Conformational Transition Accompanying the Binding of Ca^{2+} to the Protein Activator of 3′,5′-Cyclic Adenosine Monophosphate Phosphodiesterase†

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ABSTRACT: The Ca^{2+}-dependent protein activator of 3′:5′-cyclic adenosine monophosphate phosphodiesterase is shown to undergo a conformational transition upon binding of 2 mol of Ca^{2+}/mol of activator. Circular dichroic studies indicate that Ca^{2+} induces an increase of 5–8% in α-helix content with a concomitant decrease in the amount of random coil. In the absence of Ca^{2+} and in the presence of [ethylenebis(oxoethylenenitrilo)]tetraacetic acid (EGTA), the protein contains 30–35% α helix, 50% random coil, and 15–20% β-pleated sheat. Spectrophotometric titration indicates that the two tyrosyl residues have pK's of 10.4 and 11.9 and are therefore in different environments. The Ca^{2+}-induced conformational change is accompanied by an increased exposure to protons of the partially exposed tyrosine, as shown by a shift in its pK from 10.4 to 10.1. Increased solvation is also consistent with a negative difference spectrum at 287 and 279 nm as seen upon Ca^{2+} binding. Modification in the environment of all or some of the phenylalanine residues also is part of the conformational change accompanying Ca^{2+} binding. A new and rapid purification procedure which yields large amounts (25–30% yields) of homogeneous protein activator and a direct and sensitive assay procedure for cAMP phosphodiesterase and its activator are also described.

The protein activator of 3′:5′-cyclic adenosine monophosphate phosphodiesterase, which has recently been shown to stimulate soluble brain adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975), was first reported by Cheung (1967) and characterized by Cheung (1970, 1971) and Kakiuchi et al. (1970) in brain extracts and by Goren and Rosen (1972) in heart extracts. It was purified to homogeneity from bovine heart by Teo et al. (1973) and from bovine brain by Lin et al. (1974a,b). The activation of the enzyme by its activator protein is dependent upon Ca^{2+} (Kakiuchi et al., 1973; Teo and Wang, 1973). Ca^{2+} binds to the activator in the absence of enzyme, and induces changes in UV[1] absorption and fluorescence spectra (Wang et al., 1975). The Ca^{2+}-activator complex is the functional form of the protein (Kakiuchi et al., 1973; Teo and Wang, 1973; Lin et al., 1974a,b).

The Ca^{2+} activation of cAMP phosphodiesterase by the activator protein has been compared to the Ca^{2+}-dependent stimulation of actomyosin ATPase by troponin (Wang et al., 1975). The similarity in the physical and chemical properties of troponin C and the activator protein led Wang et al. (1975) to propose that the two proteins may be specified by genes derived from a common ancestral gene. Despite its structural similarity, troponin C fails to replace the activator protein in stimulation of the diesterase (Wang et al., 1975). Other differences between the two proteins that may be related to their different biological activities emerge from the spectral data provided in the present study.

In the present paper, the UV-absorption changes which accompany the binding of Ca^{2+} to the activator protein are shown to be correlated with the perturbation of the environment of one specific tyrosine residue. In the Ca^{2+}-free protein, this tyrosine is partially buried. Upon binding of Ca^{2+}, exposure of this tyrosine residue to solvent is increased as part of a large conformational change that affects some or all of the phenylalanine residues as well, and results in a small increase in α-helix content.

A new, high yield, purification procedure for the activator protein and a direct and sensitive assay procedure for cAMP phosphodiesterase and its activator are also described in this communication.

Materials and Methods

Adenosine 5′-monophosphate (AMP), 3′,5′-cyclic adenosine monophosphate (cAMP), [8-^{3}H]cAMP, and [8-^{14}C]AMP were products of Schwarz/Mann. To ensure low blanks in the assays, the labeled compounds were purified under the assay conditions, using the same columns and deionized water as eluant. They can be stored frozen for 2 months. [^{14}C]AMP was tested for purity on PEI-cellulose plates using two different solvents and was of more than 99% isotope purity. Solutions of imidazole (Gallard-Schlesinger Chemical Corp.) were filtered through acid-washed charcoal prior to use. Methanesulfonic acid, 4 N, containing 0.2% of 3-(2-aminoethyl) indole was a product of Pierce Chemical Co.

Circular Dichroism. Circular dichroic measurements were performed using a Cary 6001 attachment to a Cary 60 spectropolarimeter. The measurements were made at 26 °C in 0.01 M Tris-HCl (pH 7.5), 0.05 M NaCl, in cells with a light path of 1 mm. Scans were performed in duplicate at very low speed using a 10-s pen period and the sensitivity was 0.04°/100% of the chart. No time-dependent modification was observed. The MRW calculated from the amino acid analyses was 112.9.

UV-Absorption Spectra. UV-absorption spectra were measured with a Cary, Model 118 C, spectrophotometer equipped with a microcell holder (Model 1643400) and a reference beam attenuator (Model 14473475). Measurements

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[1] Abbreviations used are: UV, ultraviolet; MRW, mean residue weight; CD, circular dichroism; PDE, phosphodiesterase; cAMP and cGMP, cyclic adenosine and cyclic guanosine monophosphates; ATPase, adenosine triphosphatase; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

were made at 26 °C in a cell with a light path of 1 cm at a speed of 0.2 nm/s. Absorbance ranges of 0.02–0.2 were routinely used.

Spectrophotometric Titrations. The UV spectra were recorded twice, at 5-min intervals after each addition of NaOH. The pH was measured directly in the experimental cuvette and corrected for the effect of sodium ions at pH values above 11.0. The titrations were also done in 0.1 M piperidine buffers and corrected for time-dependent changes at high pH. Below pH 11, no time-dependent change was observed. The 100% Δ_{294nm} M^{-1} cm^{-1} was calculated from the protein concentration assuming two tyrosines/mol and an $\epsilon_{294nm}{}^{M}$ for the difference spectrum of 2357 per tyrosine (Mihalyi, 1970). Reversibility was monitored at the end of the experiment by bringing the pH to 6.5 with HCl.

Amino Acid Analysis. The protein samples were first dialyzed against 0.05 M $(NH_4)HCO_3$ and lyophilized three times to dryness in hydrolysis tubes (Pierce Chemical Co.) to remove $(NH_4)HCO_3$. Hydrolysis in constant boiling HCl or 4 N methanesulfonic acid (Simpson et al., 1976) was performed at 110 °C under nitrogen. One crystal of phenol was added to ensure good recoveries of tyrosine. Performic acid oxidation was as described by Hirs (1967). Analyses were performed on a Beckman, Model 120 C, amino acid analyzer equipped with a Durrum single-column system using the Durrum Pico-buffer system II developed by Benson (1972) with a DC-6A resin.

Disc Gel Electrophoresis. Gel electrophoresis was performed with a 7.5% polyacrylamide gel according to the method of Davis (1964). Gel electrophoresis in the presence of sodium dodecyl sulfate and 1% β-mercaptoethanol was as described by Neville (1971) using an 11% gel. Proteins were stained with Coomassie blue.

Removal of Contaminating Metals. The activator was freed of contaminating metals by passage through a chelex column as described by Teo and Wang (1973). A 1.5-mL column was used for 3-mL solution of activator (0.5 mg/mL) in 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl. The eluted protein was readjusted to pH 8.0 by addition of 40 μL of 0.9 M chelex-treated Tris-HCl buffer (pH 8.0) per mL. Alternatively, the activator was treated with 1 mM [^{14}C]EDTA and dialyzed repeatedly against the appropriate buffers to remove the chelating agent. Radioactivity was used to measure the residual concentration of EDTA. Teflon, polyethylene containers, glassware, and dialysis tubing were treated as described previously (Klee, 1972).

Enzymatic Assays

Phosphodiesterase activity was assayed by direct measurement of the product of the reaction, 5′-AMP. The assay is based on the fractionation of 3′,5′-cAMP and 5′-AMP by Dowex-50 columns (Sutherland and Rall, 1958; Schultz et al., 1974). The two nucleotides were eluted sequentially with water. cAMP, with a pK of 3.6,[2] was eluted first as a sharp peak, at a pH of 3.5. 5′-AMP (pK 3.8 (Dunn and Hall, 1970)) was eluted later, free of cAMP, as a broad peak. cGMP and GMP (pK values of 2.0[2] and 2.4 (Dunn and Hall, 1970), respectively) were eluted earlier and did not separate from each other with water as eluant.

cAMP phosphodiesterase assay; AG-50 W × 8, 200–400 mesh, H^+ form (Bio-Rad) was suspended in water; enough slurry was poured into disposable polypropylene columns (0.7 × 4 cm, Bio-Rad) to provide a column volume of 1 mL. Prior to use, the columns were washed with 2 mL of 1 N NaOH, then with 4 mL of 1 N HCl, and finally with 8 mL of water. After use, the columns were washed with 2 mL of 1 N NaOH and stored at room temperature. During storage, the tips were immersed in NaOH to avoid drying. The columns can be reused over a period of at least 1 year. The reaction mixtures (0.1 mL) routinely contained 0.04 M Tris-HCl, pH 8.0, 0.05 mM $CaCl_2$, 3 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin (Pentex), [^{3}H]cAMP at concentrations between 0.4×10^{-6} and 2×10^{-3} M (30 000–50 000 cpm), and 10^{-7} M [^{14}C]AMP (1000 cpm). Incubation, at 30 °C, was started by addition of enzyme ($7–10 \times 10^{-5}$ units of enzyme measured with a saturating concentration of activator) and the reaction was stopped by addition of 1 mL of 5% trichloroacetic acid containing 1 mM AMP and 1 mM cAMP. [^{14}C]AMP was present during the assay to permit estimation of the recovery of AMP after chromatography and correction of assay values for losses due to contaminating enzymes. The sample was applied to the columns which were washed with successive additions as follows: twice with 1 mL of water and three times with 3 mL of water. To ensure low blank values, no liquid was allowed to be present at the top of the column prior to each addition. The eluate from these washes, which contained the cAMP, was discarded and AMP was eluted with 4 mL of 0.4 M sodium citrate (adjusted to pH 7.5 with citric acid): the 4-mL eluate was collected directly into a scintillation vial and counted as a gel after addition of 12 mL of Aquasol/4-mL sample (Salomon et al., 1974). The procedure takes 60–80 min regardless of the number of samples. Radioactivity was measured in a Beckman LS 335 liquid scintillation spectrometer using the narrow ^{3}H and ^{14}C windows. The nanomoles of [^{3}H]AMP produced during the reaction was calculated from the ^{3}H counts corrected for ^{14}C spillover, blank subtraction, and AMP recovery.[3] The blank values (^{3}H counts in the AMP fraction) with reaction mixtures stopped at zero time or incubated without enzyme were reproducibly low (0.1–0.6% of the starting [^{3}H]cAMP counts). The elution pattern of adenosine, a product of degradation of AMP, was also tested; although 30% was eluted with AMP, most remained on the column and was washed off by the NaOH wash.

To eliminate variations in the column-elution patterns resulting from the wide range of concentrations of substrate (cAMP) and product (AMP) anticipated in experiments, high concentrations of both were added to reactions at the end of the enzymatic reactions. Addition of various other reagents such as 5 mM EDTA, 5 mM Ca^{2+}, 5 mM Mg^{2+}, or imidazole to the reaction mixture did not significantly affect AMP recoveries or cAMP blanks. Similar results were obtained with 0.2 mL of incubation mixture, instead of the standard 0.1 mL. This assay procedure, which measures the product of the reaction catalyzed by cAMP phosphodiesterase directly, avoids the potential problems caused by a coupled enzymatic system. The great sensitivity, low-blank values and the inclusion of a correction procedure for AMP recoveries makes it suitable for quantitative studies over a wide range of substrate concentrations (10^{-7}–10^{-3} M).

One unit of cAMP phosphodiesterase is the amount of enzyme which catalyzes the hydrolysis of 1 μmol of cAMP/min

[2] The pK' values of 3′,5′-cAMP and 3′,5′-cGMP were determined by spectrophotometric titration in citrate phosphate buffer at 25 °C.

[3] With purified enzyme preparations the [^{14}C]AMP recoveries, at cAMP concentrations between 10^{-3} and 5×10^{-7} M, varied between 65 and 80%. The loss corresponds to the 5′-AMP eluted with cAMP during the washes. With crude homogenates, the [^{14}C]AMP recoveries varied between 40 and 50%, indicating the presence of degradative enzymes.

TABLE I: Purification of Pig Brain Protein Activator.

Purification Steps	Vol (mL)	A_{280} (units)	Units $\times 10^{-6}$	Sp Act. (Unit/A_{280})	Yield (%)
(1) Acetone powder extract[a]	2700	16200	6.7	410	100
(2) DEAE-cellulose, 0.3-0.7 M NaCl	800	3700	3.6	980	54
(3) $(NH_4)_2SO_4$ 50-85%, pH 4.5, Sephadex G-100 (44-68)[b]	110	433	2.6	6 000	39
(4) Hydroxylapatite (81-100)[b]	395	30	2.5	83 000	37
(5) DEAE-cellulose (47-56)[b]	48	5.1 (29 mg)[c]	1.6	314 000 (55 000)[d]	24

[a] The data are based on 100 g of acetone powder. [b] The numbers in parenthesis corresponds to the number of the pooled fractions. [c] Protein measured according to Lowry et al. (1951). [d] Units/mg of protein.

at a concentration of 0.2×10^{-3} M cAMP under the assay conditions described above.

Assay of Activator Protein. The activator was assayed by its effect in the assay described above (at 0.2 mM cAMP), using a partially purified bovine brain cortex enzyme prepared according to Cheung and Lin (1974). One unit is the amount of activator needed to give half-maximal activation in the assay described above. Before the Sephadex G-100 step, the activator fractions were tested for diesterase contamination in the absence of added enzyme. If diesterase was present, the activator was boiled for 3 min and centrifuged for 10 min at 0 °C at 2000 rpm. The resulting supernatant fluid was diluted in a 1 mg/mL solution of bovine serum albumin prior to assay. The presence of carrier protein was necessary to avoid losses due to adsorption on the walls of the containers and was routinely added when the activator concentration was less than 0.1 mg/mL.

Purification of Protein Activator of cAMP Phosphodiesterase.

All operations were carried out at 0-4 °C; pH measurements were at 25 °C, except when indicated otherwise. All buffer solutions containing phenylmethanesulfonyl fluoride were prepared 24 h earlier at room temperature and subsequently chilled to 0-4 °C. The purification procedure is summarized in Table I.

(1) Acetone Powder Extraction. Pig brain acetone powder (Sigma, 100 g) was mixed with 1500 mL of 0.1 M Tris-HCl buffer, pH 7.5, containing 2.25 mL of solution of phenylmethanesulfonyl fluoride in dimethyl sulfoxide (50 mg/mL) and stirred for 1 h at 0-4 °C. The suspension was centrifuged for 30 min at 9000 rpm (8900*g*) in a Beckman refrigerated, Model J-21B, centrifuge. The supernatant fluid (volume: 1200 mL) was filtered through glass wool and diluted with 1500 mL of cold deionized water to bring the conductivity to 3.5 mS (measured at 0 °C).

(2) DEAE-Cellulose Concentration Step. The acetone powder extract was mixed with 500 mL of a slurry of 60 g of DEAE-cellulose (Whatman DE-23) equilibrated with 0.02 M Tris-HCl, pH 7.5, 0.001 M $MgCl_2$, and 0.05 M NaCl, and stirred occasionally for 15 min at 0-4 °C. The mixture was filtered through a coarse scintered glass 3-L funnel without vacuum (2 h). The packed resin was washed with 800 mL of the above buffer made 0.25 M in NaCl. Both eluates were discarded (13% of activity). The activator was eluted with 800 mL of the above buffer containing 0.7 M NaCl. The recovery of activity at this step was 60-80%.

(3) Ammonium Sulfate Fractionation. The DEAE-cellulose eluate (fraction 2) was brought to 50% saturation by addition of 29 g/100 mL of ammonium sulfate (Schwarz/Mann, ultrapure). The 50% ammonium sulfate supernatant fluid (900 mL) was brought to pH 4.55 (0 °C) by addition of 30 mL of 5% acetic acid, and then brought to 85% saturation by addition of 213 g of ammonium sulfate. The precipitate was collected and redissolved in 50 mL of 0.01 M Tris-HCl, pH 7.5, containing 0.05 M NaCl and 0.001 M $MgCl_2$. If the pellet did not dissolve readily, the pH was raised to 7.5 by addition of 1 M Tris base. This material was then applied to a (2.5 × 90 cm) Sephadex G-100 column equilibrated and eluted with 0.02 M Tris-HCl, pH 7.5, containing 0.05 M NaCl and 0.001 M $MgCl_2$. The flow rate was 8 mL/h and 5-mL fractions were collected. The activator was eluted after the bulk of the UV-absorbing material, between fractions 40 and 70. After this step, contamination of the activator by the enzyme was negligible and the boiling step prior to assays was omitted.

(4) Hydroxylapatite Chromatography. Fractions 44-68 from the Sephadex G-100 column were pooled and applied to a 2.6 × 30 cm hydroxylapatite column[4] (0.37 mL/A_{280} unit) equilibrated with 0.02 M Tris-HCl buffer, pH 7.4, containing 0.001 M imidazole and 0.1 M NaCl. The sample was washed into the column with 70 mL of equilibrating buffer and the column was eluted with a linear gradient made with 2 L of the above buffer and 1.7 L of the same buffer containing 150 mL of saturated $(NH_4)_2SO_4$ solution (0 °C)/L (0.6 M). The flow rate was maintained at 2 mL/min and 20-mL fractions were collected in tubes containing 0.2 mL of 0.1 M $MgCl_2$. The column was monitored at 280 and 230 nm. Since the activator protein contains no tryptophan (Teo et al., 1973; Lin et al., 1974a,b), the absorbance at 280 nm decreased with purification and absorbance at 230 nm was a more sensitive assay. Activity was eluted between tubes 80 and 120 (0.14-0.23 M $(NH_4)_2SO_4$), as shown in Figure 1.

(5). DEAE-Cellulose Chromatography. Fractions 81-100 of the hydroxylapatite column were pooled (225 A_{230} units, conductivity 27 mS at 0 °C), diluted with 2 volumes of equilibrating buffer, and applied to a 2.6 × 32 cm Whatman DE52 column (0.75 mL/A_{230} unit) equilibrated with 0.02 M Tris-HCl buffer (pH 7.5), 0.001 M $MgCl_2$. The dilution was done in 100-mL batches in polyethylene containers. The flow rate was 140 mL/h. The first eluate contained 64 A_{230} units which did not bind to the column and were devoid of activity. The column was washed with 120 mL of buffer made 2% saturated in $(NH_4)_2SO_4$ (0.08 M) and 200 mL of the same buffer, 3.6% saturated in $(NH_4)_2SO_4$ (0.144 M) which eluted 17 more A_{230} units and no activity. A gradient of 260 mL of the last buffer (0.144 M) and 250 mL of 30% saturated buffer (1.2 M

[4] I wish to thank Mr. David Rogerson for the preparation of hydroxylapatite according to Levin (1962).

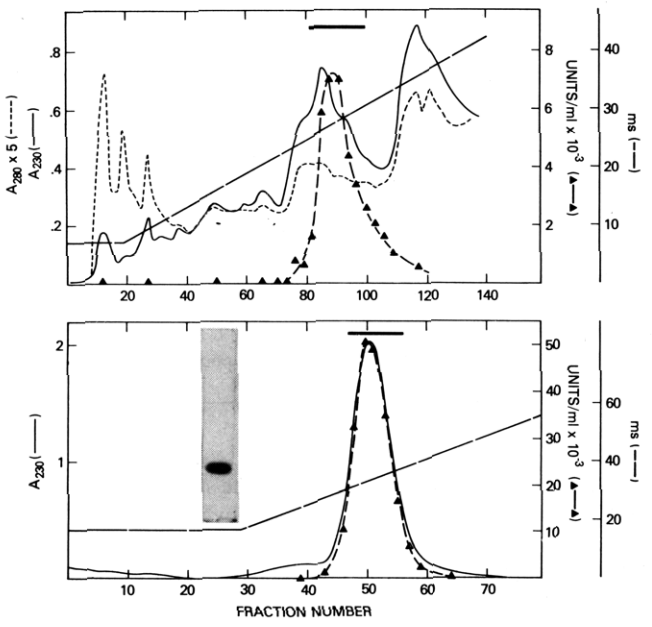

FIGURE 1: Elution diagrams from the hydroxylapatite chromatography, step 4 (top) and DEAE-cellulose chromatography, step 5 (bottom). The bar at the top of the chromatogram indicates the fractions that were pooled. The insert is a polyacrylamide disc gel electrophoresis pattern under native conditions of the pooled fractions from step 5 stained with Coomassie blue.

TABLE II: Amino Acid Composition of Protein Activator.

Amino Acid	Pig Brain	Bovine Heart[a] (residues/mol)[c]	Bovine Brain[b]
Lys	7	9	8
His	1	1	1
Arg	6	6	7
Asp	24–25	25	24
Thr[d]	13	12	14
Ser[d]	6	3	5
Glu	30–29	30	30
Pro	2	2	2
Gly	12–13	12	13
Ala	11–12	12	13
Cys	0	0	0
Val[e]	8	9	8
Met	9	9	11
Ile[e]	9	8	9
Leu	11	10	11
Tyr	2	2	2
Phe	8	9	10
Trp[f]	<0.1	0	0
γ-carboxyGlu[g]	<1	ND	ND

[a] Values from Wang et al. (1975). [b] Values from Lin et al. (1974a,b). [c] The number of residues per mole was normalized to two tyrosines per 16 500 mol wt polypeptide chain. [d] Values obtained after extrapolation to zero-time hydrolysis (the hydrolysis times were 12, 24, 37, and 71 h). [e] Values obtained after 71-h hydrolysis. [f] Tryptophan was determined spectrophotometrically. [g] γ-carboxyglutamic acid was determined according to Fernlund and Stenflo (1975).

$(NH_4)_2SO_4$) was applied to the column and 5-mL fractions were collected. The elution profile is shown in Figure 1. The overall recovery of protein was 85%. The ratio of absorption at 230 and 280 nm increased from 7.5 to 14. This ratio was constant in the pooled fractions (47–56). A good correspondence between activity and absorption at 230 nm was also observed in the peak fractions. A small amount of contaminating protein was found on each side of the activity peak. The fractions from the two sides of the peak were pooled separately and rechromatographed subsequently on the same column (3 mg).

The activator protein is stable at −20 or 0 °C in the presence of 1% toluene. It could be dialyzed in the absence of $MgCl_2$ without loosing activity, as reported by Lin et al. (1974b). Very dilute solutions, however, showed significant losses of activity due to absorption on glass containers, as observed during the spectrophotometric measurements. The activator could be concentrated on hydroxylapatite, or by lyophilization after dialysis against 0.05 M $(NH_4)HCO_3$, without loss of activity.

Comments on the Purification. Two previously published procedures which also yield homogenous protein (Teo et al., 1973; Lin et al., 1974a,b) require large amounts of starting material and result in very poor yields (14–7%). The procedure described above, starting from 100 g of acetone powder (the equivalent of 750 g of brain), eliminated the large scale early-purification steps and the preparative electrophoresis and gave reproducibly 25–30% overall yield of pure protein. The heat step commonly used was omitted because of losses which were probably caused by the trapping of the activator in large precipitates of denatured protein. (The DEAE-cellulose (step 2) fraction lost 50% of its activity after heating.) Bovine acetone powder stored at −20 °C gave slightly higher yields in the purification procedure. Aged preparations of acetone powder stored at −20 °C gave the same yield of activator but contained less cAMP phosphodiesterase.

The physicochemical properties of activator obtained by this method, including its amino acid composition, its sodium dodecyl sulfate gel electrophoretic mobility, its elution on Sephadex G-100, its heat and acid stability, and its Ca^{2+} dependence, were very similar to those described by Teo et al. (1973) and Lin et al. (1974a,b) for the bovine heart and brain activators. Taking into account the differences in units and assay conditions (volume, substrate concentration), the specific activities are also comparable for all these preparations.

Results

The material eluted from DEAE-cellulose was tested for homogeneity by disc gel electrophoresis in polyacrylamide under native conditions (Figure 1, insert) and in the presence of sodium dodecyl sulfate and β-mercaptoethanol. In both cases, a single protein band was observed. The molecular weight of this protein, determined by sodium dodecyl sulfate gel electrophoresis, was 16 500 ± 500 (phosphorylase a, bovine serum albumin, fumarase, lactate dehydrogenase, and α-lactalbumin as molecular-weight markers). The amino acid composition of the protein is shown in Table II. The number of residues per mole of activator was normalized to two tyrosines/mol of protein (determined spectrophotometrically as described below and using a molecular weight of 16 500). The amino acid content was found to be very similar to those published for bovine heart and bovine brain activator proteins (Wang et al., 1975; Lin et al., 1974a,b (Table II)). The absence of tryptophan was confirmed by two independent techniques, spectrophotometrically according to Edelhoch (1967) and by chromatographic analysis after acid hydrolysis in methanesulfonic acid. No significant amount of γ-carboxyglutamic acid was detected after alkaline hydrolysis (the small ninhydrin-positive peak that was eluted at the position of cysteic acid was not characterized further as γ-carboxyglutamic acid, since much less than 1 mol/mol of activator was detected, Table II). No decrease in glutamic acid content was observed in alkaline

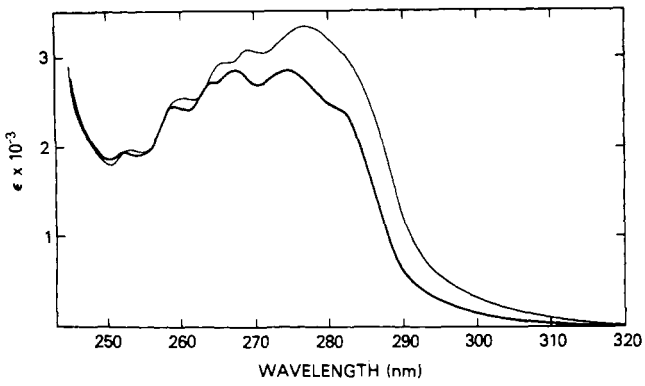

FIGURE 2:. UV-absorption spectrum of protein activator (mol wt 16 500) in 0.01 M Tris-HCl buffer, pH 7.5, containing 0.05 M NaCl (—) and in the same solvent containing 6 M guanidinium chloride (—). Prior to the experiment, a solution of protein, 0.5 mg/mL, was dialyzed against 1000 volumes of 0.04 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 0.001 M EDTA to remove contaminating metal ions and then dialyzed against the same solvent in the absence of EDTA. The spectrum was measured in the dialyzed sample after dilution 1:4 with H_2O or 8 M guanidinium chloride. The protein concentrations were calculated from the absorption at 230 nm in the absence of denaturant.

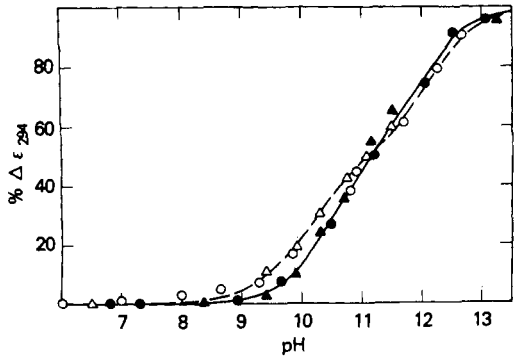

FIGURE 3: Spectrophotometric titration of activator protein. The percent change at 294 nm is plotted as function of pH. EDTA-treated activator in: 0.05 M NaCl (0.13 mg/mL) (●); 0.05 M NaCl, 0.1 M piperidine buffers (0.2 mg/mL) (▲); 0.05 M NaCl, 0.5 mM Ca^{2+} (0.3 mg/mL) (○); 0.05 M NaCl, 0.1 M piperidine buffer, 0.5 mM Ca^{2+} (0.2 mg/mL) (△). The points were determined experimentally. The lines are calculated assuming two titration curves (Henderson–Hasselbach equation) with pK's of 10.4 and 11.9 (—) and pK's 10.1 and 12 (- - -).

digests as compared to acid hydrolysates. This residue has been found at the Ca^{2+}-binding site of vitamin K dependent Ca^{2+}-binding proteins (Fernlund and Stenflo, 1975).

A new ninhydrin-positive peak resistant to performic acid oxidation (1 residue/mol) was consistently found in the acid hydrolysate (24- and 72-h hydrolysates). Its elution profile corresponded to that of a weakly basic amino acid that does not have the titration behavior of an imidazole derivative. An unknown basic amino acid has also recently been described in the activator protein by Vanaman and Watterson (1976). The minimal molecular weight calculated from the amino acid analyses was 18 000.

UV-Absorption Spectra. The concentration of activator was routinely based on the absorption at 230 nm because of the low absorption at 280 nm. The extinction coefficient based on the protein measured by the method of Lowry et al. (1951), using serum albumin as a standard, was $\epsilon_{230nm}{}^{1\%} = 25 \pm 0.5$. No significant difference in ϵ was found between pH 6 and 9 in phosphate, borate, Tris-HCl, or bicarbonate buffers, or in 0.05 M NaCl at pH 6.0. The molecular weight used to determine the molar absorption coefficients was 16 500.

The UV-absorption spectrum of the activator shown in Figure 2 reflects the low tyrosine/phenylalanine ratio (2:8) determined by amino acid analyses. A maximum at 277 nm ($\epsilon_{M,277nm} = 3300 \pm 100$) and a shoulder at 282 nm correspond to the tyrosine spectrum. The multiple peaks at 253, 258.5, 265, and 268.5 nm are characteristic of the fine structure of the absorption band of phenylalanine. At pH 7.5, the ratio of A_{277}/A_{260} was 1.3 under native conditions and 1.2 in 6 M guanidinium chloride. However, this ratio may sometimes be lower because of light scattering. The spectrum in 6 M guanidinium chloride (Figure 2) showed a blue shift of 2 nm accompanied by a 15% decrease in the intensity of the peak absorption. These changes are usually indicative of the unfolding of the polypeptide chain and the negative $\Delta\epsilon_{287nm}$ of $-750\ M^{-1}\ cm^{-1}$ corresponds to the exposure of one tyrosine residue. The spectrum of the protein in guanidinium chloride was almost identical with that of an aqueous solution containing the same relative concentrations of *N*-acetyltyrosineamide and *N*-acetylphenylalanineamide (2:8) as the corresponding amino acid residues in the activator, except for a 0.5–1-nm red shift as is usually observed (Edelhoch, 1967). A slightly greater absorption at 290 nm in the UV spectrum of the activator when compared with the model compounds could be due to a small amount of contamination by protein-containing tryptophan (<0.1 residue/mol) or by a metal ion–protein complex. A similar observation has been reported for troponin C (Greaser and Gergely, 1973), a protein very similar to the activator but is not yet understood. A tyrosine residue with an abnormally low pK could also have resulted in such a spectrum. Spectrophotometric titration curves for the activator in the presence and absence of Ca^{2+} are shown in Figure 3. The experimental data were described well by the sum of two titration curves with pK's of 10.1 and 12 or 10.4 and 11.9 (in the presence and absence of Ca^{2+}, respectively). The data indicate that, although the two tyrosines present in the molecule have different environments, no abnormally low pK was determined and only a negligible fraction of the residues is ionized at pH 7.5. Interestingly, one of the tyrosines appears buried in the interior of the protein molecule (pK of 11.9–12) and is probably the one primarily responsible for the difference spectrum observed in 6 M guanidinium chloride. The second tyrosine residue, which is at or near the surface of the protein, is affected by Ca^{2+} binding.

Effect of Ca^{2+} on the UV Spectrum. The UV-absorption spectrum of bovine heart activator protein has been reported to show a small decrease upon addition of Ca^{2+} (Wang et al., 1975). The addition of 0.5 mM $CaCl_2$ also resulted in an 8% decrease of the peak absorption of the pig brain activator (data not shown). This decrease was completely reversed by 2.0 mM EGTA. Removal of Ca^{2+} by EGTA was also followed by a slow, time-dependent, increase in absorption which was apparently due to light scattering. Addition of 1.5 mM $MgCl_2$ resulted only in a negligible change in the spectrum and did not prevent the Ca^{2+}-induced change upon subsequent addition of 0.9 mM $CaCl_2$, in agreement with the results of Lin et al. (1974b) on the lack of effect of Mg^{2+} on the binding of Ca^{2+}. The small response to Mg^{2+} could reflect a difference in the binding constant of the activator for the two metal ions. It certainly indicated that the Donnan effects are not responsible for the spectral changes observed. Indeed, a similar Ca^{2+}-dependent decrease of absorption was observed in the presence of 0.3 M $(NH_4)_2SO_4$.[5]

[5] In the presence of 0.3 M $(NH_4)_2SO_4$, the same changes were observed but higher Ca^{2+} concentrations were required.

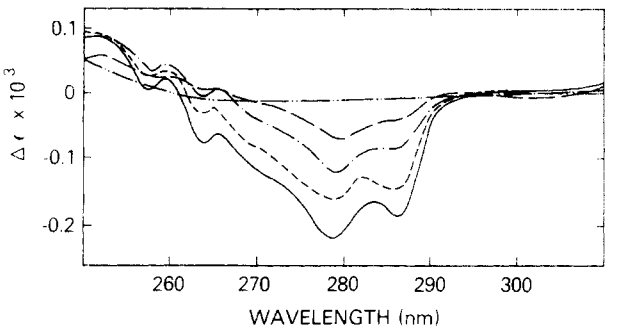

FIGURE 4: Difference spectra of activator protein produced by increasing concentrations of $CaCl_2$. Chelex-treated activator in 0.01 M Tris-HCl, pH 8.0, 0.05 M NaCl (0.5 mg/mL) was used for these studies. The difference spectra were recorded after successive additions of $CaCl_2$ against a reference cell containing the same protein solution with successive additions of water. The data were corrected for dilution and are expressed as difference in molar absorption, $\Delta\epsilon$. The temperature was 25 °C (— —), 19×10^{-6} M Ca^{2+}; (— • —), 31×10^{-6} M Ca^{2+}; (- - -), 51×10^{-6} M Ca^{2+}; (——), 72×10^{-6} M Ca^{2+}; (— •• —) 0.2×10^{-3} M EGTA.

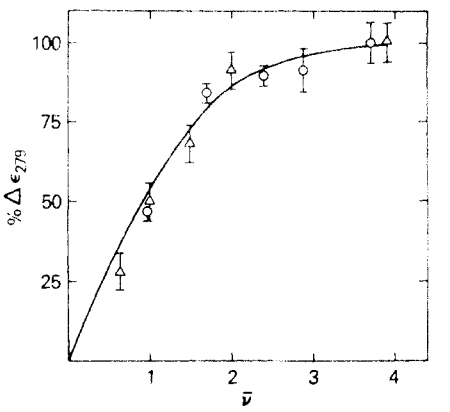

FIGURE 5: Changes in $\epsilon_{279nm}{}^{M}$ as a function of Ca^{2+} binding to protein activator. The different symbols correspond to two different experiments (△), described in legend to Figure 4. (○) The difference spectra of activator protein (0.44 mg/mL) in 0.02 M Tris-HCl, pH 7.5, 0.05 mM NaCl at 26×10^{-6}, 52×10^{-6}, 78×10^{-6}, 100×10^{-6}, and 440×10^{-6} M Ca^{2+}. The number of moles of Ca^{2+} bound per mole of activator ($\bar{\nu}$) was calculated as indicated in the text.

Ultraviolet difference spectra upon addition of increasing amounts of $CaCl_2$ to a solution of activator freed of contaminating Ca^{2+} by passage through a chelex column are shown in Figure 4. Both tyrosine and phenylalanine chromophores underwent an alteration in their environment. The appearance of a double negative trough at 286 and 279 nm is characteristic of a blue shift of the tyrosine-absorption band (Donovan, 1973). The higher amplitude of the 279-nm change reflects a decrease in absorption intensity, as well as the blue shift. Alternatively, the small intensity of the 287-nm trough relative to the 279-nm peak may reflect the decreased steepness of the curve due to the high absorption above 280 nm. A negative difference spectrum at 279–287 nm is usually interpreted as an increased exposure of tyrosine residues to the solvent (Donovan, 1973). The small molar absorbance change of −170 M^{-1} cm^{-1} at 287 nm indicates that only a small change in the environment of the two tyrosine residues has occurred. These data, taken together with the fact that Ca^{2+} decreases the pK of the partially exposed tyrosine (pK 10.4 to 10.1) without significantly affecting the buried tyrosine (pK of 11.9–12), suggest that Ca^{2+} modifies the environment of the exposed tyrosine. This may occur through a conformational change which also affects the environment of some or all of the phenylalanine residues (note a sharpening of the fine structure of the absorption bands below 270 nm). Also shown in Figure 4, addition of EGTA abolished all the difference spectrum; the absence of any positive peak is evidence that the starting material in the control cuvette was not significantly contaminated by Ca^{2+} ions.

The same preparation of activator was tested by equilibrium dialysis under similar conditions (26 °C, 0.01 M Tris-HCl, pH 8.0, 0.05 M NaCl) for its ability to bind Ca^{2+}. Two classes of nonequivalent or interacting sites with dissociation constants of 4×10^{-6} and 1.2×10^{-5} M, respectively, were observed. Each class of sites bound 2 mol of Ca^{2+} (data not shown). Slightly different results were obtained by Lin et al. (1974b) at somewhat different temperatures and ionic strengths. The constants obtained by equilibrium dialysis were used to determine the moles of Ca^{2+} bound per mole of activator ($\bar{\nu}$) in the UV-absorption experiments shown in Figure 4. The percent of the maximal change in absorption at 279 nm is plotted as a function of $\bar{\nu}$ in Figure 5. Most of the UV-absorption change accompanied the binding of the first 2 mol of Ca^{2+}.

Effect of Ca^{2+} on the CD spectrum. Since the effect of Ca^{2+} on the UV-absorption spectrum appeared to reflect a conformational change, the far-UV circular dichroic spectrum of the protein activator was measured in the presence and absence of Ca^{2+} (Figure 6). In the absence of any addition (but without prior removal of adventitious Ca^{2+}), the measured mean residue ellipticity was found to be $[\theta]_{221nm} = -12\,000 \pm 500$ (deg cm^2) $dmol^{-1}$. Addition of 1.2 mM EGTA reduced $[\theta]_{221nm}$ to $-11\,500 \pm 500$ (deg cm^2) $dmol^{-1}$. Using the values derived from CD curves obtained with poly-L-lysine by Greenfield and Fasman (1969) and with proteins of known structures (Saxena and Wetlaufer, 1971), it may be estimated that the activator is approximately 30–35% α helix, 50% random coil, and 15–20% pleated sheet. Upon addition of 0.3 mM Ca^{2+}, an 18–20% increase of $[\theta]_{221nm}$ was observed ($[\theta]_{221nm} = -15\,000 \pm 500$ (deg cm^2) $dmol^{-1}$), a change that correspnnds to an increase of α-helix content of 5–8% with a corresponding decrease of random coil. In contrast to poly(glutamic acid) (Cassim and Yang, 1967), a decrease in pH from 8 to 3.6 failed to induce a large increase in molar ellipticity (data not shown). Circular dichroic changes as a function of Ca^{2+} concentrations were measured using a chelex-treated protein with results as shown in Figure 7. As had been observed by difference spectroscopy,

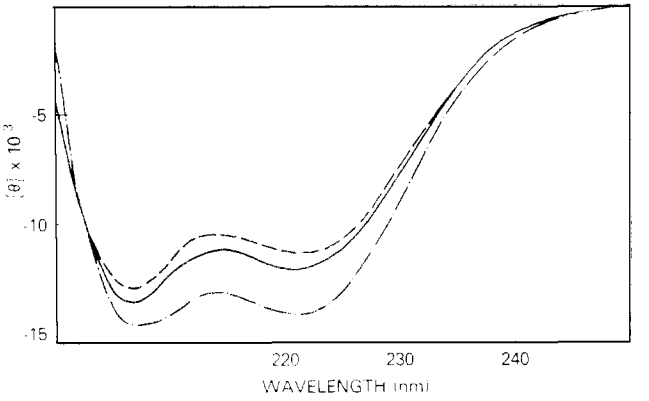

FIGURE 6: Circular dichroism of activator protein. The circular dichroism of EDTA-treated activator (——), after addition of 1.2×10^{-6} M EGTA (- - -) and 0.3×10^{-3} M $CaCl_2$ (— • —), expressed as mean residue ellipticity. The concentration of protein measured directly in the cuvette by its absorption at 230 nm was 0.18 mg/mL in 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl.

the bulk of the CD changes occurred upon binding of the first 2 mol of Ca^{2+}/mol of activator.

Discussion

The phosphodiesterase activator interacts with phosphodiesterase (PDE) in a reaction sequence involving at least two steps (studies on the mechanism of activation are reviewed by Wang et al., 1975) as follows:

$$\text{activator} + Ca^{2+} \rightleftharpoons \text{activator} \cdot Ca^{2+} \quad (1)$$

$$\text{activator} \cdot Ca^{2+} + \text{PDE} \rightleftharpoons \text{PDE} \cdot \text{activator} \cdot Ca^{2+} \quad (2)$$

Evidence presented here suggests that 2 mol of Ca^{2+} are bound per mole of activator in reaction 1, since both spectral and circular dichroic changes associated with Ca^{2+} binding to the purified isolated activator are closely correlated with the binding of 2 mol of Ca^{2+} to the high-affinity sites (K_{dissoc} = 4×10^{-6} M). In addition, the correspondence between the affinity of Ca^{2+} for these sites and the concentration required for half-maximal stimulation of activator-dependent PDE activity (2–5 $\times 10^{-6}$ M (Teo and Wang, 1973; Kakiuchi et al., 1973, and Lin et al., 1974b)) suggests that these sites, rather than the low affinity ones, are required for the activated complex shown in reaction 2. The functional significance, if any, of the low affinity Ca^{2+} binding sites remains to be elucidated. Conceivably, the enzyme may increase the affinity of these sites for Ca^{2+} and thus allow them to play a role in complex formation. On the other hand, the activator is an acidic protein which might be expected to bind cations, and Ca^{2+} in particular, to COO^- groups on its surface.

It has been suggested by Wang et al. (1975) that reaction 1 is accompanied by conformational changes in the protein. The circular dichroic measurements reported here show that Ca^{2+} induces very extensive changes in conformation resulting in increased amounts of α helix. These conclusions are consistent also with the ultraviolet difference spectra, which show changes both in the environment of tyrosine and phenylalanine residues associated with Ca^{2+} binding.

The two tyrosine residues of the activator are in very different environments, as shown by the spectrophotometric titration studies reported here. One tyrosine residue with a pK' near 12 is apparently buried within the hydrophobic interior of the protein and is not significantly affected by the conformational changes accompanying Ca^{2+} binding. It is presumably this tyrosine residue that is responsible for the large difference spectrum induced by 6 M guanidinium chloride ($\Delta\epsilon_{287}$ = −750).

The second tyrosine residue has the more normal pK' value of 10.4, which is on the high side of the usual range of pK values of exposed tyrosine residues of proteins (9.5–10 (Nozaki and Tanford, 1967)). The slightly elevated pK value could be explained if this residue were located on the surface of the protein but hydrogen bonded to a carboxyl group, as in the case of the tyrosine-25 residue of pancreatic ribonuclease which is hydrogen bonded to aspartic-14 (Richards and Wyckoff, 1971). Binding of Ca^{2+} could break this hydrogen bond and thereby reduce the pK (pK 10.1) as observed here and increase the tyrosine fluorescence as reported by Wang et al. (1975). The direction of the change in pK shows that this tyrosine residue becomes more easily acessible to protons during the conformational transition. Evidence arising from the difference spectra, discussed below, suggests that this tyrosine residue is more highly exposed to solvent in the Ca^{2+}-liganded conformation and makes it unlikely that the change in pK is due primarily to changes in charge density in its vicinity.

The difference spectrum generated by 6 M guanidinium chloride shows major peaks at 287 and 279 nm in a ratio of 3:2 and with a molar absorption difference of −750 M^{-1} cm^{-1} as expected for the transfer of a single tyrosine from the hydrophobic interior of a protein to essentially unhindered contact with solvent (Bigelow, 1961). The Ca^{2+}-induced difference spectrum, on the other hand, is larger at 279 than at 287 nm. Since phenylalanine absorbance is negligible above 270 nm (Herskovitz, 1967), the anomalous difference spectrum must reflect some particular properties of the tyrosine chromophores. The UV-absorption spectrum, as a composite of the vibronic transitions of the two tyrosine residues, will reflect their discrete environments and, as has been shown for ribonuclease (Horwitz et al., 1970), the buried tyrosine in its hydrophobic environment will be the predominant contributor to the absorption in the long-wavelength (287 nm) range. Thus, the relatively high intensity of $\Delta\epsilon_{279}$ in the Ca^{2+}-induced difference spectrum indicates a selective modification of the more exposed tyrosine and supports the conclusion drawn from the spectrophotometric titration that it is the surface tyrosine that is primarily affected by Ca^{2+} binding.

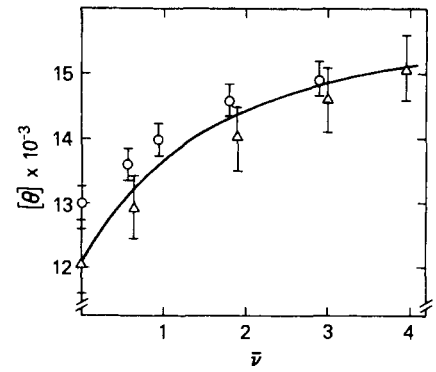

FIGURE 7: Changes in mean residue ellipticity at 221 nm ($[\theta]_{221nm}$) as a function of Ca^{2+} binding to protein activator. The conditions of the experiment are as described in legend to Figure 6. The two symbols correspond to two different experiments. The protein concentration was 0.17 mg/mL and after the successive additions the Ca^{2+} concentrations were 6×10^{-6}, 11×10^{-6}, 28×10^{-6}, and 22×10^{-6} M. The residual EDTA concentration was 10^{-7} M (○). The protein concentration was 0.09 mg/mL, and the Ca^{2+} concentration 6×10^{-6}, 22×10^{-6}, 53×10^{-6}, and 330×10^{-6} M with 10^{-7} M EDTA (△). The number of moles of Ca^{2+} bound per mole of activator, $\bar{\nu}$, was calculated as described in the text.

Interestingly, Ca^{2+} binding to troponin C is also associated with a large conformational change in which the helical content of the protein is apparently increased (Murray and Kay, 1972; Van Eerd and Kawasaki, 1972; McCubbin and Kay, 1973). In the case of troponin C, however, the tyrosines are made less available to solvent rather than more so, since a positive UV difference spectrum is observed (Head and Perry, 1974; Van Eerd and Kawasaki, 1972). Thus, despite their apparent similarities in some structural features (Wang et al., 1975), the two proteins may undergo different kinds of conformational transitions upon binding of Ca^{2+}. Although not a logical necessity, it seems likely that a surface tyrosine residue, the environment of which is drastically changed in reaction 1, may play a role in the activated complex formation of reaction 2. Direct studies of PDE·activator·Ca^{2+} complex formation, currently being carried out, are necessary to answer this and other questions that have arisen as a result of the present study.

Added in Proof

Since submission of this manuscript, conformational changes upon binding of Ca^{2+} have been reported by Liu and

Cheung (1976), although they did not observe any effect on the UV-absorption spectrum. An alternate purification procedure has recently been published by Watterson et al. (1976). Their overall yield, the amino acid composition, and the UV spectrum of the protein are very similar to those reported here.

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