An Unusual Adenosine 3′,5′-Phosphate Dependent Protein Kinase from *Dictyostelium discoideum*†

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ABSTRACT: The cAMP-dependent protein kinase from *Dictyostelium discoideum* was extracted from cells at the stage of culmination. Less than 50% of the enzyme remains as a cAMP-dependent holoenzyme in the extracts, and the rest is recovered in the form of dissociated regulatory and catalytic subunits that were purified. The regulatory subunit is a monomeric protein of M_r 42 000 that carries only one cAMP binding site (K_d = 3 nM). The catalytic subunit is also a monomer of M_r 40 000 with a sedimentation coefficient of 3.3 S. The cAMP-dependent holoenzyme is a dimer consisting of one regulatory and one catalytic subunit, and the same structure is found for the holoenzyme reconstituted from the isolated subunits. Whereas cAMP binding to the regulatory subunit is independent of pH, both the catalytic activity and its ability to be inhibited by addition of regulatory subunit are increased very strongly between pH 5.5 and 7. The differences in molecular and catalytic properties of this cAMP-dependent protein kinase with those from mammalian origin are discussed in relation with the possibility that the enzyme from *Dictyostelium* represents an early form of the molecule in the evolutionary process.

Vegetative amoebae from the cellular slime mold *Dictyostelium discoideum* are induced by food deprivation to initiate a developmental program leading to the formation of a multicellular aggregate that further differentiates into a fruiting body composed of two major cell types, stalk cells and spores (Loomis, 1975). cAMP[1] plays a crucial role both in the aggregation process as an extracellular chemoattractant (Robertson & Grutsch, 1981) and as an intracellular effector in the transcription and stability of mRNAs specific to the steps occurring after the establishment of tight cellular contacts (Landfear & Lodish, 1980; Mehdy et al., 1983; Mangiarotti et al., 1983).

Several cAMP binding proteins that could be intracellular targets for cAMP in *D. discoideum* cells have been described (Cooper et al., 1980; Leichtling et al., 1981a; de Gunzburg & Véron, 1981; Arents & van Driel, 1982). Two types of proteins have been distinguished on the basis of their function, molecular structure, and developmental regulation. One of these proteins has recently been identified as the enzyme *S*-adenosyl-L-homocysteine hydrolase (de Gunzburg et al., 1983), and current studies in our laboratory indicate that cAMP might play a role in the regulation of this enzymatic activity (Hohman & Véron, 1984). However, the only well-documented system in eukaryotic cells through which cAMP is known to play a regulatory role is via the activation of cAMP-dependent protein kinases [reviewed in Flockhart & Corbin (1982)]. These enzymes are generally found in the form of a catalytically inactive R_2C_2 holoenzyme complex composed of two regulatory (R) and two catalytic (C) subunits. Activation proceeds via the binding of cAMP to the regulatory subunits, thereby inducing the complex to dissociate into an R_2 dimer of regulatory subunits and two free and active catalytic subunits. cAMP stimulation of protein kinase activity in *D. discoideum* extracts has been difficult to demonstrate, especially when histones were used as substrates (Véron & Patte, 1978; Rahmsdorf & Gerisch, 1978). However, one of the cAMP binding proteins has been identified as the regulatory subunit of a cAMP-dependent protein kinase (Leichtling et al., 1981b; Leichtling et al., 1982; Rutherford et al., 1982).

We have recently reported the presence of a cAMP-dependent protein kinase activity in differentiating *D. discoideum* cells and purified the regulatory subunit (de Gunzburg & Véron, 1982). We now describe the molecular properties of the holoenzyme from *D. discoideum* and of its isolated subunits.

Materials and Methods

Chemicals and Buffers. [5′,8-^{3}H]cAMP (43 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham International Ltd. N^6-(Aminoethyl)-cAMP–agarose was from P-L Biochemicals, Inc. (Milwaukee, WI) and 8-[(β-hydroxyethyl)thio]cAMP was synthesized according to Muneyama et al. (1971) and coupled to epoxy-activated Sepharose 6B as previously described (Weber et al., 1979). P81 cellulose phosphate paper was purchased from Whatman (U.K.) and Selectron filters (Type BA85, 0.45 μm, sheets or 25 mm diameter circles) from Schleicher & Schüll (West Germany). Nucleotides and kemptide were purchased from Sigma.

Buffer A was 10 mM MOPS, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, and 10 mM benzamidine and was adjusted to pH 7.5 with KOH. Buffer B consisted of 10 mM MOPS (pH 7.5 unless otherwise stated) containing 2 mM EDTA, 50 mM KCl, 5 mM 2-mercaptoethanol, 0.01% Triton X-100, and 5 mM benzamidine. Buffer C was similar to buffer A except that it contained 0.01% Triton X-100 and was adjusted to pH 6.5 with KOH.

Growth and Differentiation. D. discoideum amoebae of the axenic strain AX2 were grown at 22 °C in HL5 broth (Watts & Ashworth, 1970) to a density of (4–6) × 10^6 cells/mL. The cells were collected, washed free of nutrient medium, and plated for differentiation at 22 °C on 50 × 60cm trays con-

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[1] Abbreviations: cAMP, adenosine 3′,5′-phosphate; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DIF, differentiation inducing factor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene.

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taining 2% nonnutrient agar as previously described (de Gunzburg & Véron, 1982). After 18 h, cellular masses had reached the early culmination stage and contained mostly prestalk and prespore cells; a few mature fruiting bodies were also present. Cells were harvested, washed once, collected by centrifugation, frozen in liquid N_2, and stored at −80 °C. In a typical preparation, 12 L of culture yielded 20–30 g of frozen cells.

Preparation of Extracts. A total of 50 g of frozen cells was thawed in 300 mL of buffer A containing 0.4 mM phenylmethanesulfonyl fluoride (PMSF). The suspension was homogenized by five strokes in a Dounce homogenizer and centrifuged at 4 °C for 15 min at 16000*g*. The pellet consisting of mature spores and cellular debris was discarded. The supernatant was adjusted to pH 7.5 with 1 M KOH and centrifuged for 45 min at 143000*g*. This second supernatant was diluted 2.5-fold with cold distilled water, EDTA was added to a final concentration of 0.1 mM, and the pH was readjusted to 7.5 with KOH. The extract was immediately loaded on a DEAE-Sephacel column as described in the text. Meanwhile, this procedure was repeated with another batch of 50 g of cells until a total of 250 g, corresponding to 6.6×10^{11} cells, was processed.

The "crude soluble extract" for immunoblotting experiments was made by thawing 0.5 g of frozen cells in 1 mL of buffer A containing 0.4 mM PMSF. The extract was homogeneized as described above and centrifuged at 4 °C for 10 min at 12000*g* and then for 30 min at 180000*g*. The "total cell lysate" was obtained by direct immersion of 5×10^7 frozen cells in 0.25 mL of boiling SDS-PAGE sample buffer.

Assays. Measurements of cAMP binding were performed as previously described (de Gunzburg & Véron, 1981) with [^{3}H]cAMP (43 Ci/mmol). Briefly, samples were incubated at 0 °C for 30 min in a final volume of 50 μL with 0.1 μM [^{3}H]cAMP and filtered through Selectron filters, which were washed, dried, and counted in toluene–POPOP scintillant. For the determination of the association rate constant, purified regulatory subunit was incubated at 0 °C in a final volume of 1 mL of buffer B (pH 6 or 7.5) with 5 nM [^{3}H]cAMP. After increasing periods of time, the extent of cAMP binding was measured by filtration. In order to determine the dissociation rate constant, purified regulatory subunit was preincubated for 3 h at 0 °C with 0.1 μM [^{3}H]cAMP in buffer B (pH 6 or pH 7.5). A 50-μL aliquot of this mixture was added to 10 μL of 0.5 mM nonradioactive cAMP, and residual [^{3}H]cAMP binding was measured by filtration at increasing times at 0 °C after isotopic dilution. Equilibrium dialysis experiments were performed in 0.5-mL dialysis cells with a final concentration of 50 nM [^{3}H]cAMP.

Protein kinase activity was determined by using the synthetic heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (kemptide) as substrate (Kemp et al., 1977). Assays were carried out at 30 °C in a final volume of 50 μL containing 50 mM MOPS buffer, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 20 mM NaF, 0.2 mM kemptide, 0.2 mM [γ-^{32}P]ATP (0.15 μCi/nmol), and the appropriate amount of enzyme. The reaction was initiated by the simultaneous addition of [γ-^{32}P]ATP and kemptide and stopped after 5–20 min with 25 μL of glacial acetic acid. Under these conditions, the assay was linear with respect to time and enzyme concentration. The phosphopeptide was separated from unreacted [γ-^{32}P]ATP on 2 × 2 cm phosphocellulose papers (Glass et al., 1978) and counted in toluene–POPOP scintillant. One unit of protein kinase activity will transfer 1 nmol of phosphate/min at 30 °C to kemptide. Protein concentrations were determined either according to Bradford (1976) with bovine γ-globulin as a standard or with the amidoschwartz dye method (Schaffner & Weissmann, 1973) with bovine serum albumin as a standard.

Sucrose Gradient Centrifugation. Samples in buffer A (20–60 μL) were layered on top of 4-mL linear 5–20% sucrose gradients prepared in the same buffer. After centrifugation for 15 h at 54000 rpm in a Beckman SW56 rotor at 4 °C, 130-μL fractions were collected from the bottom of the tube and assayed for [^{3}H]cAMP binding and protein kinase activity in the presence of 20 μM cAMP.

Gel Electrophoresis. Nondenaturing gel electrophoresis was carried out at 4 °C in 7.5% polyacrylamide tube gels according to Davis (1964). Gels were either stained with Coomassie brilliant blue or sliced into 3-mm fractions. The slices were crushed, eluted overnight at 4 °C in 0.11 mL of 50 mM MOPS buffer (pH 7.5) containing 0.1 mM EDTA, 15 mM 2-mercaptoethanol, 0.01% Triton X-100, and 0.11 M HCl (which was necessary to compensate the alkalinity of the gel), and assayed for protein kinase activity. SDS gels were performed on 10% polyacrylamide slab gels according to Laemmli (1970) and stained with 0.125% Coomassie brilliant blue.

Preparation of Antibodies and Immunoblotting Experiments. Antibodies against the regulatory subunit were raised in two female Buscat rabbits. The rabbits were injected subcutaneously with 50 μg of purified regulatory subunit in Freund's complete adjuvant at a 6–8-week interval. The third injection was performed in Freund's incomplete adjuvant. The rabbits were bled from the ear 7, 14, and 21 days following the second and third injections. For immunoblotting, proteins separated on SDS gels were electrophoretically transferred to nitrocellulose sheets (Towbin et al., 1979). Blots were incubated for 30 min at 37 °C with 2% bovine serum albumin in 20 mL of Tris–saline buffer (10 mM Tris-HCl buffer, pH 7.3, containing 0.9% NaCl) and for 1 h at 37 °C with antiserum diluted 50-fold in 20 mL of Tris–saline containing 2% bovine serum albumin. The blots were then washed 3 times with 20 mL of Tris–saline, 3 times with 20 mL of Tris–saline containing 0.05% Nonidet NP-40, and 3 times with 20 mL of Tris–saline. The nitrocellulose sheets were incubated for 1 h at 30 °C with 1 μCi of ^{125}I-labeled protein A in 20 mL of Tris–saline containing 2% bovine serum albumin, washed as described above, dried, and exposed at −80 °C to Kodak X-Omat AR films with a Cronex Lightning Plus intensifying screen (E. I. Du Pont de Nemours, Wilmington, DE).

Results

Separation of Holoenzyme from Dissociated Subunits. Figure 1 shows the elution profile of the chromatography of the soluble extract on DEAE-Sephacel. No activity was recovered in the flow-through. Two peaks of protein kinase activity (A and B) were eluted with the salt gradient, and only peak B was activated by cAMP. Whereas the bulk of the cAMP binding activity (peak b) was devoid of protein kinase activity, a minor fraction (peak a) coeluted with the cAMP-dependent protein kinase activity. Hence, the activities present in peak B correspond to the holoenzyme composed of regulatory (R) and catalytic (C) subunits. Reconstitution experiments showed that the protein kinase activity in peak A and the cAMP binding activity in peak b correspond to the dissociated catalytic and regulatory subunits, respectively. Indeed, addition of cAMP binding protein purified from the material in peak b (see below) results in the inhibition of the protein kinase activity from peak A, and this inhibition is relieved by cAMP (Table I, line 1).

In order to further separate the holoenzyme from the dissociated catalytic subunit, the fractions from the DEAE

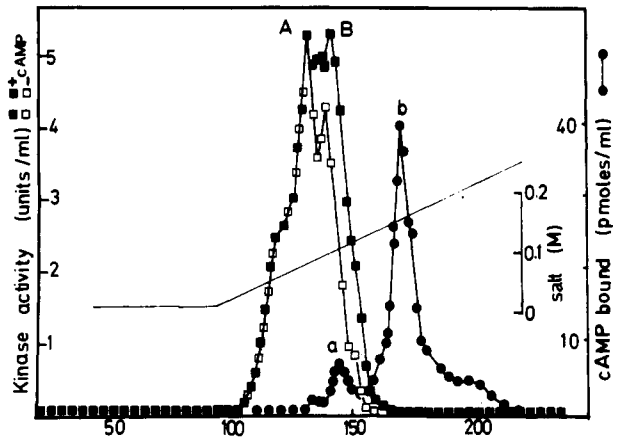

FIGURE 1: Chromatography of crude extract on DEAE-Sephacel. The crude soluble extract was loaded at a flow rate of 600 mL/h onto a 6.7 × 30 cm DEAE-Sephacel column equilibrated at 4 °C in buffer A. The column was washed at 140 mL/h with buffer A and developed with a linear 0–0.4 M KCl gradient in the same buffer. Fractions (25 mL) were analyzed for cAMP binding (●) and protein kinase activity in the absence (□) or in the presence (■) of 20 μM cAMP.

Table I: Inhibition of Protein Kinase Activity by Purified Regulatory Subunit

sample	addition[a] cAMP	purified R	purified R + cAMP
DEAE-Sephacel, peak A (fraction 110)	100	3.93	98.4
hydroxylapatite, peak A (fraction 55)	120	3.76	110
gel filtration a, peak II (fraction 80)	194	13.0	163
gel filtration b, peak II (fraction 80)	100	4.81	118

[a] Samples (20 μL) of the appropriate fraction were preincubated for 30 min at 0 °C in a final volume of 40 μL with 20 μM cAMP or 0.36 pmol of regulatory subunit and then assayed. Protein kinase activities are expressed as percent of the activity determined with no addition.

column containing protein kinase activity (fractions 100–150 in Figure 1) were pooled and chromatographed on hydroxylapatite. Figure 2 shows the elution profile in which two peaks of kinase activity can again been distinguished. The activity in peak B was strongly stimulated by cAMP and coeluted with the cAMP binding activity. Upon gel filtration of the corresponding fractions (Figure 3a), more than 80% of the cAMP binding component remained associated with the protein kinase activity that eluted from the column at a position corresponding to an apparent M_r of 88 000 (peak I). This represents the protein kinase holoenzyme, since the activity is highly dependent on cAMP. A second peak of protein kinase activity also appeared (peak II, Figure 3a) with an apparent M_r of 44 000, probably reflecting partial dissociation of the subunits during the ammonium sulfate precipitation that preceded the gel filtration step. Peak II contains the free catalytic subunit and some regulatory subunit. The stimulation of kinase activity by cAMP indicates that the dissociated subunits are able to reassociate in the column fractions or in the assay (see also Figure 6).

The kinase activity in peak A from the hydroxylapatite column depicted in Figure 2 was essentially devoid of cAMP binding activity and not stimulated by the addition of cAMP. Analysis of these fractions by gel filtration (Figure 3b) shows that the bulk of the protein kinase activity elutes at the same position as peak II in Figure 3a. This peak represents free

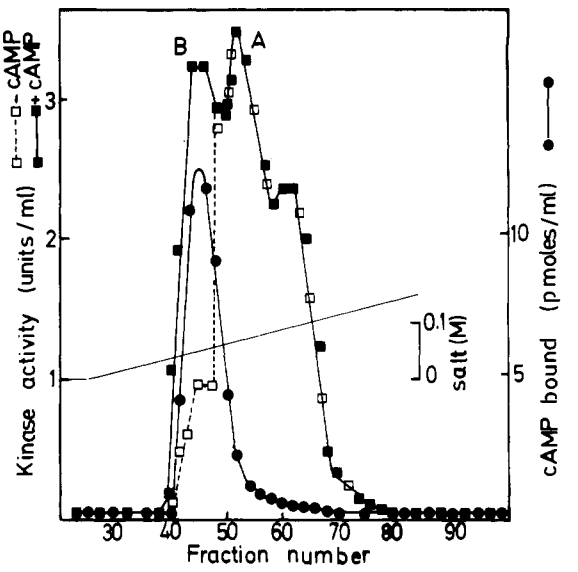

FIGURE 2: Chromatography of the protein kinase on hydroxylapatite. Fractions 100–156 containing protein kinase activity eluted from the DEAE-Sephacel column depicted in Figure 1 were pooled and loaded onto a Bio-Gel HTP column (4.5 × 26 cm) equilibrated in buffer A. The column was developed with a linear 0–0.4 M gradient of potassium phosphate buffer, pH 7.5, in buffer A at a flow rate of 90 mL/h. Fractions (25 mL) were assayed for cAMP binding (●) and protein kinase activity inthe absence (□) or in the presence (■) of 20 μM cAMP.

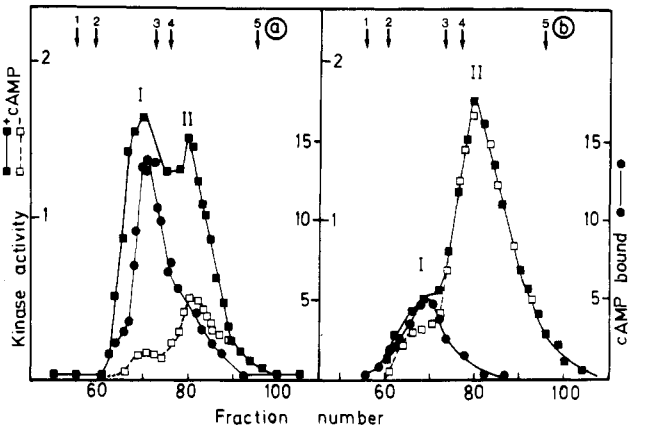

FIGURE 3: Gel filtration on Ultrogel AcA44 of the cAMP-dependent protein kinase. (a) Fractions from peak B (40–50) of the hydroxylapatite column described in Figure 2 were pooled and precipitated with 80% ammonium sulfate. The pellet was redisolved in 10–20 mL of buffer A containing 50 mM KCl and loaded onto a 2.5 × 93 cm calibrated column of Ultrogel AcA44. Elution was carried out at 20 mL/h with the same buffer and fractions (4 mL) were assayed for cAMP binding (●) and protein kinase activity in the absence (□) or in the presence (■) of 20 μM cAMP. The arrows indicate the positions of the peak activities of the following marker enzymes: (1) *S*-adenosyl-L-homocysteine hydrolase from *D. discoideum* (de Gunzburg et al., 1983); (2) aldolase; (3) hemoglobin; (4) bovine serum albumin; (5) chymotrypsinogen. (b) The same experiment was performed with fractions from peak A (51–70) of the hydroxylapatite column described in Figure 2.

catalytic subunit as demonstrated by its ability to be reversibly inhibited upon addition of purified regulatory subunit (Table I, lines 2–4).

Purification of the Regulatory Subunit. Purification was carried out at 4 °C by a slight modification of the method previously described (de Gunzburg & Véron, 1982). Fractions corresponding to peak b in the DEAE column (Figure 1, tubes 160–180) were pooled and loaded onto a column (1 × 7.5cm) made of N^6-(aminoethyl)-cAMP–agarose in buffer A. The

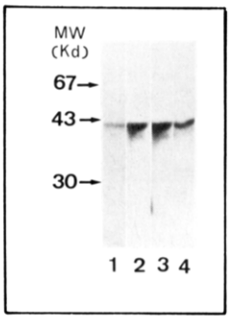

FIGURE 4: Immunoblotting of the regulatory subunit. Extracts were submitted to SDS–PAGE in 10% gels and either stained with Coomassie brilliant blue (lane 1) or electrophoretically transferred to nitrocellulose sheets (lanes 2–4). Immunoblotting was carried out as described under Materials and Methods: (lanes 1 and 2) 3–5 μg of purified R; (lane 3) 150 μg of protein from a crude soluble extract; (lane 4) 150 μg of protein from a total cell lysate (see Materials and Methods).

column was washed with buffer B and then with the same buffer containing 2 M KCl. The regulatory subunit was eluted with 30 mM cAMP in buffer B. After dialysis against buffer B for 5 days with one change per day, the fractions with cAMP binding activity were pooled and loaded onto a second column (0.7 × 5 cm) containing 8-[(β-hydroxyethyl)thio]-cAMP–Sepharose 6B in buffer B. The column was washed, and the regulatory subunit was eluted with cAMP and dialyzed as described above. The pure protein (450 μg of regulatory subunit corresponding to a 12 500-fold purification with a 40% yield) was stored at 4 °C with 0.02% sodium azide. As shown in Figure 4, lane 1, it gave a single band of 42 kDa on SDS gels.

In order to compare the molecular weight of the purified regulatory subunit to that present in the cell and thus to eliminate the possibility that the purified regulatory subunit was a product of proteolytic degradation, antibodies against the purified regulatory subunit were used to identify the corresponding protein in crude extracts. The immunoblotting experiment shown in Figure 4 demonstrates that the antiserum indeed recognizes the regulatory subunit either purified or in a crude extract of differentiating cells. No band was detected with preimmune serum (not shown). The molecular mass of the purified protein (lanes 1 and 2) is identical with that of the regulatory subunit from a crude soluble extract (lane 3). In order to further eliminate the possibility of proteolysis during preparation of the extract analyzed in lane 3, differentiating cells were lysed by direct immersion in boiling SDS immediately followed by immunoblotting. The results (lane 4) show that the same 42-kDa polypeptide was detected. Such an experiment was repeated with cells from various stages of development, and in no cases was any evidence found for a regulatory subunit with a higher molecular weight. These results demonstrate that the regulatory subunit present in *D. discoideum* cells in indeed a protein of 42 kDa.

Purification of the Catalytic Subunit. The protein kinase activity recovered from gel filtration (fractions 75–90 in Figure 3b) was loaded onto a column (1.5 × 15cm) of Affi-Gel Blue equilibrated in buffer A. The catalytic activity recovered in the flow-through (referred to as "partially purified") was further purified by chromatography on a column (0.7 × 5 cm) of CM-Sephadex equilibrated in buffer C. The column was developed with a gradient (10 mL) of KCl (0–0.4 M), and the protein kinase activity was eluted as a homogeneous species at 0.2 M KCl. Demonstration that the purified protein actually corresponds to the catalytic subunit came from the experiment depicted in Figure 5, in which nondenaturing

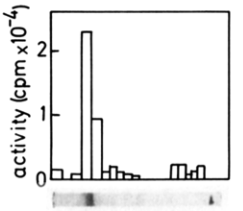

FIGURE 5: Electrophoresis in nondenaturing gels of the purified catalytic subunit. Purified catalytic subunit was subjected to electrophoresis in duplicate nondenaturing 7.5% polyacrylamide tube gels. (Top) One of the gels was sliced into 3-mm fractions. The protein was eluted and the activity in the eluates measured. (Bottom) Coomassie blue staining. Migration was from left to right; the mark at the far end of the gel corresponds to the final position of the tracking dye.

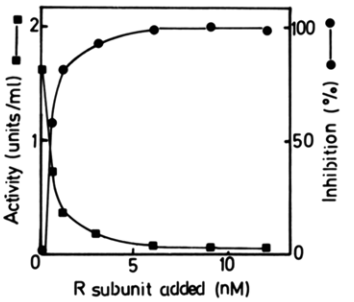

FIGURE 6: Inhibition of the catalytic subunit by the addition of regulatory subunit. Partially purified catalytic subunit was preincubated for 15 min at 22 °C with increasing concentrations of purified regulatory subunit in a final volume of 40 μL containing 80 mM MOPS buffer, pH 7.5. Residual activity was measured at 22 °C. The final concentration of regulatory subunit was calculated by assuming an M_r of 42 000.

polyacrylamide gels were used to show that the only protein band present also carried the protein kinase activity. The pure enzyme has a molecular specific activity of 7 units/μg, when protein is measured with Coomassie blue. The catalytic subunit has a Stokes radius of 29 Å as measured by gel filtration on Ultrogel AcA44 (Figure 3) and a sedimentation coefficient of 3.3 S (see Figure 7). Assuming a partial specific volume of 0.73 g/cm^3, the molecular mass of the catalytic subunit was calculated to be 41 000 by the method of Siegel & Monty (1966). Since SDS gels of the purified catalytic subunit also gave a single band of 40 kDa (not shown), we conclude that the isolated catalytic subunit is a monomer.

Subunit Structure of the Holoenzyme. Comparison of the molecular weight of the holoenzyme (see Figure 3) with that of the dissociated catalytic and regulatory subunits leads to the conclusion that the cAMP-dependent protein kinase from *D. discoideum* is a dimer of 82 000 daltons composed of one regulatory (42 kDa) and one catalytic subunit (40 kDa).

Since an important fraction of the cAMP-dependent protein kinase was isolated in the form of dissociated subunits, it was necessary to assess the structure of the reconstituted holoenzyme. Indeed, Figure 6 shows that the catalytic subunit interacts with the purified regulatory subunit. At pH 7.5 and in the absence of cAMP, addition of regulatory subunit results in complete inhibition of the catalytic activity. Given a molecular specific activity of 7 units/μg pure catalytic subunit (see above), 95% inhibition is obtained with a 1:1 molar ratio of regulatory to catalytic subunits. The inhibition is reversed by cAMP with a half-maximal effect at 40 nM (see below). This reversal is highly specific for cAMP and has the same requirements as cAMP binding to the regulatory subunit (de Gunzburg & Véron, 1982). Figure 7 shows an analysis of the inhibited complex by sucrose gradient sedimentation. The

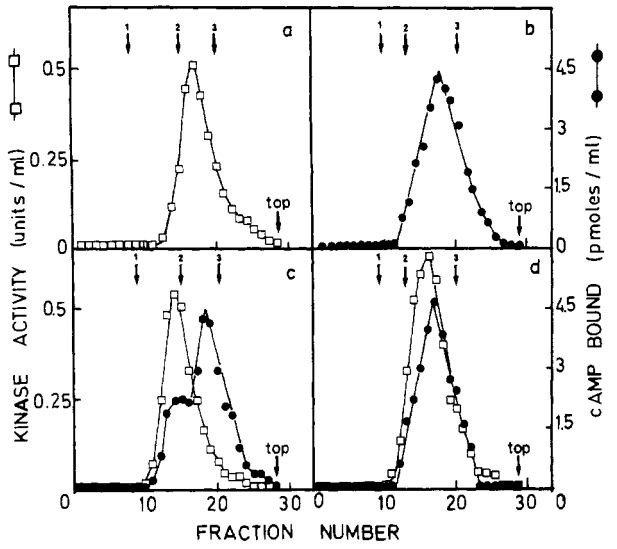

FIGURE 7: Sucrose gradient centrifugation analysis of reconstituted cAMP-dependent protein kinase. Sucrose gradient centrifugation was performed as described under Materials and Methods. Fraction 1 corresponds to the bottom of the tube. (a) Partially purified catalytic subunit (0.44 unit); (b) purified regulatory subunit (3.5 pmol); (c) partially purified catalytic subunit (0.44 unit) together with purified regulatory subunit (3.5 pmol); (d) the same sample as in (c) preincubated for 30 min at 4 °C with 1 μM cAMP and sedimented in a gradient also containing 1 μM cAMP. In the latter case, nonradioactive cAMP bound to the regulatory subunit exchanged during the cAMP binding assay with both free nonradioactive cAMP contained in the sample and radioactive cAMP added in the assay. The resulting isotopic dilution of the ligand was taken into account for activity calculations. The arrows show the positions of marker proteins added to the sample prior to centrifugation: (1) lactic dehydrogenase from rabbit muscle (7.3 S); (2) human hemoglobin (4.2 S); (3) soybean trypsin inhibitor (2.3 S).

monomeric regulatory and catalytic subunits sedimented behind hemoglobin at 3.2 and 3.3 S, respectively (Figure 7a,b). In contrast, when the two types of subunits were mixed together (Figure 7c), both the cAMP binding and the protein kinase activities sedimented slightly ahead of hemoglobin. The additional peak of cAMP binding activity in Figure 7c is due to the large excess regulatory over catalytic subunit in this experiment. Preincubation and sedimentation of both subunits in the presence of 1 μM cAMP restored the sedimentation pattern of the isolated subunits (Figure 7d). Therefore, inhibition of the activity of the catalytic subunit upon addition of the regulatory subunit results from the formation of a stable reassociated complex. The sedimentation coefficient of the reconstituted holoenzyme (4.4 S) is that expected for a dimer of one catalytic and one regulatory subunit. No indication of a heavier complex that could correspond to a tetramer has been observed, even when the ratio of regulatory to catalytic subunits was much larger than that in the experiment shown in Figure 7c.

Dependence of Protein Kinase and cAMP Binding Activities upon pH. Figure 8 shows that the activity of the isolated catalytic subunit is highly dependent on pH within a physiological range. The activity is undetectable at pH 5 and increases 15-fold from pH 5.5 to 7.0. The Michaelis constants of the protein kinase activity for its substrates were measured at pH 6.0 and 7.5. The K_m for ATP (50 μM) remained unchanged whereas the K_m for kemptide increased from 60 μM at pH 7.5 to 400 μM at pH 6.0. In contrast, cAMP binding to the regulatory subunit did not vary significantly between pH 5.5 and 8.0 (Figure 8). Binding at 0 °C and with 5 nM cAMP was saturated within 30 min, and the association rate constant (k_1 = 3.7 × 10^5 M^{-1} s^{-1}) was the same at pH

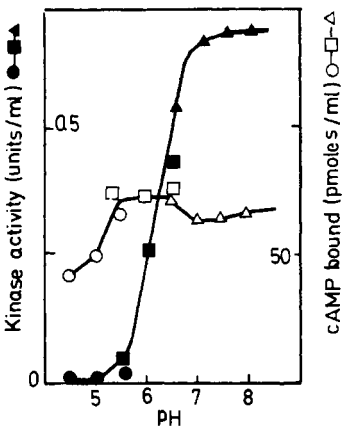

FIGURE 8: pH dependence of cAMP binding to the regulatory subunit and activity of the catalytic subunit. Purified regulatory subunit (1.4 pmol in 20 μL of buffer B) was incubated in a final volume of 50 μL with 160 mM buffer at the indicated pH and 0.1 μM [^{3}H]cAMP (45 Ci/mmol). After 1 h at 0 °C, cAMP binding was measured by the filter assay (open symbols). Partially purified catalytic subunit (20 μL in buffer A) was preincubated for 1 h at 0 °C with 400 mM buffer (20 μL) at the indicated pH. Activity was measured during a 20-min reaction at 22 °C at the corresponding pH (filled symbols). The buffers used to set the pH were (○, ●) potassium acetate at pH 4.5, 5.0, and 5.5, (□, ■) MES buffer at pH 5.5, 6.0, and 6.5, and (△, ▲) MOPS buffer at pH 6.5, 7.0, 7.5, and 8.0. All determinations were performed in duplicate.

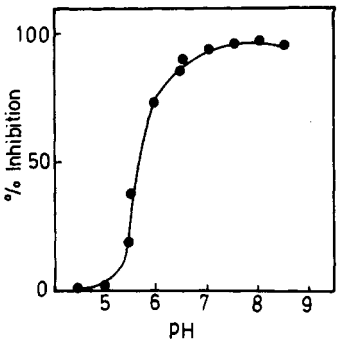

FIGURE 9: pH dependence of the interaction between regulatory and catalytic subunits. Partially purified catalytic subunit (0.01 unit) was preincubated with a large excess of purified regulatory subunit (0.63 pmol) for 1 h at 0 °C in a final volume of 40 μL containing 20 μL of buffer A, 5 μL of buffer B, and 160 mM buffer at the indicated pH. Buffers and determination of residual activity were as described in the legend to Figure 8. Results are expressed as percent inhibition compared to the control incubated at the same pH in the absence of added regulatory subunit.

6.0 and 7.5. Similarly, the dissociation kinetics of bound cAMP were similar whether the experiment was performed at pH 6.0 or 7.5. In both cases, a single class of binding sites was detected with a dissociation rate constant of k_{-1} = 7.3 × 10^{-4} s^{-1}. The half-life of the cAMP–regulatory subunit complex was 17 min at 0 °C and 65 s at 22 °C. At the physiological temperature of 22 °C, binding of 2 μM cAMP to the regulatory subunit was essentially complete after 1 min, whether the catalytic subunit was present or not.

We also examined the effect of pH on the interaction between regulatory and catalytic subunits. Reassociation of the RC complex was measured by the extent of inhibition of the catalytic activity upon addition of regulatory subunit. Figure 9 shows that this interaction was highly pH dependent and that only at high pH could the protein kinase activity be fully inhibited by the regulatory subunit. Dissociation of the reconstituted holoenzyme by rising concentrations of cAMP was investigated at pH 6 and 7. In both cases, half-maximal effect was reached at 40 nM cAMP, and no inhibition of the catalytic

Table II: Stoichiometry of cAMP Binding to the Regulatory Subunit Measured by Equilibrium Dialysis[a]

expt	R concn (nM)	bound cAMP concn (nM)	stoichiometry (sites/R)
1[b]	6.00	6.18	1.03
1[c]	7.50		0.82
2[b]	12.00	11.15	0.93
2[c]	15.00		0.74
3[b]	18.00	17.47	0.97
3[c]	22.50		0.78

[a] Purified regulatory subunit (0.5 mL) in buffer B was dialyzed against 0.5 mL of the same buffer containing 100 nM [^{3}H]cAMP (45 Ci/mmol). After establishment of equilibrium (24 h at 4 °C with gentle rotation), two 0.1-mL aliquots were removed from each compartment and counted. [b] Protein concentration was measured by the amidoschwartz dye method. [c] Protein concentration was measured by the method of Bradford.

activity by the regulatory subunit remained in the presence of 1 μM cAMP (not shown).

Stoichiometry of cAMP Binding to the Regulatory Subunit. Binding experiments were performed by two different methods: the filtration assay and equilibrium dialysis. The concentration of purified regulatory subunit (assuming an M_r of 42 000) was also measured by two methods, and values obtained with Coomassie blue were 25% higher compared to those obtained with amidoschwartz. The Scatchard plot of the cAMP binding data obtained by the filtration assay was linear, showing the presence of a single class of binding sites with K_d = 3 nM (not shown) and a stoichiometry of 0.7–0.8 equiv of cAMP bound per subunit. Since the filtration method has yielded underestimates of cAMP binding to the regulatory subunits isolated from mammals (see Discussion), binding was also determined by equilibrium dialysis. Table II shows that no more than 1 equiv of cAMP could be bound per subunit at various protein concentrations, even though the ligand concentration was well above the dissociation constant. We thus conclude that the regulatory subunit from *D. discoideum* only carries one cAMP binding site.

Discussion

The crucial role played by cAMP in the control of development in *D. discoideum* makes it an attractive eukaryotic system for studying regulation by cAMP of enzymatic activities and differentiation processes. Since the early description of a histone phosphorylating activity that could be stimulated by cAMP in soluble extracts from *D. discoideum* cells (Sampson, 1977), numerous attempts have been made to isolate cAMP-dependent protein kinases. It now appears that the major problem in this task arises from the fact that the oligomeric holoenzyme dissociates easily into its subunits, leading to a protein kinase activity that is no longer stimulated by cAMP. In the present work, the large quantity of cells used to prepare the extract provided enough material to purify the dissociated subunits and also to characterize the holoenzyme, although the latter represented only a fraction of total enzyme.

Our results show that the regulatory and catalytic subunits are isolated as monomeric proteins of respective M_r 42 000 and 40 000 and the cAMP-dependent holoenzyme is a dimer composed of one subunit of each type. When the isolated subunits are mixed, the reconstituted inactive holoenzyme has the same molecular weight and is activated under the same conditions as the native holoenzyme. It thus appears that a single cAMP-dependent protein kinase is present in the soluble fraction of *D. discoideum* cells. The multiple peaks of kinase activity found upon DEAE chromatography or gel filtration of crude extracts reflect various states of association of the subunits rather than the presence of different enzymes.

cAMP-dependent protein kinases from mammalian origin (Flockhart & Corbin, 1982) as well as from lower eukaryotes (Brochetto-Braga et al., 1982; Moreno & Passeron, 1980) are generally proteins of M_r 170 000–200 000 with an R_2C_2 tetrameric structure. Type I and II regulatory subunits from mammalian cells are R_2 dimers of respective subunit M_r 49 000 and 55 000 (Hofmann et al., 1975), each monomer carrying two intrachain binding sites for cAMP (Builder et al., 1980; Rannels & Corbin, 1980). Similar characteristics have been described for regulatory subunits of cAMP-dependent protein kinases from lower eukaryotes such as *Neurospora crassa* (Trevillyan & Pall, 1982), *Blastocladiella emersonii* (Brochetto-Braga et al., 1982), and *Mucor rouxii* (Moreno & Passeron, 1980).

The molecular weight and monomeric structure of the catalytic subunit from *D. discoideum* are similar to those of that purified from mammalian tissues (Bechtel et al., 1977). However, there are probably structural differences between the two proteins since the heat-stable inhibitor of the mammalian catalytic activity (Ashby & Walsh, 1974) has little effect on the catalytic subunit from *Dictyostelium* (J. de Gunzburg, unpublished observations). The cAMP binding component that we have isolated is different from both type I and type II regulatory subunits. It is a monomer of 42 kDa, as opposed to a dimer of 2 × 49 000 daltons or 2 × 55 000 daltons. In addition, the regulatory subunit from *D. discoideum* appears to have only one cAMP binding site. The Scatchard analysis yields a stoichiometry of 0.8 and shows only one class of binding sites with a dissociation constant of 3 nM, a value in good agreement with that calculated as the ratio of the dissociation to the association rate constant (k_{-1}/k_1 = 2.0 nM). Similar conclusions were drawn from another study involving the regulatory subunit of *D. discoideum* cells based on a kinetic analysis of cAMP binding (de Wit et al., 1982). These results are unlikely to be due to an underestimate of cAMP binding measured by the filtration technique (Builder et al., 1980) since (i) similar values were obtained when the stoichiometry of cAMP binding to the regulatory subunit was measured by equilibrium dialysis and (ii) dissociation kinetics, which usually reveal heterogeneity among cAMP binding sites (Rannels & Corbin, 1980), show a single dissociation rate. It is possible that the unusually fast dissociation of bound cAMP from the regulatory subunit is related to the absence of a second cAMP binding site. In this connection, it would be interesting to assess whether the regulatory subunit from bakers' yeast cAMP-dependent protein kinase exhibits similar rapid dissociation kinetics since this protein also carries only one cAMP binding site per subunit but, in contrast to the *Dictyostelium* regulatory component, is isolated as an R_2 dimer (Hixson & Krebs, 1980).

The dimeric RC structure of the *D. discoideum* cAMP-dependent protein kinase holoenzyme deserves comment. Dissociation of the enzyme into its subunits is not merely a consequence of ion-exchange chromatographies, as is the case of the enzyme from the insect *Ceratistis capitata* (Garcia et al., 1983). Indeed, when a crude soluble extract from differentiating *D. discoideum* cells is directly analyzed by gel filtration (not shown) the cAMP-dependent protein kinase is separated into two fractions corresponding to a dimeric RC holoenzyme and to the dissociated subunits, as upon DEAE chromatography (Figure 1). This is in contradiction with the report by Rutherford et al. (1982) of an M_r 500 000 cAMP-dependent protein kinase activity. However, these authors might have dealt with an aggregated form of the enzyme since

addition of cAMP did not result in dissociation of the "holoenzyme". The possibility that upon gel filtration the peak corresponding to the RC dimer (apparent M_r of 88 000) might actually reflect a rapid equilibrium between an R_2C_2 tetramer and the dissociated subunits was considered. In such a hypothesis, the apparent molecular weight of the cAMP-dependent activity would depend on the concentration of the protein kinase. This is not the case since the elution position of the cAMP-dependent activity remained unchanged upon gel filtration of a crude soluble extract and rechromatography of the fractions corresponding to the dimeric holoenzyme on the same column. Furthermore, gel filtration of extracts from various stages of the purification (see Figure 3) shows that cAMP-dependent protein kinase activity consistently elutes at a position corresponding to an RC dimer.

We propose that the dimeric RC structure of the *D. discoideum* holoenzyme is a consequence of the absence in the regulatory subunit of the structural domains involved in the formation of R_2 dimers. It is tempting to relate this property along with the absence of a second cAMP binding site to the lower molecular weight of *D. discoideum* regulatory subunit. One might argue that partial proteolysis occurred in the extract and that we have purified a degraded form of the protein. Indeed, proteolytic products from both type I and type II mammalian regulatory subunits have been isolated that had lost the ability to form dimers but retained cAMP binding capacity (Potter & Taylor, 1980). Other proteolyzed subunits, which had lost one cAMP binding site, were usually incapable of inhibiting the activity of the catalytic subunit (Weber & Hilz, 1979). Recently, it has been shown that the regulatory subunit of the cAMP-dependent protein kinase from *Blastocladiella emersonii* zoospores was readily degraded in crude extracts from a 59-kDa form to a 43-kDa form that could still bind cAMP (Gomes et al., 1983). Since the use of protease inhibitors may not be sufficient, we have addressed this crucial question as to whether proteolysis occurred by using antibodies raised against the purified regulatory subunit. The same 42-kDa polypeptide was recognized in high-speed supernatants of crude extracts and pure protein preparations. Overexposure of the autoradiograms showed minor bands at 36 and 22 kDa, which are likely to correspond to degradation products of the 42-kDa subunit. In the past, cAMP binding proteins have been reported with M_r of 35 000–38 000 on the basis of sucrose gradient sedimentation of partially purified proteins (de Gunzburg & Véron, 1981), or photoaffinity labeling of crude extracts with 8-azido-[^{32}P]cAMP (Wallace & Frazier, 1979; Cooper et al., 1980). It is likely that those lighter species correspond to proteolytic products since the experiments were carried out with cells from early stages of differentiation where proteolytic activity is high (North, 1982) and with only PMSF as a protease inhibitor. Demonstration that the 42-kDa regulatory subunit itself is not a proteolytic product of a larger precursor has come from immunoblots of total cellular lysates obtained by direct immersion of the cells in boiling SDS, a treatment that would certainly prevent any proteolytic degradation. In conclusion, although proteolysis of the regulatory subunit does occur under certain conditions—always leading to polypeptides of M_r smaller than 40 000—the 42-kDa regulatory subunit that we have characterized cannot be distinguished from the protein actually present in the cell.

It has been proposed that the presence of two intrachain cAMP binding sites on the mammalian type II regulatory subunit might result from a tandem gene duplication, since these sites are located in highly homologous regions of the molecule (Takio et al., 1982). Such sites seem to have been highly conserved through evolution since they also exhibit significant homology with the cAMP binding domain of the catabolite gene activator protein (CAP) from *Escherichia coli* (Weber et al., 1982). On the other hand, domains responsible for the interaction between regulatory and catalytic subunits of cAMP-dependent protein kinases seem to have also been highly conserved, since functional hybrid enzymes can be reconstructed by using catalytic subunit from bovine heart and regulatory subunits from bakers' yeast (Hixson & Krebs, 1980) or *D. discoideum* (Leichtling et al., 1981a; de Gunzburg & Véron, 1982). One could thus imagine that the regulatory subunit from *D. discoideum* that is monomeric and carries only one binding site for cAMP represents an "ancestral" form of the molecule. If this is the case, the selective advantage for the regulatory subunits to carry two binding sites for cAMP and to be able to form dimers leading to a tetrameric R_2C_2 holoenzyme would then remain to be understood.

Another interesting property of the cAMP-dependent protein kinase from *D. discoideum* is its susceptibility to pH changes. Both the catalytic activity and its ability to be regulated by cAMP are strongly enhanced at pH 7 compared to pH 5.5, whereas cAMP binding to the regulatory subunit is essentially not affected. As a consequence, the specific activity of the protein kinase is very low at pH 5.5 and is not subject to inhibition by the regulatory subunit. In contrast, activity of the catalytic subunit is maximal at pH 7.0. Full interaction between subunits ensures that the activity of the holoenzyme is highly dependent upon cAMP. The effects of pH variation on both the activity and the association state of the protein are different from those reported with cAMP-dependent protein kinases from mammalian origin (Reimann et al., 1971; Shizuta et al., 1975), and it is tempting to propose that they are somehow linked to the unusual dimeric structure of the holoenzyme. This speculation is especially interesting in view of the possible role that the cAMP-dependent protein kinase could play in the differentiation of starved *D. discoideum* amoebae into spore and stalk cells. A low molecular weight morphogen (differentiation inducing factor) has been isolated that, applied together with cAMP to isolated cells, induces their differentiation into stalk cells (Gross et al., 1981); DIF also inhibits spore formation and could therefore regulate the choice between differentiation pathways in the *Dictyostelium* slug (Kay & Jermyn, 1983). Furthermore, there are indications that DIF acts via a modification of intracellular pH (Gross et al., 1983) in the same range (pH 5–7) as that which affects the cAMP-dependent protein kinase. Since our experiments were performed with differentiating cells including both prestalk and prespore types, we propose that the effects of pH and cAMP on gene expression during terminal differentiation could be mediated, at least in part, by the regulation of the activity of the cAMP-dependent protein kinase.

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Registry No. cAMP, 60-92-4; ATP, 56-65-5; kemptide, 65189-71-1; protein kinase, 9026-43-1.

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