no discrete intermediates are liberated into the medium.

From the examination of the interactions between the synthetic tripyrranes and the enzymatic system it becomes clear that the former are not substrates of the co-synthase in the presence of porphobilinogen. This again falls in line with our suggestion that there are not discrete intermediates formed by the deaminase which are taken up by the cosynthase. The specific incorporation of tripyrrane **8** into isomer III, as well as its strong inhibitory effect on the formation of isomer I (Figure 4a), are further evidence that both uroporphyrinogen isomers follow separate pathways during the enzymatic polymerization of porphobilinogen.

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Purification of Cyclic 3′,5′-Nucleotide Phosphodiesterase Inhibitory Protein by Affinity Chromatography on Activator Protein Coupled to Sepharose†

C. B. Klee* and M. H. Krinks

ABSTRACT: The Ca^{2+}-dependent, reversible, interaction of cyclic adenosine 3′,5′-monophosphate (cAMP) phosphodiesterase with its activator has been used to purify the enzyme by affinity chromatography. Activator-dependent cAMP phosphodiesterase is only a minor component of the proteins specifically adsorbed in the presence of Ca^{2+} by the Ca^{2+}-dependent activator protein coupled to Sepharose and subsequently released by [ethylenebis(oxyethylenenitrilo)]tetraacetic acid. The major protein component can be partially resolved from the enzyme by gel filtration on Sephadex G-200. This protein has been purified to apparent homogeneity and shown to be composed of two polypeptide chains with molecular weights of 61 000 and 15 000, respectively. This protein is, by itself, devoid of phosphodiesterase activity and inhibits the activation of cAMP phosphodiesterase by its activator without affecting the basal activity. Thus, activation of cAMP phosphodiesterase by the Ca^{2+}-dependent activator protein may be controlled by interactions with yet a third component of the enzyme complex.

Cyclic nucleotide phosphodiesterase from mammalian tissues has been shown to exist in multiple molecular forms (for a review of the literature, see Appleman et al., 1973; Amer and Kreigbaum, 1975). At least one of them, the one constituting most of the soluble phosphodiesterase of the cerebrum (Weiss, 1975), interacts specifically with the Ca^{2+}-dependent activator protein[1] first reported by Cheung (1967) and characterized by Cheung (1970, 1971) and by Kakiuchi et al. (1970). The activator protein has been recently purified to homogeneity from several sources (Teo et al., 1973; Lin et al., 1974; Watterson et al., 1976; Klee, 1977; Wolff et al., 1977). The Ca^{2+}-dependent interaction of the enzyme with its activator, which is specific and reversible (Kakiuchi et al., 1975; Lin et al., 1975), has been used in the purification procedure of soluble cAMP phosphodiesterase of bovine brain (Watterson and Vanaman, 1976; Miyake et al., 1977). The results presented here, however, indicate that the enzyme is a minor component of the proteins specifically retained on a column of activator protein coupled to Sepharose in the presence of Ca^{2+}, and released by EGTA. The major protein component released by EGTA can be resolved from the enzyme by Sephadex G-200 gel filtration and it inhibits the activation of the enzyme by its

† From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received July 21, 1977.*

[1] The cAMP phosphodiesterase activator protein has also been called modulator protein (Watterson et al., 1976) and calcium-dependent regulator protein.

activator. This protein appears to be responsible for the inhibitory activity recently described by Wang and Desai (1976, 1977).

Materials and Methods

Cyanogen bromide was a product of Fluka AG. Dimethyl suberimidate dihydrochloride was obtained from Sigma. Glycerol "AnalaR" was a product of Gallard-Schlesinger Chemical Corp. [8-^{3}H]cAMP was from ICN Pharmaceuticals Inc. and could be used in the enzymatic assays without prior purification. Tos-PheCH$_2$Cl[2]-treated trypsin and soybean trypsin inhibitor were from Worthington Biochemical Corp. All other chemicals were as previously described (Klee, 1977). Pig or calf brain activator protein was prepared as described previously (Klee, 1977).

Activator–Sepharose Preparation. The activator was coupled to cyanogen bromide activated Sepharose 4B prepared according to Cuatrecasas and Anfinsen (1971), by incubating 1.2 mg of protein with 4 mL of activated Sepharose for 18 h at 4 °C in 0.1 M borate buffer, pH 8.2, 1 mM $MgCl_2$, and 0.02 mM $CaCl_2$. No residual activator activity was detected in the supernatant fluid after this incubation. After washing with the above buffer, the activator–Sepharose was resuspended in 0.5 M aminoethanol, adjusted to pH 8 with HCl, and mixed at 4 °C for 24 h to ensure complete reaction of the remaining cyanogen bromide activated Sepharose. The gel was then washed with 0.02 M Tris-HCl buffer, pH 8, containing 1 mM $MgCl_2$ and 0.02 mM $CaCl_2$. It could be stored for at least 1 year at 4 °C in the presence of 0.02% sodium azide without significant loss of binding capacity. The amount of activator protein bound to Sepharose was determined by amino acid analysis after acid hydrolysis; it was 60–70% of the starting material.

Preparation of Crude Activator-Dependent Enzyme. The activator-dependent enzyme was prepared by a modification of the method of Cheung and Lin (1974). All buffers contained 1.5 mL of a solution of phenylmethanesulfonyl fluoride (50 mg/mL in dimethyl sulfoxide) per L. All operations were carried out at 4 °C. Bovine brain cerebral cortex (100 g) was homogenized in 300 mL of 0.1 M Tris-HCl, pH 7.5. After centrifugation at 8000*g* for 30 min, the supernatant fluid was collected, the pellet was reextracted with 150 mL of the above buffer, and the two supernatant fluids were pooled (crude extract). The 30–60% ammonium sulfate fraction of this crude extract was dialyzed overnight against 12 volumes of 0.02 M Tris-HCl buffer, pH 7.5, containing 0.05 M $(NH_4)_2SO_4$ (conductivity at 0 °C, 6.5 mS); the dialysis fluid was changed once. The dialyzed sample was freed of endogenous activator by passage through a Whatman DE23 cellulose column (160 mL/1000 A_{280} units) equilibrated in the dialysis buffer. The column was washed with 1 volume of 0.02 M Tris-HCl, pH 7.5, containing 0.05 M $(NH_4)_2SO_4$ followed by 1 volume of the same buffer made 0.08 M $(NH_4)_2SO_4$. The enzyme was then eluted in a 50–60% yield with 1.5 volumes of the same buffer made 0.15 M $(NH_4)_2SO_4$. The stimulation of the enzyme by activator was eight- to tenfold. The specific activity was 0.1 unit/mg. A similar preparation was obtained when EGTA (2×10^{-5} M) was added to the column buffers.

Purification of Inhibitory Protein and Purified Phosphodiesterase. Since the inhibitory protein is a major component of the material bound to the activator–Sepharose in the presence of Ca^{2+}, it was purified as a side fraction of cAMP phosphodiesterase. The affinity chromatographic step described in Figure 1A was carried out on a preparative scale using a 40-mL column of activator–Sepharose (0.15 mg of activator protein per mL of Sepharose) and 40 units of enzyme. Prior to elution with the buffer containing EGTA the column was washed with 60 mL of loading buffer made 0.2 M NaCl. This step did not elute the enzyme or the inhibitory protein but eluted some high molecular weight contaminating proteins. The combined EGTA eluates of two columns were concentrated under vacuum in a collodion bag (Schleicher and Schuell) and made 50% in glycerol. The concentrated protein (5 mL) was applied to a 1.5 × 80 cm column of Sephadex G-200 superfine equilibrated in 0.04 M Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$, 0.1 M NaCl, 0.1 mM dithioerythritol, and 20% glycerol. The flow rate was 2 mL/h. The column was monitored for A_{280} absorption and enzyme activity. Fractions enriched in phosphodiesterase activity were pooled and were used as Sephadex–enzyme (specific activity 10–15 units/mg). The UV-absorbing material that eluted just after the enzyme was concentrated as above. This material contained some residual activity (0.1 unit/mg) and was rechromatographed on the same column. A peak of UV-absorbing material containing two-thirds of the material applied to the column was eluted at the position of the low molecular weight fraction corresponding to a molecular weight of 90 000–110 000 by comparison with proteins of known molecular weights. This peak contained the inhibitory activity. Since phosphodiesterase activity was eluted with the first half of this peak, the second half only was pooled and referred to as inhibitory protein; the yield was 0.2 mg/300 g of brain. The contamination by phosphodiesterase was less than 0.01 unit/mg. One-third of the protein applied to the column eluted prior to the inhibitory protein and was shown by gel electrophoresis to be similar to the material eluted later, indicating that this protein undergoes aggregation.

Enzyme Assays. The enzyme was assayed as described previously (Klee, 1977). The incubation mixture contained 0.04 M Tris-HCl, pH 8, 3 mM $MgCl_2$, 0.1 mM dithioerythritol, 0.2 mM cAMP, 0.01 mg of bovine serum albumin, enzyme, 50 000 cpm of [^{3}H]cAMP, and 1500 cpm of [^{14}C]AMP in a final volume of 0.1 mL. Basal activity was measured in the presence of 0.05 mM EGTA. Activator-dependent activity was measured in the presence of 0.05 mM $CaCl_2$ and appropriate amounts of activator. One unit of enzyme catalyzes the formation of 1 μmol of AMP per min at 30 °C under these conditions. The inhibitory protein was assayed at subsaturating concentrations of activator (7×10^{-9} M).

Gel Electrophoresis. Electrophoresis under nondenaturing conditions was performed as described by Davis (1964), using a 7.5% gel. Disc gel electrophoresis in the presence of NaDodSO$_4$ was carried out in 5–15% or 5–10% gradients of acrylamide, using the Laemmli system (1970). The molecular weight markers were microtubulin-associated proteins (280 000 and 270 000), chicken gizzard filamin (240 000), rabbit skeletal muscle myosin (210 000), β and β′ subunits of *E. coli* RNA polymerase (165 000 and 155 000), phosphorylase *a* (97 000), bovine serum albumin (68 000), brain tubulin (56 000), rabbit skeletal muscle actin (42 000), lactic dehydrogenase (35 000), and β-lactoglobulin (17 500). A linear relationship between the R_f and the log of molecular weight was obtained within this molecular weight range. The proteins cross-linked with dimethyl suberimidate were electrophoresed as described by Davies and Stark (1970). Catalase and fumarase treated in a similar fashion were used as markers. Treatment of proteins with dimethyl suberimidate was done at 30 °C for 90 min. The protein concentration was 0.5–1 mg/mL in 0.2 M triethanolamine hydrochloride, pH 8.5. The

[2] Abbreviations used are: Tos-PheCH$_2$Cl, L-1-tosylamido 2-phenylethyl chloromethyl ketone; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate; UV, ultraviolet.

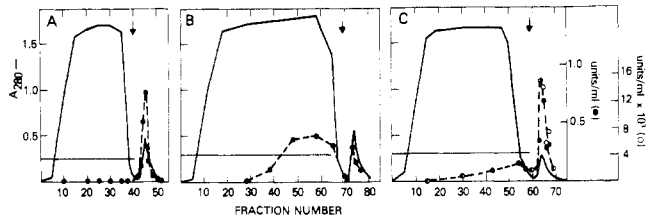

FIGURE 1: Affinity chromatography of cAMP phosphodiesterase on activator-Sepharose columns. The same column of activator-Sepharose (1 × 5 cm) was used for the three experiments. The equilibrating buffer was 0.04 M Tris-HCl, pH 7.5, 0.05 M NaCl, 0.2 mM $CaCl_2$, 3 mM $MgCl_2$, and 0.1 mM dithioerythritol. The eluting buffer was 0.04 M Tris-HCl, pH 7.5, 2 mM EGTA, 1 mM $MgCl_2$, 0.2 M NaCl, and 0.1 mM dithioerythritol. The flow rate was 8 mL per h and the fraction size 1 mL. Prior to loading on the column, the enzyme was dialyzed against the loading buffer. The column was washed with equilibrating buffer until no more A_{280} units were detected in the wash. The column was then washed with eluting buffer. (A) Twenty-five milliliters of enzyme containing 50 A_{280} units with a specific activity of 0.1 unit/A_{280} was loaded onto the column. (B) Fifty milliliters of the same enzyme solution was loaded onto the column. (C) The pooled fractions 30–70 from column B were loaded onto the column. The ordinate scale on the left indicates the absorbance at 280 nm (solid line). The ordinate on the right indicates the units per mL measured at 0.2 mM cAMP (●) and at 10^{-6} M cAMP (○). The arrows indicate the start of elution with EGTA buffer. The horizontal line indicates the concentration of enzyme in the starting material.

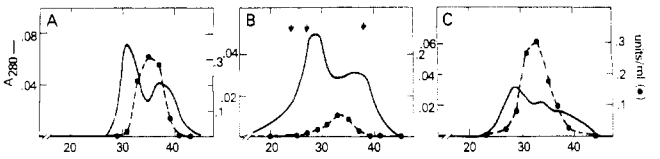

FIGURE 2: Gel filtration of cAMP phosphodiesterase. Samples in 0.02 M Tris-HCl, pH 7.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 M NaCl, 0.05 mM dithioerythritol, and 50% glycerol were applied to a Sephadex G-200 superfine column (0.9 × 57 cm) equilibrated with 0.04 M Tris-HCl buffer, pH 7.5, containing 1 mM $MgCl_2$, 0.1 M NaCl, 0.1 mM dithioerythritol, and 10% glycerol. The flow rate was 1 mL/h and the fraction size 0.7 mL. The ordinate on the left indicates absorbance at 280 nm. The ordinate on the right indicates the number of enzyme units/mL measured at 0.2 mM cAMP. (A) Preparation A, tubes 44 and 45 from Figure 1A. (B) Preparation B, tubes 73 and 77 from Figure 1B. (C) Preparation C, tubes 63–66 from Figure 1C. The arrows reading from left to right indicate the elution positions of blue dextran, phosphorylase *a*, and bovine serum albumin, respectively.

reagent concentration was 4 mg/mL.

Spectrophotometric measurements and amino acid analyses were performed as previously described (Klee, 1977). Protein concentrations were determined by UV absorption or by the method of Lowry et al. (1951).

Results

Affinity Chromatography of Activator-Dependent cAMP Phosphodiesterase on Sepharose Coupled to Activator Protein. Activator-dependent phosphodiesterase was retained on a column of activator-Sepharose in the presence of 0.2 mM $CaCl_2$ (Figure 1A), and was thereby separated from the bulk of the UV-absorbing material (96%) and some activator-independent enzyme (5% of the units applied). Most of the enzyme, together with 3.5% of the protein, was eluted with a buffer containing 2 mM EGTA. The specific activity of the pooled fractions 44 and 45 (preparation A, Figure 1) was 2 units/A_{280} unit and the stimulation by activator (10^{-7} M) was 15- to 16-fold. When twice as much enzyme was applied to the same column (Figure 1B), the enzyme was also adsorbed by the activator-Sepharose (fractions 1–30 were devoid of activity), but most of it was then displaced even in the presence of Ca^{2+}. Thus, fractions 45–65 contained more units/mL than did the starting material. Subsequent washing of the column with the EGTA buffer eluted some UV-absorbing material (preparation B) with a low specific activity (0.4 unit/A_{280} unit) that was stimulated only 7-fold by activator (10^{-7} M). These data suggested that some proteins devoid of enzyme activity, but able to interact with the activator protein in the presence of Ca^{2+}, were bound to the column even more tightly than was the enzyme and displaced the latter from the column. When the enzyme that had been displaced from the column (Figure 1B, fractions 30–70) was applied again to the column (Figure 1C), a large fraction of it was retained in the presence of Ca^{2+} and eluted with buffer containing 2 mM EGTA, yielding material with a specific activity of 3–4 units/A_{280} unit and a 15- to 20-fold stimulation by activator protein (preparation C). The enzyme recoveries from these columns were between 40 and 50% when the enzyme was assayed after the chromatography. When preparations A, B, and C were diluted with an equal volume of glycerol and stored for 2–5 days at −70 °C a 1.5–2-fold increase in activity was observed. This activation was stable over a period of at least 2 months. The overall purification by the affinity chromatography was 20- to 40-fold. The 15- to 20-fold stimulation by exogenous activator indicated that no significant leakage of activator from the column had occurred (in the absence of exogenous activator, the basal activities measured in the presence of Ca^{2+} or EGTA were identical).

Gel Filtration of cAMP Phosphodiesterase Purified by Affinity Chromatography. The material eluted from the affinity columns was fractionated further by gel filtration, as shown in Figure 2. Aliquots of preparations A, B, and C were applied, separately, to columns of Sephadex G-200, superfine. The three protein elution profiles were similar (Figures 2A, B, and C) and indicated the presence of at least two major protein components, a high molecular weight component, at about fraction 30, and a low molecular weight component at about fraction 39. The enzyme activity eluted between the two protein peaks and in all cases the recovery of enzyme was 80–105% of the input activity. The peak activity fractions of the experiment described in Figure 2C (tubes 30–36) were pooled and this fraction is referred to as Sephadex-enzyme. Its specific activity was 12.5 units per mg. The overall purification over crude brain extract was 400-fold.

Inhibition of Enzyme Activation by a Protein Component Which Binds to the Activator-Sepharose. In view of the high recovery of enzyme activity after gel filtration mentioned above, we considered the possibility that an inhibitory factor was removed from the enzyme at this step. The activation of the Sephadex-enzyme by increasing concentrations of activator protein is shown in Figure 3A. The activation curve is hyperbolic[3] in contrast to the sigmoidal curve observed with crude brain extracts and attributed to the presence of an inhibitory factor by Wang and Desai (1976). At limiting concentrations of activator the extent of activation was independent of enzyme concentration. The enzyme activity associated with the low molecular weight proteins eluted after the enzyme on Sephadex G-200, when tested under similar conditions displayed a markedly reduced affinity for the activator and exhibited sigmoidal kinetics (Figure 3A): the extent of stimulation was dependent on enzyme concentration. This different behavior could be explained by the presence of an inhibitory factor in this fraction. As shown in Figure 3B, when the Sephadex-enzyme was mixed with the low molecular weight

[3] The hyperbolic nature of the activation curve was determined by an independent experiment using lower concentrations of activator protein.

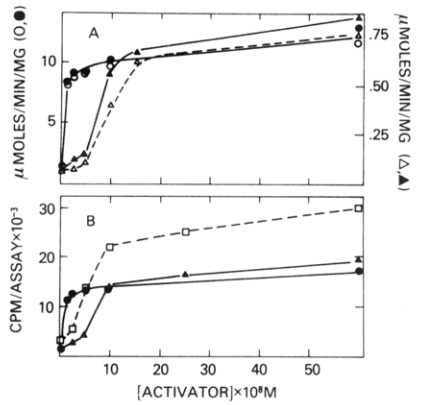

FIGURE 3: Inhibition of activation of phosphodiesterase by a low molecular weight component eluted from Sephadex G-200. Activity was measured as described in Materials and Methods. The incubation time was 8 min; the various protein fractions are described in the text. (A) The Sephadex–enzyme was assayed at 0.5 μg/mL (●) and 1 μg/mL (○). The residual enzyme activity associated with the low molecular weight fraction was assayed at 10 μg/mL (▲) and 17 μg/mL (△) (these protein concentrations exhibited levels of activity comparable to those obtained with the "Sephadex enzyme"). The results are expressed as moles of AMP produced/mg of enzyme. (B) Prior to assays the two protein fractions were mixed to give a final concentration of 0.5 μg/mL of Sephadex–enzyme and 10 μg/mL of the low molecular weight fraction in the assay (□); activity of the two fractions assayed alone as described in A expressed as cpm per assay (● and ▲).

component prior to assays, a large inhibition of the activation was observed at low activator concentrations. The inhibition was removed by high concentrations of activator. The low molecular weight protein(s) eluted after the enzyme must compete with the enzyme for the activator and therefore prevent activation of cAMP phosphodiesterase. In the absence of activator no significant inhibition was observed (Figure 3B). Furthermore, as shown in Table I, when the activity of the Sephadex–enzyme was measured in the presence of EGTA with increasing concentrations of the low molecular weight fraction, no significant inhibition was detected. The high molecular weight fraction eluted before the enzyme from the Sephadex G-200 column had no effect on the enzyme activity measured in the presence of EGTA (Table I) or in the presence of activator and Ca^{2+} (data not shown).

Characterization of Inhibitory Factor. It seemed likely that the inhibitory factor is one (or some) of the proteins which binds to the activator–Sepharose more tightly than does the enzyme and therefore displaces the enzyme from the column (Figure 1B). Preparation B, which is enriched in this protein, showed a major protein band with a R_f of 0.4 and some Coomassie blue staining material which did not enter the gel after electrophoresis under native conditions (Figure 4, gel 1). The low molecular weight protein from the Sephadex G-200 showed a single protein band with the same R_f (0.4) as shown in Figure 4, gel 2. The proteins which did not enter the gel in preparation B were eluted from the Sephadex G-200 with the high molecular weight proteins. Despite the apparent electrophoretic homogeneity, the low molecular fraction is still associated with residual enzyme activity (0.1 unit/mg) which was removed by rechromatography on Sephadex G-200 as described in Materials and Methods. After this step the protein, analyzed by gel electrophoresis on gradients of acrylamide in the presence of $NaDodSO_4$, was found to be composed of two polypeptides with molecular weights of 61 000 and 15 000, respectively (Figure 4, gel 1S). The relative proportion of these two components was not determined, but, when the protein was treated with dimethyl suberimidate prior to electrophoresis

TABLE I: Effect of Sephadex G-200 Fractions on Basal Activity.

Additions	μg/mL	cpm per min per assay Obsd[b]	Theor[b]	Inhibition (%)
Low mol wt component[c]	7	210	230	9
	10.5	280	280	0
	17.5	360	370	1
High mol wt component[c]	2.3	250	280	10
	3.5	290	320	10
	5.8	380	400	6

[a] Assays were performed under standard conditions in the absence of activator and Ca^{2+}. The incubation mixture contained 0.05 mM EGTA. Incubation time was 60 min. [b] A constant amount of Sephadex enzyme (1 μg/mL) was assayed in the presence of increasing amounts of Sephadex fractions as indicated (observed values). Enzyme and Sephadex fractions were also assayed alone and the theoretical enzyme activity in the combined fractions was calculated on the basis of additivity. Enzyme activity in all fractions was linear with protein concentration. [c] High and low molecular weight components from the Sephadex G-200 column are defined in the text. The specific activities of the Sephadex enzyme, high and low molecular weight components, under these assay conditions, were 0.65, 0.25, and 0.05 units/μg, respectively.

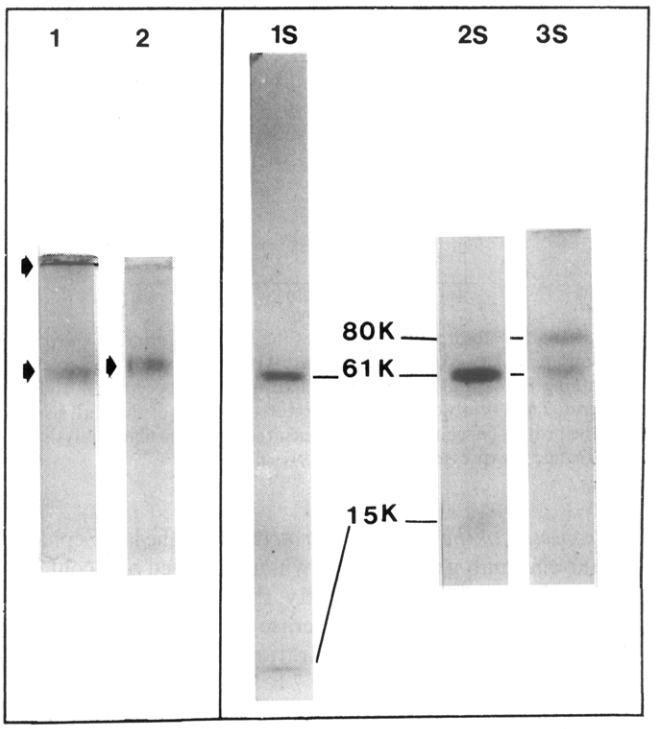

FIGURE 4: Disc gel electrophoretic pattern of inhibitory protein. (Left) Disc gel electrophoresis under native conditions. (Gel 1) Seven micrograms of preparation B; (gel 2) 3 μg of the low molecular weight fraction (tube 39, Figure 2B). (Right) Gel electrophoresis under denaturing conditions. (Gel 1S) One microgram of inhibitory protein purified as described in Materials and Methods. Electrophoresis was performed in a 5–10% acrylamide gradient; (gel 3S) protein treated with dimethyl suberimidate prior to electrophoresis performed as described in Materials and Methods, 10 μg; (gel 2S) control sample incubated in the absence of reagent, 10 μg.

under denaturing conditions, a new band corresponding to a molecular weight of 80 000 was present (Figure 4, gel 3S). Its formation was accompanied by a decrease in the intensity of the 61 000 molecular weight band and the disappearance of the 15 000 molecular weight component (Figure 4, gels 2S and 3S). These data suggest that the two polypeptides are the subunits of the inhibitory protein.

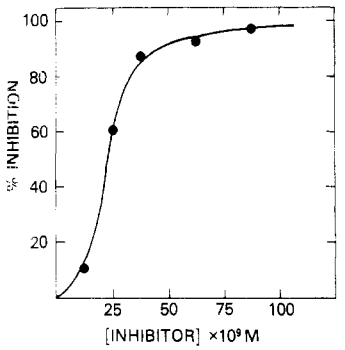

FIGURE 5: Inhibition of the activation of cAMP phosphodiesterase by purified inhibitory protein. The Sephadex-enzyme was assayed under standard conditions in the presence of a subsaturating concentration of activator protein (7×10^{-9} M) and increasing concentrations of purified inhibitory protein prepared as described in Materials and Methods. The concentration of inhibitory protein is based on a molecular weight of 80 000. The maximal inhibition (100%) is defined as the level of activity equal to that observed when EGTA is present in the assay.

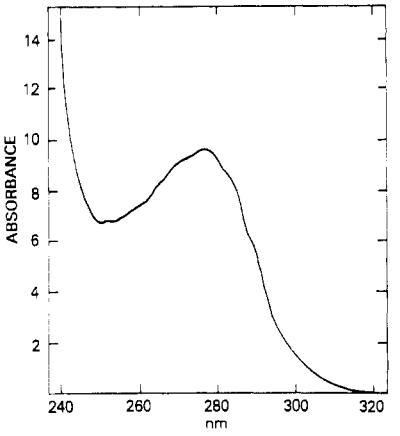

FIGURE 6: UV absorption spectrum of the inhibitory protein. The protein (0.1 mg per mL) was dialyzed against 0.04 M Tris-HCl buffer, pH 8, containing 1 mM $MgCl_2$, 0.1 M NaCl, 0.1 mM dithioerythritol, and 10% glycerol. The UV absorption was corrected for that of the dialysis fluid and the protein concentration was measured by amino acid analysis. The absorbance is expressed as that of a 1% solution.

Properties of the Inhibitory Protein. After the last Sephadex G-200 chromatographic step, which removed the contaminating phosphodiesterase activity, the inhibitory protein still inhibited cAMP phosphodiesterase activation, as shown in Figure 5. At saturating concentrations of this protein, the enzyme activity in the presence of 7×10^{-9} M activator protein was reduced to the basal level (obtained in the presence of EGTA) and could not be decreased further. At this concentration of activator protein, addition of 1.8 μg of inhibitory protein per mL (2×10^{-8} M, assuming a molecular weight of 80 000) resulted in 50% inhibition. Assuming a one to one complex of this protein with the activator protein, a dissociation constant of 4×10^{-9} M was estimated. This value is slightly higher than the K_m of the enzyme for the activator. It should be pointed out, however, that the K_m value is greatly affected by cAMP concentrations[4] (Teo et al., 1973; Brostrom and Wolff, 1974) and under the conditions of the affinity chromatography (in the absence of cAMP) the affinity of the enzyme for the activator could be lower.

The inhibitory activity is sensitive to heat and acid treatment. Some activity (30%) was lost after dilution of the protein with an equal volume of 0.2 M HCl. Heating for 3 min at 100 °C resulted in a 50% loss of activity. Exposure of the inhibitory protein to trypsin (0.1 μg/mL) for 10 min at 30 °C resulted in complete loss of inhibitory activity. The trypsin treatment did not result in activation of a latent phosphodiesterase activity. Limited proteolysis was accompanied by a change in the size of the large subunit which is converted first to a 58 000 and later to a 41 000 molecular weight polypeptide. Within the limits of sensitivity of the gel electrophoresis technique, no significant change in the size of the small subunit was observed.

TABLE II: Amino Acid Composition of Inhibitory Protein.

Amino acid	Residues/mol[a]
Lys	47
His	30
Arg	29
Asp	73
Thr	38
Ser	73
Glu	100
Pro	48
Gly	65
Ala	58
Val	41
Met	12
Ile	29
Leu	55
Tyr	19
Phe	27
Trp	ND[b]
Cya	12[c]

[a] These values correspond to a molecular weight of 80 000. [b] Not determined. [c] Cysteic acid was measured after performic acid oxidation.

The UV absorption spectrum of the inhibitory protein is shown in Figure 6. An extinction coefficient of $\epsilon_{278nm}^{1\%} = 9.6$ was derived from these data. The amino acid composition is summarized in Table II. Although the tryptophan content was not determined, the UV-absorption spectrum indicates that it is a tryptophan-containing protein.

In view of the ability of the activator protein to interact with other enzymatic systems, the inhibitory protein was tested for adenylate and guanylate cyclase activities and none was detected. It was also devoid of ATPase activity measured as described by Puszkin and Kochwa (1974). Since the inhibitory protein could be the activator-binding subunit of phosphodiesterase, it was also tested for its ability to restore activator dependence to a Sephadex-enzyme treated with trypsin. Sephadex-enzyme incubated at 30 °C for 20 min in the presence of 1 μg of trypsin per mL was activated twofold and lost its ability to be stimulated by activator protein. Addition of inhibitory protein directly in the assay mixture or to the enzyme prior to assay did not restore the ability of the enzyme to be activated.

The inhibitory protein appears to be the major contaminant of the enzyme preparation after purification by affinity chromatography on activator coupled to Sepharose as shown in Figure 7, gel 2. The two polypeptides associated with it are major components of the protein bound to the column. The high molecular weight material seen on the Sephadex G-200 profile (Figure 2) has a large UV absorption due to light scattering. This fraction was shown by $NaDodSO_4$ gel electrophoresis to contain the two subunits of the inhibitory protein together with a heterogeneous mixture of large polypeptides

[4] After the affinity chromatography step the enzyme had K_m values for the activator protein of 6×10^{-9} M and 0.5×10^{-9} M at 10^{-7} and 2×10^{-4} M cAMP, respectively.

(mol wt 225 000, 150 000, and 80 000) and two minor small ones (mol wt 41 000 and 38 000). The nature of these proteins is presently under study. It is also clear from the gel electrophoretic pattern (Figure 7, gel 2) that the subunits of the inhibitory protein are present in large excess over the enzyme whose subunits are not detectable at this step. Although the two polypeptides of the inhibitor are present in large amount in the EGTA eluate of the activator–Sepharose column, they are not present in large amount in brain since they are not detectable in the gel electrophoretic pattern of the crude brain extract (Figure 7, gel 4) or of the DEAE-cellulose fraction (gel 3).

Discussion

In our attempt to purify the activator-dependent cAMP phosphodiesterase by affinity chromatography on a column of activator coupled to Sepharose, we observed that the enzyme is a minor fraction (less than 10%) of the protein which binds to the column in the presence of Ca^{2+} and is released by EGTA. Another protein, composed of two subunits with molecular weights of 61 000 and 15 000, respectively, was the major component of the EGTA eluate. The two subunits of this protein are also the major polypeptides found in the enzyme prepared by affinity chromatography by Watterson and Vanaman (1976). This protein, although present in large excess over the enzyme, appears to be a minor fraction of crude brain extract.

As shown in Figure 2B this protein forms a Ca^{2+}-dependent complex with the activator protein and the formation of this complex does not require the presence of the enzyme. The ability of the protein to interact with the activator protein explains the specific inhibition of the stimulation of cAMP phosphodiesterase by activator and the lack of inhibition of the basal activity measured in the presence of EGTA. An inhibitory factor with a similar effect on the activation of phosphodiesterase has recently been described and partially purified from bovine brain by Wang and Desai (1976, 1977). This factor has been called modulator binding protein on the basis of its ability to bind to the phosphodiesterase activator as measured by Sephadex gel filtration. The purified inhibitory protein described here exhibits the same inhibitory properties, similar heat and acid lability and a comparable apparent molecular size (90 000–110 000 by Sephadex chromatography) as does the modulator binding protein of Wang and Desai. The two proteins are probably identical. Another inhibitory factor of cAMP phosphodiesterase has been reported in ox retina by Dumler and Etingof (1976) but this factor is heat and acid stable and has an estimated molecular weight of 38 000; it probably represents a different protein from the one described in this paper.

The relationship of the inhibitory protein to cAMP phosphodiesterase cannot yet be ascertained. The fact that it binds to the activator in a manner similar to the way the enzyme does and coelectrophoreses with the enzyme under native conditions[5] may indicate that it is an inactive form of the enzyme. Preliminary evidence, however, indicates that its subunit molecular weight is greater than that of the enzyme. The Sephadex enzyme analyzed by $NaDodSO_4$ gel electrophoresis was shown to contain three polypeptides of molecular weights 61 000, 59 000, and 15 000.[6] Although it could be an inactive precursor of the phosphodiesterase, we were unable to activate this protein by limited proteolysis.

[5] Under native conditions, enzyme activity was eluted from the gel described in Figure 4 (gel 1) at a position corresponding to the protein band (R_f 0.4).

[6] C. B. Klee and M. H. Krinks, manuscript in preparation.

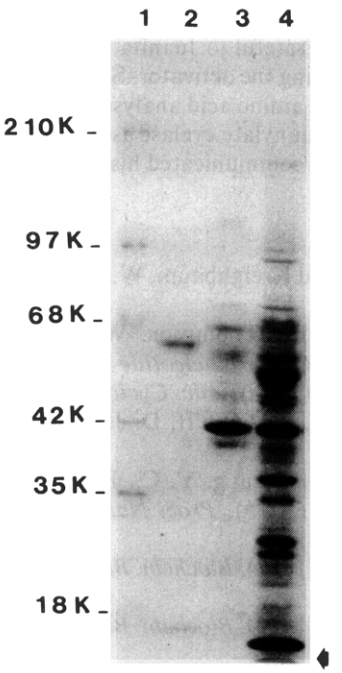

FIGURE 7: Sodium dodecyl sulfate gel electrophoresis of crude brain fractions in gradients (5–15%) of acrylamide. (Gel 1) Molecular weight markers, rabbit skeletal muscle myosin, phosphorylase *a*, bovine serum albumin, actin, lactic dehydrogenase, activator protein; (gel 2) EGTA eluate of activator–Sepharose column, 2×10^{-3} A_{280} units; (gel 3) crude activator-dependent phosphodiesterase (DEAE-cellulose fraction), 24×10^{-3} A_{280} units; (gel 4) crude brain extract, 80 μg.

Although we cannot rule out the possibility that this protein catalyzes a reaction which is not yet characterized, a more attractive hypothesis would be that it is a regulatory subunit of the enzyme. The inhibitory protein coelectrophoreses with phosphodiesterase in gels run under native conditions. Our most highly purified enzyme preparation, with a specific activity of 150 units/mg, was shown to be contaminated with this protein.[6] During the last purification steps which resulted in partial removal of this protein a partial loss of stimulation by activator protein was observed. The failure of the inhibitory protein to restore activator dependence may reflect our inability to reconstitute the activator-dependent enzyme. The large proportion of this protein compared with phosphodiesterase could be explained if the inhibitory protein were a subunit common to a number of enzyme complexes all under the control of the Ca^{2+}-dependent activator protein. There is considerable evidence that the activator protein is a multifunctional modulator (Smoake et al., 1974; Egrie and Siegel, 1975; Waisman et al., 1975; Hait and Weiss, 1976; Watterson et al., 1976). The activator was shown to activate partially purified brain adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975) and more recently it has been identified as the activator of calcium transport (MacIntyre and Green, 1977) and ATPase of erythrocyte membranes (Berridge, 1975; Jarrett and Penniston, 1977; Gopinath and Vincenzi, 1977). Because of its similarity to other Ca^{2+}-binding proteins it was also proposed as a potential regulator of nonmuscle cell contractile proteins (Vanaman et al., 1976).

Thus brain cAMP phosphodiesterase activity is regulated by at least two different proteins in Ca^{2+}-mediated processes, one of which is a stimulator and the other an inhibitor of phosphodiesterase activity. The functional state of the enzyme in the cell therefore can be controlled both by Ca^{2+} levels and by the availability of each of these proteins to the catalytic subunit of the cAMP phosphodiesterase complex.

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