration through a Sephadex G-25 column pre-equilibrated with 0.02 M Tris-HCl buffer (pH 7.5) containing 1 mM imidazole and eluted with the same buffer. The protein activator was concentrated by ultrafiltration through a UM-2 membrane. Protein activator (180 units) was diluted into 5 ml of 0.02 M Tris-HCl and 1 mM imidazole buffer (pH 7.5) containing different concentrations of magnesium acetate. These solutions were stored at 4°, and aliquots of 0.1 ml were used for the determination of activator activity at different time intervals. Other conditions for the phosphodiesterase assay were as in Fig. 1. Activator activity at zero time, ○; first day, □; second day, △; sixth day, □.

day. Samples in low concentrations of Mg**+ (1 mM or lower) also lost their activity but at slower rates. After 5 days of incubation, these samples lost approximately 30% of their original activities. These results suggest that the activator may be a labile protein and that this may account for the loss in activity during its purification. There is, however, no significant difference in the activator stability between samples incubated with 1 mM Mg**+ and without Mg**+.

One possible explanation is that the protein activator contains tightly bound Mg**+ which dissociates from the protein only to a slight extent. The tight binding of Mg**+ may account for the stability of the activator in the absence of added Mg**+, whereas the slight dissociation may explain the loss in activity during Sephadex G-100 chromatography in the absence of Mg**+. To test further the possibility that the protein activator contains tightly bound Mg**+, the effect of low levels of EDTA on the stability of the activator was examined. Fig. 7 shows that inclusion of 1 mM EDTA in the incubation solution results in a rapid and extensive loss in the activator activity during storage at 4°. In contrast, the activator activity decreases gradually in the same buffer in the absence of EDTA. In all of the experiments described in Figs. 6 and 7, a small amount of EDTA (approximately 0.1 mM) and additional Mg**+ (maximally 3 mM) were carried over from the activator sample into the assay mixture. Separate experiments indicated that phosphodiesterase activity was not affected by increasing the Mg**+ concentration from 20 to 30 mM nor by inclusion of 0.1 mM EDTA in the assay mixture.

Attempts to maintain the activator activity at 4° have not been successful. Slow decrease in activity was observed with samples in 1 mM Mg**+ at pH values ranging from 4 to 10, or at pH 7.5 with protein concentrations ranging from 10 μg per ml to 2 mg per ml. The activator activity could be maintained for over half a year when samples were stored frozen at -20 to -15°.

**Activation Properties**—Recently, we have shown that progress curves for reactions catalyzed by bovine heart cAMP phosphodiesterase often exhibit initial nonlinear regions followed by linear reaction rates. From a study on the factors affecting the characteristics of these curves, it has been suggested that the interaction between the activator and the enzyme is a slow process relative to the enzyme catalysis (8). Thus, the slow enzyme-activator interaction may account for the nonlinear regions of the progress curves, whereas the linear regions represent the steady state rates of the enzyme reactions. To study further the mechanism of activation of cAMP phosphodiesterase by the protein activator, the steady state kinetic properties of the enzyme reaction were examined. The steady state velocity, v, was determined from the slopes of the linear regions of the progress curves. Each reaction was analyzed for product formation at three or four times intervals. The first analysis was usually at 10 min, since it was shown that the nonlinear regions lasted less than 10 min (8). The product formation was then plotted as a function of the reaction time to obtain the slope for the calculation of the steady state velocity.

Other workers (3, 4) have observed that the activator activates the enzyme by both enhancing the V_max and decreasing the K_m for cAMP. Similar results have been obtained using the purified activator. Fig. 8 shows that addition of 1.7 units of the protein activator to the enzyme reaction mixture results in a more than 3-fold increase in the maximum velocity of the reaction. This low level of the activator shows little effect on the K_m for cAMP. If the activator concentration is increased to 3.3 and 11 units, the K_m for cAMP decreases to 0.67 and 0.4 μM, respectively. However, no further increase in V_max is observed. Thus, although both V_max and K_m of the cAMP phosphodiesterase are influenced by the protein activator, the two effects are observed at different concentrations of the activator.

The effect of cAMP concentration on the interaction between the enzyme and the activator has also been examined by kinetic means. Fig. 9 shows that the dependance of the steady state velocity of cAMP phosphodiesterase upon the activator concentration may be influenced by the level of cAMP in the assay mixture. As cAMP concentration increases from 0.5 to 2 mM,
FIG. 8. Reciprocal plot of reaction velocity and cAMP concentration. The reaction velocities shown here are the slopes of the linear "steady state" region of the progress curves of the enzyme reaction (see text for details). Each 0.9 ml reaction mixture contained 0.021 units of activator-deficient phosphodiesterase enzyme, 40 mM Tris-HCl (pH 7.5), 40 mM imidazole, and 20 mM magnesium acetate. The velocities plotted here are expressed as O.D. 650 per 10 min. No protein activator added, 0; 1.7 units of protein activator added, □; 3.3 units of protein activator added, ●; and 11 units of protein activator added, ■.

FIG. 9. Plot of reaction velocity against protein activator concentration at different levels of cAMP. The procedure used here is similar to that described for Fig. 8. ○: 0.5 mM cAMP, ●: 1.0 mM cAMP, and ■, 2.0 mM cAMP.

the activator concentration required for 50% maximum activation decreases from 3.1 to 1 units. The results are in agreement with a previous suggestion that the affinity of the protein activator toward the enzyme units may be enhanced by cAMP (7).

To test further the suggestion that cAMP phosphodiesterase exists as an equilibrium mixture of the enzyme, the activator and the protein complex, the dependence of the steady state velocity of the enzyme reaction on the enzyme concentration has been examined. Fig. 10 shows that, when a mixture of activator-deficient enzyme and the purified activator is used as the phosphodiesterase sample, the reaction velocity shows a

FIG. 10. Dependence of the enzyme reaction on the enzyme concentration. ●, activator-deficient phosphodiesterase enzyme with no protein activator added; ○, 40 units of protein activator mixed with 1.6 units of activator-deficient phosphodiesterase enzyme and then preincubated for 10 min. Various amounts of this mixture of enzyme and protein activator were used. The reaction velocity, V, is expressed as O.D.650 per 10 min.

Structure of the Activator

Amino Acid Composition—Amino acid composition of the purified protein activator is presented in Table II. Due to the low amount of material available for analysis, determinations for tryptophan and half-cystine were not carried out. The possible existence of non-protein material such as carbohydrate and lipid was not tested either. The fact that the protein activator was stained on polyacrylamide gel by the periodate-Schiff reagent suggests that it may contain bound carbohydrate (15).

Molecular Weight—Sedimentation and diffusion measurements have been carried out to determine the molecular weight of the purified protein activator. The sedimentation coefficient of the protein activator increases gradually with the decrease in the protein concentration. The sedimentation constant, \( s_{20, w} \), determined by extrapolation to infinite protein dilution is 2.0 S. Diffusion measurements have been carried out at three different protein concentrations. The diffusion coefficient is essentially independent of the protein concentration and an average value of \( 9.05 \times 10^{-7} \) cm\(^2\) per s has been obtained as \( D_{20,w} \). By using these values and a partial specific volume, \( \bar{\rho} \) of 0.71 ml per g, the molecular weight of the protein activator is calculated by the Svedberg equation to be 19,200. The partial specific volume is calculated on the basis of the amino acid composition of Table II as has been described by Cohn and Edsall (16).

By using the technique of gel filtration on a Sephadex G 100 column, Cheung (3) has determined the molecular weight of the brain cAMP phosphodiesterase activator to be 40,000. The
known that several glycoproteins behave anomalously on gel filtration columns (17).

The molecular weight of the bovine heart protein activator was measured by the gel filtration technique on a Sephadex G-75 column. Fig. 11 shows that the molecular weight determined by this method has a value of approximately 27,000, considerably lower than the value obtained by Cheung for the activator from bovine brain (3). It is not clear whether this difference in molecular weight values arises from the difference in tissue origins or the state of purity of these activator samples. The protein activator of Cheung's sample was relatively impure (3). In addition, the molecular weight of the bovine heart protein activator determined on the Sephadex G-75 column is significantly higher than the value obtained by the sedimentation-diffusion methods. The reason for this discrepancy is not clear. One possibility is that the activator may be a glycoprotein. It is known that several glycoproteins behave anomalously on gel filtration columns (17).

### Table II

| Amino acid | 24-Hr hydrolysis | 72-Hr hydrolysis |
|------------|-----------------|-----------------|
| Lysine     | 2.75            | 2.70            |
| Histidine  | 0.40            | 0.51            |
| Arginine   | 1.44            | 1.33            |
| Aspartate  | 6.27            | 6.02            |
| Threonine  | 3.26            | 2.87            |
| Serine     | 1.47            | 1.38            |
| Glutamate  | 7.06            | 7.03            |
| Proline    | 1.00            | 1.00            |
| Glycine    | 3.54            | 3.69            |
| Alanine    | 3.64            | 3.59            |
| Cystine    | 1.97            | 1.96            |
| Methionine | 1.40            | 0.65            |
| Isoleucine | 1.95            | 1.92            |
| Leucine    | 2.88            | 2.84            |
| Tyrosine   | 0.46            | 0.24            |
| Phenylalanine | 2.20   | 2.22          |
| Tryptophan |                |                 |

* The amino acid composition is expressed as molar ratio, with proline equivalent to 1.00.

* The amount of proline from 24 hours of hydrolysis is identical with that from 72 hours of hydrolysis.

### Table III

| Determination           | Method used   | Value          |
|------------------------|---------------|----------------|
| $s_{20,w}$             | Ultracentrifugation | 2.0S           |
| $D_{20,w}$             | Ultracentrifugation | $9.0 \times 10^{-7}$ cm² per s |
| $v$ (partial specific volume) | Calculated from amino acid composition | 0.71 ml per g |
| $M_w$ (molecular weight) | Calculated from $s_{20,w}$ and $D_{20,w}$ | 19,200         |
| $M_w$ (molecular weight) | Gel filtration | 27,000         |
| $f/m_w$ (frictional ratio) | Calculated from $D_{20,w}$ and $M_w$ | 1.35           |

* Molecular weight, $M_w$, calculated from $s_{20,w}$ and $D_{20,w}$.

The various physical parameters for the protein activator of cAMP phosphodiesterase determined in the present study are summarized in Table III. The activator appears to be a globular protein with a frictional ratio, $f/m_w$, of 1.35. The molecular weight determined by the sedimentation and diffusion method is probably the more reliable one and may be taken at present as the molecular weight for this protein activator. This value, however, is subject to modification since the partial specific volume is not definitely established in this study. To obtain a more accurate value for this parameter, the contents of carbohydrate and other nonprotein material of the activator, if present, should be quantitated.

### DISCUSSION

Several groups of investigators have encountered large losses in enzyme activity during purification of cAMP phosphodiesterase from mammalian sources (3, 9, 18). Cheung (3) has been the first to establish that this loss in enzyme activity is partly due to the removal of a specific activator from the enzyme preparations. Since this activator is susceptible to trypsin treatment, Cheung (3) has suggested that it is a protein molecule. This suggestion is fully substantiated in the present study. The activator has been purified to apparent homogeneity as judged by ultracentrifugal and disc gel electrophoretic criteria. Chemical and physical characterization of the pure activator indicate that it is a globular protein. The molecular weight of the protein activator as determined by sedimentation-diffusion method is 19,200. Gel filtration method, however, gives an estimated molecular weight of 27,000. The nature of this discrepancy in molecular weight is not clear. One possible explanation is that the protein activator contains carbohydrate moieties as prosthetic groups. Several glycoproteins have been found to give higher apparent molecular weights upon Sephadex gel filtration (17). The association of carbohydrate with the protein activator is suggested by our preliminary studies which indicate that the activator may be stained by the periodate-Schiff reagent on the polyacrylamide gels.

Since the specific activity of the purified protein activator is approximately 8,000-fold that of the crude tissue extract, this protein exists in the cardiac muscle in minute amounts. The low tissue level of the activator has made it difficult to prepare the pure protein activator in large quantities.

The fact that low concentrations of EDTA cause a rapid and extensive inactivation of the protein activator suggests that the activator is a metalloprotein. Since inclusion of Mg²⁺ in the
buffer solutions greatly enhances the recovery of the activator activity during its purification, it seems possible that Mg$^{2+}$ is the intrinsic metal ion of the protein molecule. The effects of Mg$^{2+}$ on the activity and stability of the activator, however, are complex. The metal ions do not protect the protein activator against inactivation during storage at 4° or during heat treatment at 100°. Nor do they reverse the inactivation of the protein activator brought about by EDTA. Furthermore, when Mg$^{2+}$ concentration in the buffer solution exceeds 10 mM, a rapid and metal concentration-dependent inactivation of the protein activator may be observed. These observations indicate that further investigations on the EDTA and Mg$^{2+}$ effects are needed to delineate the nature of these effects.

Butcher and Sutherland (10) showed that the activity of cAMP phosphodiesterase at pH 7.5 was activated by imidazole to more than 100%, whereas we observed only slight imidazole stimulation of the enzyme. In view of the finding that there are multiple forms of cAMP phosphodiesterase in most tissues, it may be suggested that our enzyme preparation contains predominantly one specific form of the phosphodiesterases. The fact that some of the multiple forms of cAMP phosphodiesterase have $K_m$ values for cAMP much lower than that reported in this study supports such a suggestion. It is, therefore, possible that the protein activator is acting selectively on this specific form of the enzyme rather than on all cAMP phosphodiesterase present in the cells.

Although Cheung (3) has raised the possibility that the protein activator may be a subunit of cAMP phosphodiesterase, he has also pointed out the paradoxical phenomenon that the activator concentration in crude tissue extracts appears to be in excess over the amount of enzyme. Results of the present study suggest that the enzyme and the activator are separate proteins rather than subunits of an active enzyme. The two proteins may interact reversibly with each other to establish an equilibrium mixture of the free enzyme (E), free activator (A) and the active complex (EA) as follows:

$$E + A \rightleftharpoons EA \quad (1)$$

The equilibrium conditions among the three protein species may be functions of the intracellular environment. This suggestion is supported by at least three observations. (a) Titration of the enzyme by the activator by kinetic means shows that the enzyme saturation is approached asymptotically rather than abruptly; (b) the activator concentration at the mid-point of the titration curve for a constant amount of the enzyme increases as the cAMP concentration in the assay mixture is decreased, and (c) the dependence of enzyme activity upon the protein concentration of a mixture of enzyme and the activator is nonlinear with upward curvature. Since cAMP enhances the interaction between the enzyme and the activator, the apparent excess of the protein activator in the tissue extract may be a result of the high cAMP concentrations in the assay mixture used by Cheung (3) and by us. The concentrations of cAMP in the cells are known to be much lower (19). In addition, other conditions, such as pH, temperature, ionic strength, and the presence of inhibitors, may also influence the strength of the interaction between the enzyme and the activator. Thus, it is quite possible that the enzyme is not saturated by the activator under the intracellular conditions, a situation essential for the activator to exert its regulatory effects. It appears that the activity of cAMP phosphodiesterase may be continuously modulated by its substrate which dictates the amount of the active protein complex to be formed. In addition, we have suggested previously that the establishment of the equilibrium among the enzyme, the activator, and the complex of cAMP phosphodiesterase is slow relative to the enzyme catalysis (8). This hysteretic effect (20) also appears to be advantageous in the regulation of cAMP concentration since a temporary high concentration of the cyclic nucleotide may often be desirable.

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