A Cytosolic Cyclic AMP-dependent Protein Kinase in \textit{Dictyostelium discoideum}

I. PROPERTIES*

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A cyclic AMP-dependent protein kinase was isolated and partially purified from \textit{Dictyostelium discoideum}. The cytosolic holoenzyme has an apparent $M_r = 160,000$–$180,000$; its activity was stimulated significantly by cAMP when Kemptide served as substrate. The enzyme was dissociated and the regulatory subunit purified by affinity chromatography on $\beta$-aminoethylamino-cAMP. Only one type of regulatory subunit was found; it has an apparent $M_r = 41,000$ and is a substrate for the \textit{in vitro} phosphorylation by the homologous catalytic subunit and by purified bovine catalytic subunit. Antibody against the regulatory subunit was prepared. The \textit{D. discoideum} catalytic subunit was separated from cAMP-independent protein kinase by chromatofocusing. The apparent molecular weight of the catalytic subunit of the \textit{D. discoideum} cAMP-dependent protein kinase is 33,000 and its $pI$ is 6.4. The enzyme catalyzed the phosphorylation of bovine R$_s$ but not of R$_i$ regulatory subunit and was inhibited by high concentrations of the inhibitor of mammalian cAMP-dependent protein kinase. The evolution of the functional domains of cAMP-dependent protein kinases is discussed on the basis of a comparison of the analogous \textit{D. discoideum} and vertebrate enzymes.

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A regulatory role of cAMP in both prokaryotes and eukaryotes is established. There is evidence that in the prokaryote Escherichia coli, for example, cAMP exerts its functions, probably exclusively, by the control of the synthesis of certain proteins. The cyclic nucleotide modulates the formation of these proteins by interaction with the cAMP-binding protein (referred to as catabolite gene activator protein, or CAP) which, when complexed with cAMP, has high affinity for nucleotide sequences which form part of the promoters of genes controlled in their transcription by cAMP. The binding of the CAP-cAMP complex to the promoter DNA facilitates the transcription of the cognate genes (1). In eukaryotes cAMP affects both the syntheses and the activities of a variety of proteins. There is evidence that cAMP controls the formation of at least several eukaryotic proteins at the level of transcription (2–5). The mechanism of this control, however, is not known; e.g., it is not clear whether the effect of cAMP is mediated by a CAP-like binding protein or by a cAMP-dependent protein kinase (the two possibilities are not necessarily mutually exclusive), or by yet some other mechanism. Cyclic AMP plays a central role in the development of the cellular slime mold, \textit{Dictyostelium discoideum}, and this simple eukaryote has become a favorite model for the study of the mechanism of action of cAMP in eukaryotes. The organism synthesizes and releases cAMP into its environment when deprived of nutrients. The extracellular cAMP then acts as a chemotactic, morphogenetic agent and causes the individual, vegetative amoebae to form aggregates which eventually give rise to fruiting bodies. The latter consist of moribund stalk cells and spores with the potential to germinate into new vegetative amoebae. Cyclic AMP plays a dual role; the situation is unusual insofar as the cyclic nucleotide acts as an "intracellular second messenger for the intercellular signal or "hormone" which is also cAMP; the two functions of cAMP are linked via the stimulation of the membranal adenylate cyclase by extracellular cAMP. What lends particular interest to the study of the role of cAMP in \textit{D. discoideum} is the fact that the cyclic nucleotide appears to influence development of the slime mold, presumably by its regulatory role in protein synthesis. Evidence for an effect of cAMP on development comes from several sources. Thus, for example, exogenous cAMP brings about precocious development (6, 7); in certain strains and under certain conditions exogenous cAMP leads to cellular differentiation into either stalk cells or spores even in the absence of morphogenesis, i.e., under conditions where a fruiting body is not formed (8–10). That these effects are indeed mediated by an impact, direct or indirect, of cAMP on transcription, is suggested by experiments in which multicellular slime molds of \textit{D. discoideum} were disaggregated into individual amoebae and where the persistence of certain slug-specific species of mRNA depended on the addition of cAMP to the suspension of amoebae; cAMP seemed to have an effect on both the synthesis and the stability of different stage-specific mRNAs (11, 12). In other experiments exogenous cAMP reduced the rate of transcription of the discoidin I gene (2).

In view of what has been stated earlier about possible mechanisms by which cAMP might regulate protein synthesis, it becomes relevant to examine \textit{D. discoideum} for the occurrence of cAMP-binding proteins and of cAMP-dependent protein kinases. The fact that \textit{D. discoideum} is a very primitive eukaryote, which appears to derive from the deepest known...
The occurrence of cAMP-binding proteins in *D. discoideum* has been observed in a number of laboratories (14-19). The presence of cAMP-dependent protein kinase activity in extracts of *D. discoideum* was first reported by Sampson (20); however, several investigators were unable to confirm the original observation (21, 22). Our discovery (15) in *D. discoideum* of a CAMP-binding protein with the properties of the regulatory subunit of vertebrate cAMP-dependent protein kinases and with the ability to inhibit the activity of mammalian catalytic subunit (derived from cAMP-dependent protein kinase), in a manner reversible by CAMP, led to renewed efforts to identify a CAMP-dependent protein kinase in *D. discoideum*. In a preliminary communication (23) we reported the existence of the holoenzyme in developing amoebae; more recently several other groups (24, 25) described the occurrence of the separate subunits of the cAMP-dependent protein kinase. Here we report on the properties of a cAMP-dependent protein kinase found in *D. discoideum* during vegetative growth, as well as at all stages of development. The observations supplement our earlier findings (23) on the formation of the regulatory subunit of the enzyme. The companion paper (26) describes the time course of the synthesis of the cAMP-dependent protein kinase during development.

### EXPERIMENTAL PROCEDURES

**Strain and Conditions of Growth and Development—** *D. discoideum*, strain AX3/Rc3 (obtained from D. Soll, University of Iowa), was grown axenically at 22 °C on Medium HLL (26), supplemented with 50 mM glucose, to a density of approximately 10⁶ amoebae/ml. Amoebae were harvested from 14 liters of culture, were washed successively with Buffer A, containing 1 mM cAMP, and 1 M NaCl, and again with Buffer A. The regulatory subunit, i.e. the high affinity cAMP-binding protein, was then eluted by incubation of the column at 37 °C for 30 min in Buffer A containing 1 mM cAMP followed by washing at 37 °C with the same buffer. No regulatory subunit was recovered by washing with 5 mM AMP prior to the cAMP wash and no additional subunit was released from the column by washing with 30 mM cAMP or by 6 M urea after washing with 1 mM cAMP. Identical results were obtained when a column of 6-aminomethyl-CAMP-Sepharose was employed.

The flow-through fraction, containing the protein kinase activity, was dialyzed against 20 mM MOPS buffer, pH 6.5, containing 2 mM EDTA, 4 mM mercaptoethanol, 1 mM PMSE, and 10% glycerol and passed through a 3-ml CM52 column pre-equilibrated with the same buffer. After washing, the column was eluted with 0.5-500 mM sodium chloride gradient; 4.5-ml fractions were collected and assayed for protein kinase activity. The active fractions (50 mM glucose, to a density of approximately 10⁶ amoebae/ml) were lyophilized and stored at −80 °C after the pH had been adjusted to pH 7.2. This protein kinase preparation was insensitive to stimulation by cAMP and to the mammalian inhibitor, specific for cAMP-dependent protein kinase (30); the activity of the preparation was also inhibited by the homologous free regulatory subunit obtained from the DE52 column.

**Sucrose Density Gradient Centrifugation—** Sucrose density gradients were done according to the method of Martin and Asnis (31); linear 13-mL gradients of 5-17% sucrose in Buffer A were centrifuged for 20 h at 3 °C in a Beckman SW 41 rotor; 0.3-ml fractions were collected from the bottom of the tubes with a peristaltic pump.

**Chromatofocusing—** Chromatofocusing was carried out in columns (0.5 x 10.5 cm) of Pharmacia PBE 94 gel equilibrated to pH 7.4 and eluted with Polybuffer 74, pH 4.0, diluted according to manufacturer’s instructions, and containing also 1 mM EDTA. 2 mM mercaptoethanol, 10% glycerol, and 0.5 mM PMSE.

**Gel Electrophoresis—** Gel electrophoresis was performed as described earlier (32) according to the procedures of Laemmli (33) for one-dimensional gels and of O'Farrell (34) for twodimensional gels. Proteins were stained either with Coomassie brilliant blue or silver (35). Autoradiography was done as described earlier (32). Antibody to Regulatory Subunit—The fraction eluted from the 8-aminoethylamino-CAMP-Sepharose column by 1 mM cAMP was lyophilized, redissolved in SDS electrophoresis buffer (32), and submitted to electrophoresis on a preparative 10% acrylamide gel. The bands were visualized by soaking the gel in 4 M sodium acetate according to the procedure of Higgins (36). Protein fractions corresponding to a protein of molecular weight 41,000, was excised, rinsed with water, and minced by passage through a 16-gauge needle. After elution over a period of 2 days into three changes of 0.1% SDS, containing 0.01 M ammonium bichromate, the material, i.e. the purified regulatory subunit, was lyophilized and used for injection. Free regulatory subunit, obtained from the DE52 column, was also purified by cAMP affinity chromatography and preparative gel electrophoresis and used for immunization. Rabbits were given a primary injection into the popliteal gland of 50 μg of regulatory subunit suspended in complete Freund’s adjuvant and subsequent intramuscular injections (3-4-week intervals) of 50 μg of regulatory subunit suspended in incomplete adjuvant. The serum was used without further purification after removal of the clot.

For the monitoring of cAMP-binding proteins, reactive with the antibody, extracts of *D. discoideum* were photofluorinized labeled with *Nε-[3H]cAMP at a final concentration of 200 nM and incubated with the antisera at 4 °C for 30 min. The antibody-antigen complex was then precipitated with heat-killed, formalized Staphylococcus aureus, Cowan strain, and washed according to the procedure of Erikson et al. (37). The staphylococci were prepared by the procedure of Kessler (38). The bound antigen was released by boiling in SDS electrophoresis buffer suspected to one-dimensional gel electrophoresis and autoradiography.

**Assays—** Cyclic AMP-binding activity was determined as described earlier (15); [3H]cAMP was employed at a concentration of 10 nM and as pH 7. Protein kinase activity was assayed with 10 μM Reptinamide as substrate; in addition to Reptinamide, the reaction mixture

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1 The abbreviations used are: MOPS, 4-morpholinoethanesulfonic acid; PMSE, phenylmethylsulfonyl fluoride; 8NaCAMP, 8-azido adenosine 3'5'-monophosphate; SDS, sodium dodecyl sulfate; R, and R₂, regulatory subunits of cAMP-dependent protein kinase isoforms 1 and 11 respectively; R₃C, cAMP-dependent protein kinase holoenzyme.
protein kinase activity, such activity was found; however, it was stimulated only minimally (20-50%) and variably by the addition of an excess of the mammalian inhibitor (30), and by detection of the catalytic subunit after passage of the DE52 flow-through fraction through a chromatofocusing column. The peak of CAMP-dependent protein kinase activity was eluted at a concentration of 30-50 mM NaCl, when applied to a Sephadex G-150 column. The protein kinase has an apparent molecular weight of 160,000-180,000 and is stimulated significantly by CAMP. Fig. 2 shows the behavior of the pooled fractions with CAMP-dependent protein kinase activity, obtained from the DE52 column, when applied to a Sephadex G-150 column. The protein kinase has an apparent molecular weight of 160,000-180,000 and is stimulated significantly by CAMP. Fig. 3 shows the effect of CAMP concentration on the activity of the D. discoideum holoenzyme. Half-maximal activation is achieved at approximately 30 nM CAMP; the $K_a$ for ATP is 40 $\mu$M and that for Kemptide 15-25 $\mu$M; the optimal Mg$^{2+}$ concentration is 5 mM. The pH range of optimal CAMP-dependent protein kinase activity, pH 6.3-8.0, is similar to that found for the mammalian enzyme. GTP at a concentration of 1 mM did not compete with ATP as substrate, irrespective of whether the concentration of ATP was 20 $\mu$M (limiting) or 100 $\mu$M; GMP (100 $\mu$M) did not activate the protein kinase.

**RESULTS**

**Partial Purification of CAMP-dependent Protein Kinase**—When crude extracts of D. discoideum were centrifuged at 40,000 $\times$ g and the supernatant fractions were tested for protein kinase activity, such activity was found; however, it was stimulated only minimally (20-50%) and variably by CAMP (data not shown). Consistent stimulation of protein kinase activity was detected after the extract was passed through a DE52 column. The peak of CAMP-dependent protein kinase activity was eluted at a concentration of 30-50 mM NaCl. Inspection of Fig. 1 shows that approximately two-thirds of the CAMP-binding protein, i.e., the regulatory subunit of the protein kinase, was not associated with the CAMP-stimulatable protein kinase activity. This fraction of the regulatory subunit was variable, but was usually at least one-half of the total regulatory subunit. Conversely, there was significant protein kinase activity in the flow-through fraction. This activity reflected the presence of a mixture of CAMP-independent protein kinase(s) and of free catalytic subunit of CAMP-dependent protein kinase. The presence in the mixture of variable amounts of free catalytic subunit was demonstrated by a variable decrease in kinase activity upon the addition of an excess of the mammalian inhibitor (30), specific for CAMP-dependent protein kinase (data not shown) and by detection of the catalytic subunit after passage of the DE52 flow-through fraction through a chromatofocusing column.

**Dissociation of CAMP-dependent Protein Kinase into Regulatory and Catalytic Subunits**—The holoenzyme was dissociated into its subunits by passage through an 8-aminoethylpyrimino-CAMP-Sepharose column as described under “Experimental Procedures.”

**Regulatory Subunit**—The fraction eluted by CAMP contained a major band (28%) at a position corresponding to $M_r = 41,000$ and several minor bands, as visualized by staining with silver. A similar heterogeneity of the CAMP-binding fraction in D. discoideum after one passage through a CAMP-affinity column has been observed by other workers (18, 24). The protein with an apparent $M_r = 41,000$ moved to the same position after two-dimensional SDS gel electrophoresis as we reported earlier for the material from crude cytosols or partially purified holoenzyme, photoaffinity-labeled with $8N_3-{\[^{32P}\]cAMP}$ (23). After extensive dialysis of the fraction eluted with CAMP, only the protein of $M_r = 41,000$ was photoaffinity-labeled. Antibody prepared against the regulatory subunit, as described under “Experimental Procedures,” was used to react with $8N_3-{[^{32P}]cAMP}$ photoaffinity-labeled proteins from both crude cytosols and partially purified holoenzyme. In both cases only the $M_r = 41,000$ protein and some fragments, presumably the result of proteolysis during incubation with the antisera and S. aureus, were precipitated (Fig. 4). Control preparations treated with either preimmune serum obtained from the same rabbit, which was later immunized, or exposed to the photoaffinity label in the absence of light, showed no labeling of any D. discoideum protein. The antibody did not interfere with the ability of the regulatory subunit to bind CAMP since the S. aureus precipitate of unlabeled antibody-regulatory subunit complex could be photolabeled to the same extent as an identical amount of regul.
FIG. 2. Gel filtration of the DE52 pool of cAMP-dependent protein kinase. The pooled fractions containing activity were concentrated and chromatographed on Sephadex G-150. 10-μl aliquots from the 4-ml fractions were assayed for protein kinase activity in the presence [■■■] and absence [■■■] of 1 μM cAMP. Molecular weight markers were aldolase (a), ovalbumin (b), and RNase (c).

FIG. 3. Activation of cAMP-dependent protein kinase as a function of cAMP concentration. Assay conditions (fraction purified through Sephadex G-150 step) were as described under "Experimental Procedures."

Immunoprecipitation of photoaffinity-labeled regulatory subunit of cAMP-dependent protein kinase. Holoenzyme, purified through Sephadex G-150 (Lanes 1–3) and crude cytosol (Lanes 4 and 5) were photoaffinity-labeled with 8N3-[β32P]cAMP and reacted with preimmune serum (Lane 1) or antiserum (Lanes 2 and 5) and precipitated with S. aureus, as described under "Experimental Procedures." The material was solubilized and subjected to one-dimensional SDS-gel electrophoresis; the resulting autoradiogram is shown. As controls, the photoaffinity-labeled proteins were submitted to electrophoresis without antibody treatment (Lanes 3 and 4). It is to be noted that the proteins of a molecular weight higher than 41,000 (in Lanes 3 and 4) were photoaffinity-labeled nonspecifically; i.e. cAMP at 20 μM did not compete. These proteins did not react with antibody (Lanes 2 and 5). The protein of Mt = 32,000 in the crude cytosol (Lane 4) is a photolabeled proteolytic fragment of the Mt = 41,000 regulatory subunit and reacts with the antibody (Lane 5).

FIG. 4. Phosphorylation of D. discoideum regulatory subunit of cAMP-dependent protein kinase. The regulatory subunit of the D. discoideum was purified by adsorption onto a cAMP affinity column. The fraction released from the column by 1 mM cAMP was phosphorylated by the D. discoideum catalytic subunit, obtained from the chromatofocusing column, and subjected to two-dimensional gel electrophoresis. The autoradiogram of the resulting gel shows the phosphorylated material at a position corresponding to Mt = 41,000. The area corresponding to the position of the silver-stained regulatory subunit on the same gel has been circled to show its position relative to that of the phosphorylated material.

in vitro. No physiological role for this small amount of phosphorylation has been demonstrated and attempts to show phosphorylation of the subunit in vivo have thus far been unsuccessful.

Catalytic Subunit—The fraction which ran through the cAMP-affinity column and which contained the protein kinase activity was further characterized. Fig. 6A shows the behavior of the protein kinase in a sucrose gradient; a sedimentation value of 2.7 was determined. Sephadex G-150 chromatography of the enzyme (Fig. 6B) yielded a molecular radius of 27 Å. Assuming a partial specific volume of 0.75, Mt = 33,000 and a frictional coefficient of 1.2 were calculated (44). Chromatofocusing of the catalytic subunit (Fig. 7) yielded a pI value of pH 6.4.

Fig. 8 indicates that the activity of the catalytic subunit of the cAMP-dependent protein kinase of D. discoideum, but not the activity of cAMP-independent protein kinase, was sensitive to the inhibitor of mammalian cAMP-dependent protein kinase, albeit the concentration of inhibitor required for the inhibition of the slime mold enzyme was significantly higher than that effective for the inhibition of the enzyme of mammalian origin.
D. discoideum cAMP-dependent Protein Kinase; Properties

**Fig. 6.** Sucrose density gradient (A) and gel filtration (B) of free catalytic subunit purified through the CM52 step. A, sucrose density gradient sedimentation was performed as described under "Experimental Procedures." Sedimentation coefficient standards were: a, [methyl-14C]methylated γ-globulin and b, [methyl-14C]methylated ovalbumin. B, gel filtration through Sephadex G-150 was carried out in a column (1.0 x 44 cm) and 0.55-ml fractions were collected. Standards were: c, [methyl-14C]methylated γ-globulin and d, [methyl-14C]methylated ovalbumin.

**Fig. 7.** Chromatofocusing of catalytic subunit. Free catalytic subunit, purified through the CM52 step, was applied to, and eluted from, the column as outlined under "Experimental Procedures." 300-μl fractions were collected, and 20-μl aliquots were assayed for enzymatic activity.

The substrate specificities of the holoenzyme, purified through the Sephadex G-150 step, and of the free catalytic subunit are compared in Table I. It may be seen that Kemptide was the most effective substrate for both preparations and the only one where cAMP stimulated significantly the activity of the holoenzyme. Thus, while the specificity of the D. discoideum catalytic subunit for these, nonphysiological substrates is not exactly that of the mammalian catalytic subunits, it is clear that the same amino acid sequence (Arg-Arg-X-Ser) is phosphorylated. The selectivity of the D. discoideum catalytic subunit was also tested by its ability to phosphorylate the two bovine regulatory subunits. It may be seen from Fig. 9 that the D. discoideum catalytic subunit, like

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**FIG. 8.** Effect of rabbit muscle protein kinase inhibitor on the activities of the cAMP-dependent protein kinases of bovine heart (Δ---Δ) and D. discoideum (○●○●) and of cAMP-independent protein kinase of D. discoideum (□—□). Assay conditions were as described under "Experimental Procedures"; cAMP was employed at a 1 μM concentration. 0.1 μl of the preparation contained 100 ng of inhibitor.

**TABLE I**

Substrate specificities of holoenzyme and free catalytic subunit

| Added substrate        | Holoenzyme | Catalytic subunit |
|------------------------|------------|-------------------|
|                       | -cAMP      | +cAMP             | -cAMP         |
| None                   | 0.1        | 0.1               | 0.1           |
| Kemptide               | 3.6        | 18.2              | 17.5          |
| Histone H2B            | 1.3        | 1.6               | 1.2           |
| Mixed histones         | 0.4        | 0.5               | 1.7           |
| Histone H1             | 1.9        | 4.0               | 2.2           |
| Casein (α)             | 2.0        | 1.9               | 2.6           |

**Fig. 9.** Phosphorylation of bovine regulatory subunits by the D. discoideum catalytic subunit. 3.2 μg of purified bovine R1 (Lane 1) and 4.2 μg of purified R2 (Lane 2) were phosphorylated in the presence of 5 μM cAMP by D. discoideum catalytic subunit (purified through the chromatofocusing step) and then submitted to one-dimensional gel electrophoresis; an autoradiogram of the resulting gel is shown.
its mammalian counterpart, catalyzes the phosphorylation of the R2I, but not the R1, subunit. This phosphorylation is inhibited by the mammalian inhibitor of CAMP-dependent protein kinase. We have not examined other potential mammalian substrates since we are interested primarily in the isolation of homologous, i.e. D. discoideum, substrates.

**DISCUSSION**

The findings described here confirm and extend our (15, 23) and other investigators' (24, 25) earlier observations on the occurrence of a CAMP-dependent protein kinase in the cellular slime mold, D. discoideum. We have isolated and characterized the holoenzyme and its constituent catalytic and regulatory subunits. The enzyme is soluble, at least after disruption of the amoebae by sonic disintegration. The properties of the separated subunits are similar to those which have been described recently by de Gunzburg and Vernon (24) and by Rutherford et al. (25). The latter authors also observed that CAMP-stimulatable kinase activity was not adsorbed to DE52 cellulose and had M, = 500,000 whereas we find that the purified holoenzyme has M, = 160,000-180,000. It may be that the preparation employed by Rutherford et al. contained kinase linked to some cytoskeletal component, as has been described for certain preparations of mammalian origin (46, 47). Whereas we did not find a protein kinase of such a high molecular weight, we observed occasionally that a preparation of holoenzyme, activable by CAMP (7-fold activation), was physically undissociated by even millimolar concentrations of CAMP. Photoaffinity-labeling of such preparations with 8N3-[32P]cAMP, followed by SDS-polyacrylamide gel electrophoresis yielded only the M, = 41,000 regulatory subunit. An investigation of the possibility that the non-dissociation of the protein kinase might reflect interaction with a cellular component is currently under way.

In general, the CAMP-dependent protein kinase of D. discoideum resembles the analogous enzyme of vertebrate origin. The slime mold enzyme has M, = 160,000-180,000, suggesting a tetrameric structure. Its behavior on the DE52 column (Fig. 1) is similar to that of the mammalian Type I CAMP-dependent protein kinase. Likewise, the kinetic properties of the partially purified CAMP-dependent protein kinase of Dictyostelium are similar to those of the mammalian enzyme, as is its substrate specificity (Table 1 and Fig. 9); the latter may not be a meaningful criterion in the absence of physiologically relevant substrates.

It appears that the cytosolic D. discoideum CAMP-dependent protein kinase has only one regulatory subunit of M, = 41,000. It seems that the occurrence of only one type of regulatory subunit is the rule in lower eukaryotes, albeit less primitive than D. discoideum; the molecular weight of the regulatory subunit, however, in a number of these fungi is in the range of 47,000-62,000 (43, 48-51). The occurrence of the 41,000 species in the 180,000-180,000 holoenzyme argues that 41,000 is in fact the molecular weight of the native regulatory subunit. Preparation of amoebal extracts in the presence of a variety of protease inhibitors and immediate photolabeling of crude cytosols yielded invariably the 41,000 and no higher molecular weight species. Furthermore, purified, photoaffinity-labeled bovine regulatory subunits I and II retained their molecular weights (48,000 and 52,000-54,000, respectively) when added to D. discoideum amoebae, and cytosols were then prepared in the usual manner, i.e. by sonic disintegration in the presence of inhibitors of proteolysis. If, however, the extracts were prepared by one cycle of freezing and thawing in the absence of the inhibitors of proteolysis, i.e. under conditions which brought about the degradation of the D. discoideum regulatory subunit to a molecular weight of 32,900-36,000 (63), then the mammalian regulatory subunits were degraded to fragments of molecular weight 37,000-40,000 (data not shown). These findings suggest that proteolysis of the D. discoideum regulatory subunit does not take place under our routine conditions of preparation and there is then no evidence for the occurrence in D. discoideum of regulatory subunits of M, = 48,000-58,000. Until, however, more is known about the precise nature of the subunit interactions that occur in the D. discoideum holoenzyme or until the presence of a blocked NH2 terminus (as occurs in the mammalian proteins) has been demonstrated by amino acid sequencing, the identification of the D. discoideum 41,000 species as the native form of the regulatory subunit must be considered tentative, even though the protein is fully effective in regulating the kinase activity of both the D. discoideum and bovine catalytic subunits. (We made the assumption in the preceding discussion that comparisons of estimates of molecular weights based on SDS-polyacrylamide gel electrophoresis are meaningful in the sense that the molecular weight of the regulatory subunit of the Dictyostelium CAMP-dependent protein kinase is approximately 10,000 lower than that of the analogous bovine subunits. We note, however, that the molecular weights of the bovine regulatory subunits, as derived from amino acid analyses, are much lower than estimates based on the migration of the proteins on SDS gels, i.e. 40,000 for R2I and 45,000 for R1I (52). Evidently, in those cases SDS does not completely denature the subunits. It is conceivable that the regulatory subunit of the D. discoideum CAMP-dependent protein kinase does behave as a globular protein during migration on SDS-polyacrylamide gel electrophoresis and that therefore 41,000 is the actual molecular weight. In that case the difference between the bovine and the slime mold regulatory subunits would be one of conformational form and not of size. Clearly, this question will be answered by a sequencing of the pure Dictyostelium protein kinase regulatory subunit. In the following discussion we shall assume, however, an actual difference in molecular weight).

The properties of the regulatory subunit of the CAMP-dependent protein kinase of D. discoideum may be compared with those of the two mammalian subunits. The D. discoideum resembles the R1 subunit in its behavior on DE52 and in the slight cross-reactivity of antibody against the D. discoideum subunit with the bovine R1 subunit. There is, however, also a similarity between the D. discoideum and bovine R2 subunits in that both are eluted from CAMP-affinity columns by 1 mM CAMP; furthermore, the Dictyostelium subunit is a substrate also, albeit a poor one, for autophosphorylation. We note that two-dimensional gel analysis of the regulatory subunit, purified on the CAMP-affinity column, and detected by silver staining, or of photoaffinity-labeled cytosols (23) invariably reveals at least two spots of M, = 41,000 and with similar pi values. While the two spots may represent modifications of the same gene product, they might also represent the D. discoideum equivalent of R2 and R1 with, in this case, identical molecular weights, but slightly different net charges. The difference in charge would be smaller than that found for the bovine subunits. Here too, comparisons of amino acid sequences should prove instructive. Nonetheless, at the present stage of our knowledge, it appears that the D. discoideum regulatory subunit is not analogous to either mammalian type R1 or R2; it may be more meaningful to compare the different regulatory subunits in terms of discrete domains.

Thus, it appears that the CAMP-binding site of the D. discoideum regulatory subunit is not analogous to either mammalian type R1 or R2; it may be more meaningful to compare the different regulatory subunits in terms of discrete domains.

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*E. G. Krebs, personal communication.*
protein kinase. These findings constitute an argument, albeit a tenuous one, for a common evolutionary origin of the D. discoideum regulatory subunit. The "extra" have quite different affinities for the D. discoideum cell surface cAMP receptor (54-56) and cAMP phosphodiesterase (56). By the same token, the affinities of another cAMP-binding protein of $M_r = 186,000$ found in Dictostelium (14, 18, 57) and a variety of other organisms (58-60) for analogs of cAMP and for derivatives of adenosine are quite different from that of the regulatory subunits of the cAMP-dependent protein kinase. These findings constitute an argument, albeit a tenuous one, for a common evolutionary origin of the D. discoideum and the mammalian cAMP-binding domains as well as conceivably E. coli catabolite gene activator protein (see below). Our earlier findings (15) showed that the regulatory subunit of the D. discoideum cAMP-dependent protein kinase inhibited the activity of the bovine catalytic subunit in a manner reversible by cAMP; this suggests that the domain required for the interaction with the catalytic subunit has been conserved and is presumably similar to that of the homologous mammalian subunit.

The most striking difference between the D. discoideum and the mammalian regulatory subunits is the apparent lack in the D. discoideum regulatory subunit of a significant portion of the polypeptide chain found in the mammalian regulatory subunit. The "extra" $M_r = 10,000$ fragment of the bovine subunits might harbor the second cAMP-binding site which apparently does not occur in the D. discoideum regulatory subunit. Alternatively, the additional amino acid sequences of the mammalian regulatory subunit may play a role in the anchorage of the cAMP-dependent protein kinase to a subcellular structure.

While the molecular weight of the D. discoideum catalytic subunit of the cAMP-dependent protein kinase is lower than that of its mammalian analog (61), the affinities of the two catalytic subunits for ATP and for Kemptide, respectively, are similar and the slime mold catalytic subunit catalyzes the phosphorylation of bovine regulatory subunit R$_1$, but not of R$_2$. It is likely, therefore, that the ATP-binding and the protein substrate-binding sites as well as the domains required for interaction with the respective regulatory subunits of the D. discoideum and the mammalian catalytic subunits are similar and derive from the same ancestral domains. There is evidence (13) that D. discoideum represents the deepest known eukaryotic branch of the evolutionary tree and diverged from the path leading ultimately to mammals even before yeasts did; yet, clearly those domains of the cAMP-dependent protein kinase with functions, which we were able to test, have been conserved to a high degree. This is not surprising in view of the central role of cAMP in metabolic, hormonal, and developmental regulation. In fact, significant homology in amino acid sequences between the catabolite gene activator protein of the prokaryote E. coli and the R$_1$ regulatory subunit of the cAMP-dependent protein kinase of bovine cardiac muscle has been reported recently (62). It appears that the homology resides in the cAMP-binding regions of the two proteins. There is as yet no evidence for the occurrence of amino acid sequences in the eukaryotic regulatory subunits which are homologous to the DNA-binding domains of the prokaryotic catabolite gene activator protein. The possibility that such homology exists is probably not ruled out; alternatively, the interaction of the eukaryotic cAMP-binding protein with the catalytic subunit of the protein kinase may fulfill, indirectly, the same physiological function of controlling the synthesis of proteins via the phosphorylation of a relevant protein(s) as does the catabolite gene activator protein by direct interaction with DNA. Clearly the resolution of this question is of crucial importance in arriving at an understanding of the regulation of the synthesis of eukaryotic proteins by cAMP.

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