Chromatographic Behavior of Cyclic AMP Dependent Protein Kinase and Its Subunits from *Dictyostelium discoideum*†

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ABSTRACT: An adenosine cyclic 3′,5′-monophosphate (cAMP) dependent protein kinase has recently been shown to exist in *Dictyostelium discoideum* and to be developmentally regulated. In this report we have followed the chromatographic behavior of both the holoenzyme and its subunits. A cAMP-dependent holoenzyme could be obtained from the 100000*g* soluble fraction after passage through DE-52 cellulose (pH 7.5) and Sephacryl S300. Under conditions of low pH the holoenzyme could be further purified by flat-bed electrofocusing (p*I* = 6.8). Application of the holoenzyme to electrofocusing at high pH resulted in dissociation of the holoenzyme into a cAMP binding component (p*I* = 6.1) and a cAMP-independent catalytic activity (p*I* = 7.4). Dissociation of the holoenzyme into subunits also occurred during histone affinity chromatography and gel filtration chromatography (S300) in the presence of a dissociating buffer. Although the subunit structure was clearly evident during chromatography, the holoenzyme could not be dissociated by simple addition of cAMP to the extract. The catalytic subunit could be purified further by CM-Sephadex, DE-52 cellulose (pH 8.5), histone affinity, and hydrophobic chromatography. The regulatory subunit was further purified by DE-52 cellulose (pH 8.5) and cAMP affinity chromatography. Proof that the cAMP binding activity and the cAMP-independent catalytic activity were in fact the regulatory and catalytic subunits was shown by reconstitution of the cAMP-dependent holoenzyme from the purified subunits. By using these separation procedures, one can obtain from extracts of *Dictyostelium* the subunits that are free of each other as well as free of any endogenous protein substrates.

Dictyostelium discoideum offers a model system for investigating the regulation of developmental processes by adenosine cyclic 3′,5′-monophosphate (cAMP).[1] Cyclic AMP acts as an extracellular chemotactic signal at an early stage of development in this organism and apparently coordinates movements of individual cells into a multicellular aggregate (Konjin et al., 1968; Bonner et al., 1969). In addition, cAMP may be a regulatory molecule in the subsequent stages of development, the slug stage and the culmination stage. This involvement of cAMP in the terminal stages has been described by several laboratories using a variety of experimental approaches. For example, cAMP is known to accumulate during the culmination stage (Brenner, 1978; Pahlic & Rutherford, 1979; Abe & Yanagisawa, 1983) and become localized in one cell type (Pan et al., 1974; Brenner, 1977; R. K. Merkle and C. L. Rutherford, unpublished experiments). Addition of extracellular cAMP to cell cultures causes a variety of morphological and biochemical responses. Cyclic AMP addition causes wild-type amoebas to form some stalk cells (Bonner, 1970). Similarly, addition of cAMP to a variant strain (V12M2) results in nearly complete stalk-cell differentiation (Town et al., 1976). During the culmination stage, adenylate cyclase and cAMP phosphodiesterase become localized in prespore and prestalk cells, respectively (Brown & Rutherford, 1980; Merkle & Rutherford, 1984). When the two cell types are separated on density gradients, the prestalk cells are more responsive to added cAMP than are the prespore cells (Maeda & Maeda, 1974). Similarly, Sternfield & David (1981) have shown that, in cell clumps embedded in agar, prestalk cells move toward a source of cAMP. Matsukuma & Durston (1979) demonstrated that, in mixtures of vitally stained cells, the prestalk cells will stream toward a source of cAMP. Adding cAMP exogenously to cells may induce and maintain postaggregation gene expression (Kay, 1979; Landfear et al., 1982).

Although these results provide clear evidence that cAMP is involved in processes other than chemotaxis in *Dictyostelium*, little information is available on the mechanism by which the cAMP effects are mediated to cellular metabolism. Recently, however, an adenosine cyclic 3′,5′-monophosphate dependent protein kinase (ATP:protein phosphotransferase; EC 2.7.1.37) (cAMPdPK) that accumulates during the terminal stages of development has been described (De Gunzberg & Veron, 1982; Rutherford et al., 1982; Cooper et al., 1983). Cyclic AMP dependent protein kinases are mediators of cAMP action in a wide variety of organisms (Corbin et al., 1975) and, in fact, may be the only mechanism by which cAMP exerts its regulatory properties in mammalian systems (Robinson et al., 1971). In mammalian tissues the enzyme is classified into two forms (I and II). These forms differ in both their physical and their kinetic properties (Flockhart & Corbin, 1982). Little is known, however, about the function of the two forms in regulating metabolism or their involvement in developmental processes. On the other hand, a great deal of information is available about the subunit structure of the protein. All of the cAMPdPKs that have been described contain catalytic (C) and regulatory (R) subunits. The enzyme can exist either as an inactive holoenzyme, consisting of a tetramer of the subunits (R_2C_2), or as an active catalytic subunit. In skeletal muscle, cAMP activates the enzyme by dissociating the subunits according to the equation

$$R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C$$

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; cAMPdPK, adenosine cyclic 3′,5′-monophosphate dependent protein kinase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TAMB, Tris-HCl buffer containing 2 mM mercaptoethanol, 0.02% sodium azide, and 2 mM benzamidine; TCA, trichloroacetic acid; cGMP, guanosine cyclic 3′,5′-monophosphate.

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Although a cAMPdPK has been shown to exist in *Dictyostelium* (De Gunzberg & Veron, 1982; Rutherford et al., 1982; Cooper et al., 1983) and to be developmentally regulated (Rutherford et al., 1982), little is known about its subunit structure or its physical and kinetic properties. With these goals in mind we have studied the chromatographic behavior of the holoenzyme and its subunits. We show in this report that the enzyme from *Dictyostelium* possesses both similarities to and differences from kinases in other systems and describe methods by which highly purified preparations of the two subunits can be obtained.

Materials and Methods

Reagents. Sephacryl S300, CNBr-activated Sepharose 4B, and all materials for chromatofocusing were purchased from Pharmacia. DE-52 cellulose was from Whatman, CM-Sephadex from Sigma, and cAMP–agarose from P-L Biochemicals, and all materials for preparative flat-bed electrofocusing were from LKB. All other reagents were from Sigma.

Preparation of Cell-Free Extracts. Growth and differentiation of *Dictyostelium discoideum* NC4 were carried out as previously described (Rutherford, 1976). At the slug or culmination stage of development the cells were removed from an agar surface with cold distilled water, were washed by centrifugation at 1000*g* for 3 min, and were resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 0.02% sodium azide, 2 mM 2-mercaptoethanol, and 2 mM benzamidine (TAMB) (4 mL of buffer/mL of packed cells). Most of the starting preparations contained 40–60 g wet weight of cells (approximately 1.5 g of protein). The cells were evenly distributed by two strokes of a Potter–Elvejhem tissue grinder and then were disrupted by three 45-s exposures to a 2-cm probe of a sonic cell disrupter (Model 300, Fisher) at a setting of 45. The resulting homogenate was centrifuged at 100000*g* for 60 min and the supernatant removed.

DE-52 Cellulose Chromatography at pH 7.5. The 100000*g* supernatant was applied to a DE-52 cellulose column (1.6 × 13 cm) that had been equilibrated in 50 mM TAMB (pH 7.5). The material that did not bind to the resin (flow-through) was allowed to completely elute from the column as determined by the return to the base line on a column monitor. Proteins that were bound to the resin were then eluted for 4 h with a 0–0.3 M KCl gradient in 50 mM TAMB, pH 7.5 (flow rate of 80 mL/h), as formed from a programmable chromatography pump (Dialagrad, Model 382, ISCO). The active fractions (the flow-through volume) were pooled, precipitated with ammonium sulfate (70% saturation), and then dialyzed overnight against water.

Protein Kinase and cAMP Binding Assay. Protein kinase activity was assayed in a total volume of 50 μL with 25 μL of the enzyme sample and 25 μL of a reaction mixture that contained 50 mM potassium phosphate buffer (pH 6.5), 3 mM dithiothreitol, 10 mM $MgCl_2$, 0.8 mg/mL histone VII-S (Sigma Chemical Co.) or Kemptide (60 μg/mL), and 25 μM [γ-^{32}P]ATP (0.4 Ci/mmol) either with or without 20 μM cAMP. After incubation at 25 °C the entire reaction volume was removed to 1-cm^2 pieces of filter paper (P81, Whatman). When histone VII-S was used as a substrate, the paper was immediately transferred to cold 10% TCA. After 15 min the papers were removed to hot 5% TCA (5 min), cold 5% TCA (5 min), and acetone (5 min) and then dried for determination of radioactivity. When Kemptide was used as a substrate, the papers were immediately placed in ice-cold 30% acetic acid for 5 min and were transferred through additional 5-min washes of ice-cold 30% and 15% acetic acid and then room temperature 15% acetic acid and acetone.

Cyclic AMP binding activity was measured in a total volume of 125 μL containing 100 μL of the protein sample and 25 μL of a reaction mixture containing 25 mM dithiothreitol, 5 mM $MgCl_2$, and 150 nM [^{3}H]cAMP (130 Ci/mmol) in 50 mM Tris-HCl buffer (pH 7.5). After incubation for 5 min the entire reaction mixture was removed to a Hoefer filter reservoir containing 5 mL of ice-cold 50 mM Tris-HCl (pH 7.5). The solution was immediately filtered through a Gelman GN-6 filter (0.45 μm) by vacuum filtration. The filter was washed twice in the same buffer and then removed and dried for determination of radioactivity.

Results

Gel Filtration of cAMPdPK Holoenzyme and Demonstration of Subunit Structure. The 100000*g* soluble fraction from the slug or culmination stage was passed through DE-52 cellulose (pH 7.5), concentrated by ammonium sulfate precipitation, and dialyzed overnight at 7 °C as described under Materials and Methods. The resulting sample, usually 6–8 mL, was then applied to a Sephacryl S300 column that was equilibrated in 50 mM TAMB, pH 7.5. The activity that eluted from the column could be activated by addition of cAMP to the reaction mixture (Figure 1A). Cyclic AMP binding activity showed coincident elution with the cAMPdPK. The extent of activation of the kinase activity by cAMP varied from 3- to 10-fold between different preparations of the enzyme. The molecular weight of the enzyme varied depending on the method by which the sample was prepared. We found that when the enzyme was concentrated by ammonium sulfate precipitation, followed by overnight dialysis, the activity consistently eluted from the S300 column at the same position as the catalase marker (M_r 230 000). This value may not represent the true molecular weight of the "holoenzyme", however, since several proteins that coelute with the enzyme can act as substrates for phosphorylation (L. T. Frame and C. L. Rutherford, unpublished experiments). If any of these substrates are tightly bound to the enzyme, the activity would elute from the gel filtration column at a higher molecular weight than that of the enzyme which was free of its substrates. As described below, attempts to separate the holoenzyme from its substrates resulted in dissociation of the enzyme into subunits. Therefore, determination of the true molecular weight of the holoenzyme will await the preparation of a purer sample.

The subunit nature of the "off S300" activity was demonstrated by rechromatography of the holoenzyme in "dissociation buffer". The fractions from the S300 column that contained cAMPdPK were pooled, were concentrated by membrane filtration (Amicon PM10), and then were dialyzed for 2 h against dissociation buffer (50 mM potassium phosphate buffer, pH 7.0, containing 4 mM 2-mercaptoethanol, 75 mM NaCl, and 4 mM cGMP). cGMP was used since it dissociates from most cAMP-dependent enzymes more readily than does cAMP. When this preparation was applied to an S300 column that was equilibrated in dissociation buffer, the holoenzyme dissociated into catalytic and cAMP binding activities (Figure 1B). The subunits eluted in fractions corresponding to apparent molecular weights between 40 000 and 60 000.

Although the treatment with dissociation buffer was effective in dissociating the holoenzyme, the addition of either cAMP or cGMP alone to the enzyme was not. If cAMP or cGMP (1 mM) was added directly to the "off S300" concentrated holoenzyme (in 50 mM TAMB, pH 7.5) and then reapplied to a S300 column that had been equilibrated in 50 mM TAMB, pH 7.5, containing 1 mM cAMP, the activity eluted at the same position as the holoenzyme. This failure

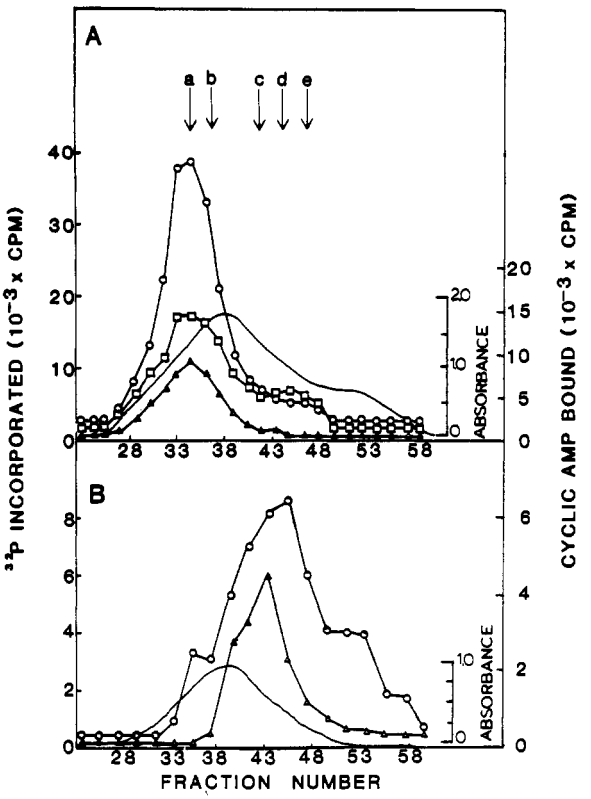

FIGURE 1: Gel filtration chromatography of cAMPdPK. Panel A: A column (1.6 × 84 cm) of Sephacryl S300 was equilibrated with TAMB (pH 7.5). Six milliliters (approximately 700 mg of protein) of the dialyzed ammonium sulfate precipitate (see Materials and Methods) was applied to the column and eluted with the same buffer. Protein kinase activity with cAMP (○) and without cAMP (□) in the reaction mixture and cAMP binding activity (△) were assayed as described under Materials and Methods. The faint solid line is absorbance. The molecular weight markers are as follows: (a) catalase, M_r 232 000; (b) aldolase, M_r 158 000; (c) bovine serum albumin, M_r 67 000; (d) ovalbumin, M_r 43 000; (e) chymotrypsinogen A, M_r 25 000. Panel B: The active fractions from the column shown in panel A were concentrated and then dialyzed against dissociation buffer (50 mM potassium phosphate buffer, pH 7.0, containing 4 mM 2-mercaptoethanol, 75 mM NaCl, and 4 mM cGMP) for 2 h. A 20-mL sample of dissociation buffer was pumped over the column, and then the enzyme was applied. In both (A) and (B) the flow rate was 30 mL/h, and 5-min fractions were collected in siliconized test tubes. Symbols are the same as in panel A.

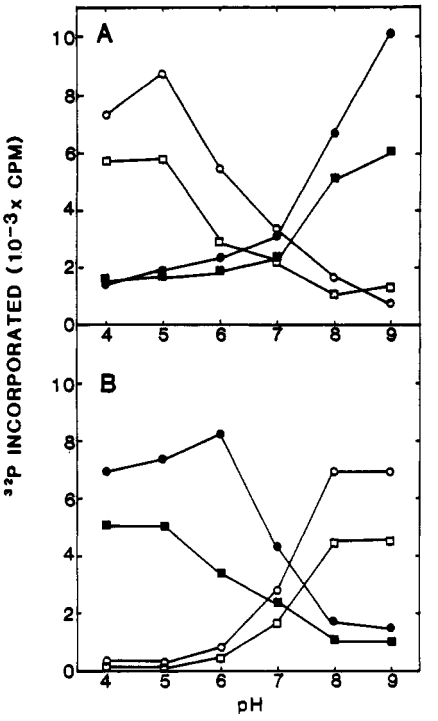

FIGURE 2: Binding and elution of cAMPdPK on anion- and cation-exchange resins as a function of pH. Panel A: The anion-exchange resin, DE-52 cellulose (Whatman), was equilibrated with 50 mM TAMB (pH 7–9) or AAMB (50 mM sodium acetate, pH 4–6). To 0.5 mL of the settled resin at each pH was added an equal volume of S300 holoenzyme that had been dialyzed against 5 mM TAMB, pH 7.5. After mixing, the pH of the resulting suspension was the same as the buffer in which the resin had been equilibrated. The samples were incubated for 15 min at 4 °C and then were centrifuged (1000*g*, 2 min), and the supernatants were removed. A 0.5-mL sample of the appropriate buffer containing 0.3 M KCl was then added to the dry resin. After incubation for 15 min at 4 °C the tubes were centrifuged (1000*g*, 2 min) and the supernatants removed. The supernatants were dialyzed overnight against 50 mM TAMB (pH 7.5) and then assayed for protein kinase as described under Materials and Methods. Circles represent the reaction mixture plus cAMP and squares the mixture without cAMP. Open symbols represent the supernatant fraction and closed symbols the KCl-elutable fraction. Panel B: The conditions were identical with those described in (A) except that CM-Sephadex was used.

of cAMP to dissociate the holoenzyme was not due to degradation of the added cAMP by endogenous cAMP phosphodiesterase, for actual measurement of cAMP levels by a radioimmunoassay revealed no loss of cAMP during the preincubation period (not shown).

Further Purification of the Holoenzyme and Demonstration of Its Subunit Structure. The "S300 holoenzyme" was tested for its behavior on both cation- and anion-exchange resins. A batch treatment was used in which a sample of holoenzyme was added to an equal volume of settled resin as described in detail in the legend of Figure 2. After incubation for 15 min the supernatant was removed and was assayed for cAMPdPK. Figure 2 shows that the enzyme behaves as a typical amphoteric protein in that it binds to the anion exchanger at high pH (Figure 2A) and binds to the cation exchanger at low pH (Figure 2B). The enzyme could be eluted from both resins by inclusion of 0.2 M KCl in the buffer. Nearly complete recovery of the original activity could be obtained, but only after dialysis to remove the KCl. We found that the activity of the enzyme was quite sensitive to both KCl and NaCl, with 80% inhibition occurring in the presence of 0.2 M salt. It should be emphasized that the holoenzyme was eluted by this batch method, for as described below, if the identical resins were placed in a column and then eluted, the catalytic and cAMP binding activities were found in separate fractions.

Purification procedures for cAMPdPK from rabbit muscle on ion-exchange resins have taken advantage of the different isoelectric points of the holoenzyme and its subunits (Bechtel et al., 1977). In these procedures, an enzyme sample was added to an ion-exchange resin at a pH at which the holoenzyme would not bind to the resin. Cyclic AMP was then added to the sample to produce subunits with isoelectric points different from that of the holoenzyme. The catalytic subunit would then bind to the resin and could be eluted in a purified form as no other proteins responded to the added cAMP. However, with the *Dictyostelium* enzyme (S300 holoenzyme) there was no difference in the binding capacity to ion-exchange resins in the presence or the absence of cAMP. In fact, when cAMP was included in all the fractions shown in Figure 2, there was no difference between the curves with or without added cAMP. Control rabbit muscle enzyme showed complete binding in the presence of cAMP. Further attempts to dissociate the holoenzyme and therefore affect catalytic subunit binding to the ion-exchange resin included various combinations of NaCl, KCl, ATP-Mg, histone, and dissociation buffer. Thus, the technique of differential binding of holoenzyme and

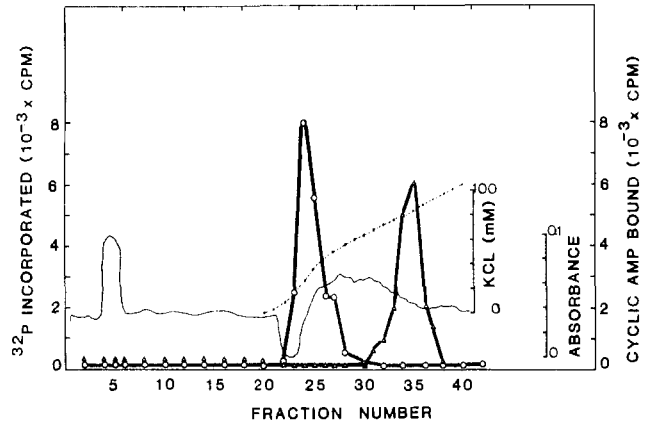

FIGURE 3: Chromatography of S300 holoenzyme on DE-52 cellulose at pH 8.5. A column (1.6 × 12 cm) of DE-52 cellulose was equilibrated with 10 mM TAMB (pH 8.5). The enzyme sample was dialyzed against this buffer and then applied to the column in a volume of 10 mL. The flow-through volume was collected, and the enzyme was eluted with a gradient to 10 mM TAMB (pH 8.5) containing 100 mM KCl. The flow rate was 80 mL/h, and 5-min fractions were collected. The KCl concentration was measured by conductance. Symbols represent (—) absorbance, (·–·) KCl concentration, (△) cAMP binding activity, and (○) protein kinase activity. No differences were detected in the kinase activity in the presence or absence of cAMP (not shown).

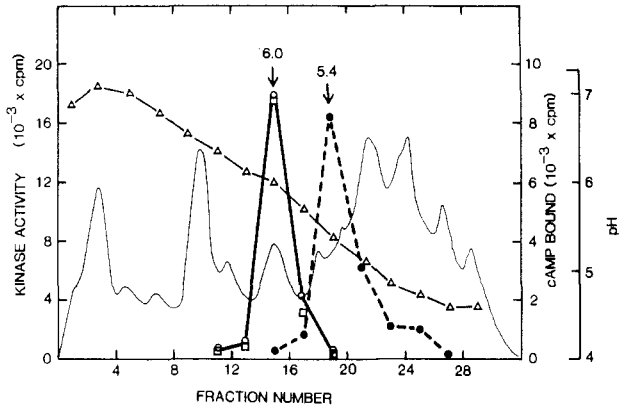

FIGURE 4: Chromatofocusing of cAMPdPK holoenzyme. A column (1.0 × 25 cm) of chromatofocusing resin (PBE94, Pharmacia) was equilibrated by washing with 1 M NaCl in 50 mM TAMB (pH 7.5), 0.1 M HCl, and then 50 mM TAMB (pH 7.5) until the pH of the eluant reached 7.5. A sample of S300 holoenzyme (10 mL in 50 mM TAMB, pH 7.5) was applied to the column, followed by polybuffer (Pharmacia) that had been brought to pH 4.0 with HCl. The flow rate was 30 mL/h, and 10-min fractions were collected. Symbols represent (—) absorbance, (△) pH, (●) cAMP binding activity, and (○) protein kinase activity with cAMP and (□) that without cAMP.

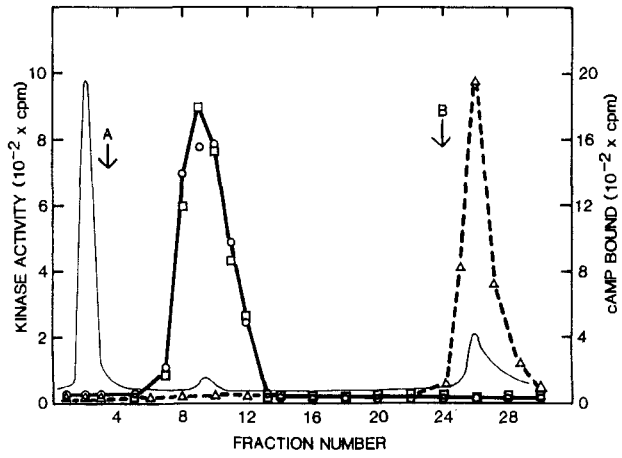

FIGURE 5: Chromatography of S300 holoenzyme on a histone VII-S affinity column. Histone VII-S (Sigma) was coupled to CNBr-activated Sepharose 4B per manufacturer's directions (Pharmacia). To a column (1.0 × 9 cm, equilibrated with 50 mM TAMB, pH 7.5) was added 10 mL of holoenzyme in 50 mM TAMB, pH 7.5. The flow-through volume was collected, and then 25 mM AAMB (sodium acetate buffer, pH 5.0) was added at arrow A. At arrow B, AAMB containing 0.3 M KCl was added. The flow rate was 80 mL/h, and 5-min fractions were collected in siliconized test tubes. Symbols represent (—) absorbance, (△) cAMP binding activity, and (○) protein kinase activity in the presence of cAMP and (□) that in the absence of cAMP.

catalytic subunit could not be used for extracts of *Dictyostelium*.

Further evidence for the subunit structure of *Dictyostelium* cAMPdPK was observed during DE-52 cellulose chromatography at pH 8.5. Since the results from Figure 2 showed that the holoenzyme could bind to the anion-exchange resin at high pH, we subjected the S300 holoenzyme to DE-52 cellulose chromatography at pH 8.5. Figure 3 shows that no activity was detectable in the flow-through volume at pH 8.5 even though at pH 7.5 (see above) the activity did not bind to the resin. Application of a KCl gradient to this column resulted in separate elution of the catalytic and cAMP binding activities. The catalytic activity eluted at 25 mM KCl and the cAMP binding activity eluted at 85 mM KCl. Note that, under these same conditions, treatment of the resin by batch elution (see above) released holoenzyme rather than catalytic subunit. It is possible that the subunits were also produced in the batch elution method but then reassociated to form the holoenzyme. We also found that the dissociation of the holoenzyme as shown in Figure 3 was not due to the pH shift from 7.5 to 8.5 (data not shown). It appears that the interaction of the resin and the gradient KCl elution at pH 8.5 resulted in dissociation of the holoenzyme. By using this method, we were able to prepare separate fractions containing the catalytic and cAMP binding activities.

Application of the S300 holoenzyme to a chromatofocusing column (Pharmacia) also resulted in separation of the catalytic and cAMP binding activities (Figure 4). The catalytic activity eluted first from the column at pH 6.0, followed by the cAMP binding activity at pH 5.4. These values do not represent the isoelectric points of the subunits, for they are quite different from the values obtained by isoelectric focusing (see below). Elution of protein from this column is a function of not only pH but also the affinity of the protein for the resin matrix as well as other unknown factors (manufacturer's description of the resin).

Chromatography of the S300 holoenzyme on a histone VII-S affinity column also caused the dissociation of the enzyme into subunits. The holoenzyme was completely bound to the affinity column when applied in TAMB (pH 7.5) (Figure 5). The catalytic activity was relatively tightly bound and could be eluted only after a large pH change to 25 mM sodium acetate buffer, pH 5.0. The activity that was eluted was not affected by addition of cAMP to the reaction mixture. Further elution of the column with acetate buffer containing 0.2 M KCl released the cAMP binding activity from the resin. We do not know whether the cAMP binding activity was bound to the histone itself or to the matrix of the resin. In any case the histone column provides an additional method for preparation of the subunits.

The dissociation of the holoenzyme into its catalytic and regulatory subunits during the chromatographic steps described above was apparently not due to proteolytic degradation of the regulatory subunit. An identical molecular weight (49 000) for the regulatory subunit was observed after spontaneous dissociation during S300 chromatography, after elution of the

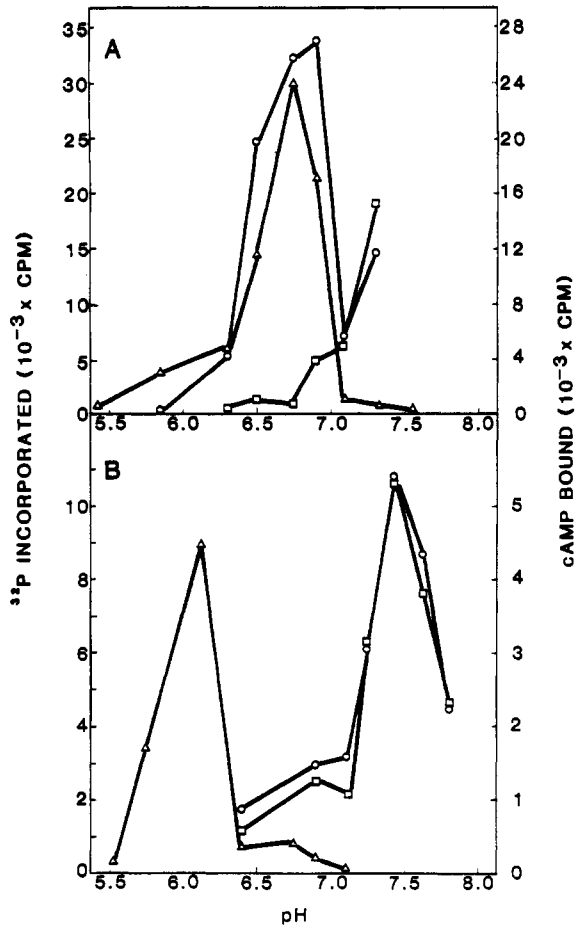

FIGURE 6: Preparative flat-bed electrofocusing in granular gel of S300 holoenzyme. The active fractions from the S300 column were pooled and concentrated to 3–4 mL on an Amicon PM10 membrane. The electrofocusing gel was removed from a trough in the gel bed, mixed with the enzyme sample, and poured back into the trough. Electrofocusing was carried out a 8-W constant power at maximum voltage and current for 14–16 h. After focusing, the gel was sectioned and the pH of each section was measured with a microelectrode. The enzyme was eluted from each section with 50 mM TAMB, pH 7.5. Protein kinase activity with cAMP (○) and without cAMP (□) in the reaction mixture and cAMP binding activity (△) were assayed as described under Materials and Methods. Panel A, low pH gel containing ampholines, pH range 5–8; panel B, high pH gel containing ampholines, pH range 5–8 and 7–9 in 3:2 v/v ratio.

free regulatory subunit from the DE-52 column, and after elution of the free regulatory subunit from the cAMP affinity column.

Although further purification of the S300 holoenzyme by any of the three methods described above resulted in dissociation of the enzyme, preparative electrofocusing yielded either holoenzyme or separated subunits (Figure 6). When the sample was applied to the dried low pH electrofocusing gel (see figure legend for details), the activity that was recovered was totally dependent on the addition of cAMP to the reaction mixture (Figure 6A). The isoelectric point of this holoenzyme was pH 6.8. In six separate experiments this value for the isoelectric point did not vary more than 0.1 pH unit. If the S300 holoenzyme was applied to the high pH electrofocusing gel, the enzyme dissociated into subunits (Figure 6B). The isoelectric points for the catalytic and cAMP binding activities were 7.4 and 6.1, respectively. As noted above, these values are quite different from the pH at which the enzyme eluted from the chromatofocusing column. The isoelectric points of the holoenzyme and catalytic subunit from *Dictyostelium* are identical with the values for rabbit muscle (Bechtel et al., 1977). The difference in the isoelectric points

Table I: Hydrophobic Chromatography of the Catalytic Subunit on Agarose–C_n or Agarose–C_n-NH_2 Series

no. of carbons (n)	enzyme units retained by resin[a]	
	C_n	C_n-NH_2
0	0 (0)[b]	1.1 (23)[b]
2	2.7 (57)	3.2 (68)
4	3.8 (81)	3.8 (81)
6	3.9 (83)	4.3 (91)
8	4.2 (89)	4.4 (94)
10	4.5 (96)	4.4 (94)

[a] Enzyme units are picomoles of phosphate incorporated into histone VII-S per minute. [b] Percentage of the total units added to the column that bound to the resin.

between the holoenzyme and the catalytic subunit in *Dictyostelium* offers the potential for purification of the catalytic subunit on an ion-exchange resin after addition of cAMP to the holoenzyme. As discussed earlier, however, the holoenzyme from *Dictyostelium* cannot be dissociated simply by adding cAMP to the medium. Attempts to dissociate the holoenzyme with cAMP-fortified electrofocusing medium were also unsuccessful. In this experiment the fractions containing cAMPdPK from preparative electrofocusing (as shown in Figure 6A) were pooled and incubated in the presence of 2 mM cAMP for 1 min. The sample was then reapplied to the electrofocusing gel and focused for an additional 14 h. Under these conditions the enzyme did not dissociate into subunits but instead was eluted as a cAMP-dependent activity in a fraction corresponding to pH 6.8.

Further Purification of the Catalytic and cAMP Binding Activities. The catalytic subunit of *Dictyostelium* cAMPdPK, as prepared by any of the above methods, was subjected to further purification by ion-exchange chromatography, hydrophobic chromatography, and affinity chromatography. Ion-exchange chromatography on CM-Sephadex was accomplished after dialysis of the catalytic subunit against 10 mM potassium phosphate buffer (pH 6.2) containing 2 mM 2-mercaptoethanol, 2 mM benzamidine, and 0.02% sodium azide. When the sample was applied to a column that was equilibrated in the same buffer, the enzyme was completely bound by the resin. The activity could then be recovered by eluting the column with a linear gradient to 0.2 M KCl. The kinase activity, which eluted at 75 mM KCl, was not affected by the addition of cAMP to the reaction mixture.

Hydrophobic chromatography also was an effective purification step for the catalytic subunit. The enzyme was applied to an agarose–C_n or agarose–C_n-NH_2 series (Miles-Yeda Ltd., Rehovot, Israel) with carbon spacers from 0 to 10 carbon atoms. Table I shows an increasing retention of the enzyme with longer carbon spacers. With both agarose–C_n and agarose–C_n-NH_2, 80% of the activity was removed with only 4-carbon spacers.

The histone affinity column proved to be the most effective step for the purification of the catalytic subunit. The kinase activity was completely bound by the resin while the majority of the applied proteins were found in the flow-through volume (Figure 7). The enzyme could then be eluted with 25 mM sodium acetate buffer, pH 5.0. Analysis of the fractions that contained activity by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and subsequent staining with silver nitrate revealed three protein bands with molecular weight between 40000 and 60000. The enzyme that eluted from the histone column was unstable, with complete loss occurring after 7 days at 7 °C. Freezing resulted in complete loss of the activity. Inclusion of bovine serum albumin (see Figure 7)

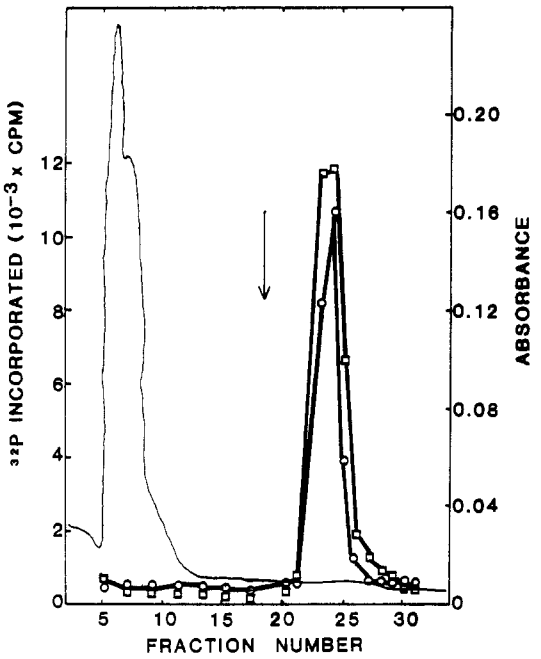

FIGURE 7: Chromatography of the catalytic subunit on a histone affinity column. The column was prepared as described in the legend to Figure 5. At the position indicated by the arrow, 25 mM AAMB (sodium acetate buffer, pH 5.0) was added to elute the enzyme. Symbols represent (○) protein kinase activity and (□) protein kinase activity in which 0.1% bovine serum albumin was included in the fractions.

or glycerol in the active fractions did not enhance their stability. However, if the sample was used within the first few days of preparation, its activity was sufficient for certain applications. For example, the catalytic subunit prepared by these methods was free of any endogenous substrates and, therefore, could be used to phosphorylate proteins that are possible substrates for the enzyme. Such an experiment has yielded several endogenous substrates that elute along with the holoenzyme during S300 chromatography (Frame and Rutherford, unpublished experiments).

The cAMP binding activity as prepared by any of the above methods was further purified by cAMP affinity chromatography. The sample was applied to 2-mL columns of cAMP–agarose (2-carbon spacers) equilibrated with TAMB, pH 7.5. All of the cAMP binding activity was retained by the resin. The column was washed with TAMB containing 1 M NaCl for 1 h to remove nonspecifically bound proteins and then rewashed with TAMB for 30 min. The cAMP binding activity was eluted from the column by incubating the resin for 60 min with TAMB containing 5 mM cAMP. The cAMP could then be separated from the binding activity by passing the cAMP–TAMB eluted material over a small DE-52 column equilibrated to pH 7.5 with TAMB. At this pH the cAMP binding activity is retained by the resin, but the cAMP is not. After the column was washed overnight with TAMB, the cAMP binding activity was eluted with a linear 0–0.2 M KCl gradient. The final product is completely free of any kinase activity.

Demonstration That the cAMP Binding Activity Is Actually Regulatory Subunit. Proof that the catalytic and cAMP binding activities as prepared by the methods described above were, in fact, the catalytic and regulatory subunits of the holoenzyme came from the reconstitution experiment, as shown in Table II. Addition of the cAMP binding activity to the catalytic activity resulted in 86% inhibition when compared to the catalytic activity alone. Inclusion of 10 μM cAMP in an identical mixture of the two subunits completely restored

Table II: Reconstitution of Holoenzyme from Catalytic and Regulatory Subunits

contents[a]	cAMP[b]	activity[c]	% control[d]
catalytic subunit	+/-[e]	352	100
catalytic plus regulatory subunit	-	50	14
catalytic plus regulatory subunit	+	341	97

[a] The catalytic subunit (10 μL) was mixed with either 15 μL of 50 mM TAMB (pH 7.5) or 15 μL of the regulatory subunit. The reaction mixture (24 μL) without Kemptide, and with or without cAMP, was added and mixtures were preincubated for 10 min at 0 °C. The reaction was started by adding 1 μL of Kemptide (1.25 mg/mL) and continued as described under Materials and Methods. The catalytic and regulatory subunits were eluted from the DE-52 column (pH 8.5) and dialyzed against 50 mM TAMB (pH 7.5) before use. [b] Final concentration of 10 μM in preincubation mixtures. [c] Activity is expressed as femtomoles of phosphate incorporated into Kemptide per minute. [d] Control is equal to activity of the catalytic subunit alone. [e] There was no difference in the kinase activity in either the presence or the absence of cAMP.

the activity of the catalytic subunit. Reconstitution of the holoenzyme could be accomplished with samples of the two subunits as prepared by any of the chromatographic methods described above.

Discussion

In this report we extend our earlier description of a cAMPdPK in *Dictyostelium* (Rutherford et al., 1982) and describe its chromatographic behavior. Conditions are presented under which the holoenzyme will bind to several chromatographic resins. With the exception of electrofocusing under certain conditions, elution of the holoenzyme from these resins resulted in dissociation of the catalytic and regulatory subunits. These results emphasize the difficulty that will be encountered in obtaining a pure form of the holoenzyme in this organism and demonstrate that the enzyme from *Dictyostelium* is more labile than the enzyme from mammalian tissues. However, both subunits are relatively stable, and several methods are described for their further purification. By using these procedures, one is able to obtain a preparation that is free of most other endogenous proteins and that contains only one of the subunits. Thus, by choosing the appropriate separation method, one should be able to obtain the catalytic subunit that is free of any endogenous substrate of interest. This result should enable the future investigation of cAMPdPK-mediated phosphorylation of specific endogenous proteins or enzymes. Likewise, the separate preparation of the two subunits will allow future research on the nature of dissociation and reassociation of the holoenzyme and, therefore, insight into the regulation of the enzyme in vivo.

The data presented here, as well as in our earlier report (Rutherford et al., 1982), show that the cAMPdPK from *Dictyostelium* resembles those enzymes that are described in the literature from other species. Similarities exist in the subunit structure of the holoenzyme, molecular weight of the holoenzyme and its subunits, isoelectric point of the holoenzyme and its subunits, and some of the kinetic constants (Flockhart & Corbin, 1982). A property that is unique to the *Dictyostelium* enzyme is its dissociation during elution from ion-exchange columns. That this property was unique to *Dictyostelium* and not due to some peculiarity of the methodology was demonstrated in control experiments using rabbit muscle as the source of the enzyme. Under the same conditions that resulted in dissociation of the *Dictyostelium* enzyme, the enzyme from rabbit muscle remained as a holoenzyme. In addition, differences exist between the enzyme

from *Dictyostelium* and the enzyme from rabbit muscle in the ease of dissociation of the holoenzyme in crude extracts. The rabbit muscle enzyme readily dissociates in the presence of cAMP while the *Dictyostelium* enzyme does not. Perhaps this result is not surprising since in another fungus, *Mucor*, cAMP was effective in dissociating the holoenzyme only in the presence of histone and/or NaCl (Moreno & Passeron, 1980). However, these treatments were also without effect on the *Dictyostelium* enzyme (data not shown).

The results of this study have also provided some insight into early reports in the literature on *Dictyostelium* protein kinase. Rahmsdorf & Gerisch (1978) and Veron & Patte (1978) reported the presence of a cAMP-independent protein kinase that could be demonstrated after elution from an anion-exchange column. Cyclic AMP binding activity from this same column eluted in fractions that were separate from the kinase activity. The results shown in Figure 3 of the present report demonstrate that if a known cAMPdPK holoenzyme is applied to an anion-exchange column, the holoenzyme will dissociate during elution and the subunits will elute in separate fractions. Thus the lack of a demonstrable cAMPdPK in these earlier studies may have been due to the use of chromatographic methods which, although they are standard approaches for the demonstration of skeletal muscle enzyme, are inappropriate for the enzyme from *Dictyostelium*. These results emphasize the necessity to understand the unique chromatographic behavior and properties of the *Dictyostelium* enzyme, as described in this report, before attempting to investigate the mechanism of regulation of cAMPdPK during development.

Registry No. Protein kinase, 9026-43-1.

References

Abe, K., & Yanagisawa, K. (1983) *Dev. Biol. 95*, 200.

Bechtel, P. J., Beavo, J. A., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 2691.

Bonner, J. T. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 110.

Bonner, J. T., Barkley, D. S., Hall, E. M., Konijn, T. M., Mason, J. W., O'Keefe, G., & Wolfe, P. B. (1969) *Dev. Biol. 20*, 72.

Brenner, M. (1977) *J. Biol. Chem. 252*, 4073.

Brenner, M. (1978) *Dev. Biol. 64*, 210.

Brown, S. S., & Rutherford, C. L. (1980) *Differentiation (Berlin) 16*, 173.

Cooper, S., Dasen, K., Lawton, M., & Ferguson, A. (1983) *Biochim. Biophys. Acta 746*, 120.

Corbin, J. D., Keely, S. L., & Park, C. R. (1975) *J. Biol. Chem. 250*, 218.

De Gunzberg, J., & Veron, M. (1982) *EMBO J. 9*, 1063.

Flockhart, D. A., & Corbin, J. D. (1982) *CRC Crit. Rev. Biochem. 12*, 133.

Kay, R. R. (1979) *J. Embryol. Exp. Morphol. 52*, 171.

Konjin, T. M., Barkley, D. S., Chang, Y. Y., & Bonner, J. T. (1968) *Am. Nat. 102*, 225.

Landfear, S. M., Chung, S., Lefebvre, P., Chung, S., & Lodish, H. F. (1982) *Mol. Cell. Biol. 2*, 1417.

Maeda, Y., & Maeda, M. (1974) *Exp. Cell Res. 84*, 88.

Matsukuma, S., & Durston, A. J. (1979) *J. Embryol. Exp. Morphol. 50*, 243.

Merkle, R. K., & Rutherford, C. L. (1984) *Differentiation (Berlin) 26*, 23.

Moreno, S., & Passeron, S. (1980) *Dev. Biol. 66*, 321.

Pahlic, M., & Rutherford, C. L. (1979) *J. Biol. Chem. 254*, 9703.

Pan, P., Bonner, J. T., Wedner, H. J., & Parker, C. W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1623.

Rahmsdorf, H. J., & Gerisch, G. (1978) *Cell Differ. 7*, 249.

Robinson, G. A., Butcher, R. W., & Sutherland, E. W. (1971) *Cyclic AMP*, Academic Press, New York.

Rutherford, C. L. (1976) *Biochim. Biophys. Acta 451*, 212.

Rutherford, C. L., Taylor, R. D., Frame, L. T., & Auck, R. L. (1982) *Biochem. Biophys. Res. Commun. 108*, 1210.

Sternfield, J., & David, C. N. (1981) *Differentiation (Berlin) 20*, 10.

Town, C. D., Gross, J. D., & Kay, R. R. (1976) *Nature (London) 262*, 717.

Veron, M., & Patte, J. (1978) *Dev. Biol. 63*, 370.