Isolation and Properties of a Cyclic AMP-binding Protein from *Neurospora*

EVIDENCE FOR ITS ROLE AS THE REGULATORY SUBUNIT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE*

James M. Trevillyan‡ and Martin L. Pali§

*From the Program in Genetics and Cell Biology, and Program in Biochemistry/Biophysics, Washington State University, Pullman, Washington 99164*

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A cyclic AMP-binding protein with a native molecular weight calculated to be 82,900 was purified 2,000-fold from *Neurospora crassa*. The apparent subunit molecular weight was 47,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, suggesting that the native protein exists as a dimer of identically sized subunits. The 8-2 cAMP-cyclic [*³²P*]AMP-labeled protein appeared as a doublet upon two-dimensional gel electrophoresis, with pl = 5.4 and 5.5. Binding studies with both noncyclic and cyclic nucleotides showed cyclic AMP to have the highest binding affinity. The *K*ₐ for cyclic AMP was 300 nM at 23 °C. The cyclic AMP-binding protein has two distinct cyclic AMP binding sites, a slowly dissociating site and a rapidly dissociating site. The cyclic AMP analog, 8-bromo cyclic AMP, was found to bind preferentially the slowly dissociating cyclic AMP binding site. The cyclic AMP-binding protein is thought to be the regulatory subunit of cyclic AMP-dependent protein kinase based upon the findings that it co-purifies with this protein kinase activity upon DEAE-cellulose chromatography, isoelectric focusing, and cyclic AMP affinity resin chromatography. On purification by the latter two procedures, no other cyclic AMP-binding activity was detected.

A number of similarities between the *Neurospora* cyclic AMP-binding protein and the cyclic AMP-dependent protein kinase regulatory subunits from yeast and mammalian tissue are discussed.

Cyclic AMP and its receptor are important regulatory elements in both prokaryotic and eukaryotic organisms. The cyclic AMP receptor proteins isolated from the bacterium *Escherichia coli* and from mammalian tissue are of two distinct types. The *E. coli* cyclic AMP receptor associates with DNA, and when complexed with cyclic AMP, promotes initiation of transcription of catabolite-repressed genes (1), whereas the mammalian receptor is a protein kinase which in turn modulates the activity of several important metabolic enzymes.

Cyclic AMP-dependent protein kinases have been reported in both vertebrate and invertebrate species (2-4) and, more recently, in eukaryotic microorganisms (5-11). Two classes of cyclic AMP-dependent protein kinase, called type I and type II, have been purified from mammalian sources (12-15). Both type I and type II isozymes are composed of catalytic (C) subunits and a regulatory dimer (R₂) to form a tetrameric holoenzyme (C₂R₂). Heterogeneity in the regulatory dimer (R₂) produces differences in several properties which distinguish the two protein kinase isozymes (15).

The mechanism of activation of these enzymes has been described (16, 17). When the catalytic (C) subunit is complexed with the regulatory dimer (R₂), the phosphotransferase activity of the catalytic (C) subunit is inhibited. Binding of cyclic AMP to the regulatory dimer causes dissociation of the catalytic subunit from the regulatory dimer and activation of the phosphotransferase activity. The balanced equation for the reversible dissociation of catalytic and regulatory subunits is:

\[
(C₂R₂) + 4cAMP = (R₂-2cAMP)₂ + 2(C)c
\]

inactive

active

Recently, cyclic AMP-dependent protein kinase activity has been reported in several fungi including Macroc (5), Blastocladia (6), Coprinus (7), Saccharomyces (8, 9), and Neurospora (10, 11, see 18 for review). These studies suggest that the cyclic AMP receptor in these microorganisms, like higher animal cells, is the regulatory subunit of a cyclic AMP-dependent protein kinase. However, unlike the dual isozymes found in mammals, each of these lower eukaryotes appears to have but a single type of cyclic AMP-dependent protein kinase activity.

It seems likely that cyclic AMP-dependent protein kinases will be found to play an equally important role in mediating the physiological effects of cyclic AMP in eukaryotic microorganisms as has been demonstrated in mammalian species. However, as yet, little is known about the function(s) of cyclic AMP-dependent protein kinases in lower eukaryotes, and only in Saccharomyces cerevisiae has the regulatory subunit of the cyclic AMP-dependent protein kinase been purified to homogeneity for closer examination at the molecular level (9).

*Neurospora crassa* presents a uniquely attractive system for the study of the function of cyclic AMP and cyclic AMP-dependent protein kinase in lower eukaryotes because of the availability of mutants deficient in cyclic AMP (19, 20) and because treatments are known which elevate cyclic AMP levels in wild type *Neurospora* but fail to do so in cyclic AMP-deficient mutants (21-23). In this paper, we report the partial purification and characterization of the cyclic AMP-binding protein associated with the cyclic AMP-dependent protein kinase from *Neurospora*. Comparison of the properties of the *Neurospora* cyclic AMP-binding protein and the cyclic AMP-dependent protein kinase regulatory subunits from yeast and mammalian tissue are discussed.

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‡ Present address, Department of Biochemistry, University of California, Riverside, CA 92509.

§ To whom correspondence should be addressed.
Neurospora protein are made with those properties reported earlier for the purified yeast and mammalian regulatory subunits.

**EXPERIMENTAL PROCEDURES**

**Materials**

All nucleotides, type I cyclic AMP-dependent protein kinase from rabbit muscle, type II protein kinase regulatory subunit from bovine heart, catalase, bovine serum albumin, ovalbumin, trypsinogen, bovine $\beta$-lactoglobulin, lysozyme, and cyclic AMP-agarose were purchased from Sigma. CO-complexed hemoglobin (horse) was a gift from Dr. Roger Calm (Washington State University, Pullman). Electrophoresis reagents including pH 5/7 and pH 3/10 Ampholines were purchased from Bio-Rad. Preswollen microgranular DE52 diethylaminoethyl cellulose was obtained from Whatman. Slightly lysine-rich histone H2B and nitrocellulose filters type HA, pore size 0.45 $\mu$m, were purchased from Millipore Corp., Bedford, MA. [3H]ATP was prepared by the method of Johnson and Walseth (24) using the STAGE 1 reaction. Cyclic [3H]AMP (32.3 Ci/mmol) was purchased from New England Nuclear. 8-N3-cyclic [32P]AMP (99 Ci/mmol) was obtained from ICN.

**Methods**

**Strains and Culture Conditions**

Wild type *N. crassa* strain St. L. 74A was cultured by inoculating $10^6$ conidia/ml in 10-liter carboys containing 2% (w/v) sucrose and 23 °C for 24 to 30 h. Vigorous aeration of the cultures was maintained by bubbling the carboys with compressed air which has been passed through a cotton filter and water trap. 300-ml aliquots of mycelial culture were collected by rapid filtration onto Whatman 1 (11 cm diameter) filter paper and immediately frozen in liquid N$_2$. Occasionally, mycelia were stored at -70 °C for up to 2 weeks before use.

**Preparation of the 47,000-dalton Cyclic AMP-binding Protein from Neurospora**

Unless otherwise stated, all procedures were performed at 4 °C. 300 to 400 grams (wet weight) of exponential phase *N. crassa* was collected and quickly frozen in liquid nitrogen. The frozen mycelial pads were ground to a fine powder in liquid nitrogen with a large mortar and pestle. The frozen mycelial powder was suspended in 800 ml of 5 mM Tris-HC1, pH 8.0, 1 mM EDTA, 1 mM EGTA, 2 mM sodium fluoride, 20 mM 2-mercaptoethanol, 1 mM sodium azide, (buffer A), plus 0.5 mM phenylmethylsulfonyl fluoride and 10 mM benzamidine. The suspension was stirred for 15 min, aliquoted into 250-ml centrifuge bottles and centrifuged at 4,920 $\times$ g for 30 min. The supernatant was decanted and added to DEAE-cellulose (500 g) previously equilibrated with buffer A. The slurry was stirred slowly for 60 min and then collected in a large Buchner funnel. The resin was washed with 1 liter of buffer A and resuspended in 1 liter of the same buffer. The resin was poured into a 5-cm diameter column (height of the packed resin was 40 cm). After packing, the resin was washed with buffer A containing 150 mM NaCl. Ten-milliliter fractions were collected and the eluted protein assayed for cyclic AMP-binding activity by the Millipore filter method (26). Those fractions containing cyclic AMP-binding activity were pooled (approximately 400 ml) and passed through a column (1 x 4 cm) of cyclic AMP-agarose (cyclic AMP attached to cross-linked agarose through N6-amino group with 8-carbon spacer) previously equilibrated with 150 mM NaCl in buffer A. A constant flow rate of 80 ml/h was maintained with a peristaltic pump. The cyclic AMP affinity resin was washed with 100 ml of 150 mM NaCl in buffer A followed by 100 ml of 750 mM NaCl in buffer A. Cyclic AMP-binding protein was eluted by one of two different methods.

**Method 1**—To generate cyclic nucleotide-free Neurospora cyclic AMP-binding protein, the resin was eluted by incubation in buffer A containing 8 mM NaCl in buffer A for 1 h at 30 °C. One mg/ml carrier protein (lysozyme, $M_\text{r} = 14,000$) was also included in the elution buffer as a precaution against protein modification of the cyclic AMP-binding protein by the urea treatment and against possible loss of binding protein to nonspecific adsorption. Much of the lysozyme was found to precipitate upon subsequent removal of the urea by dialysis against buffer A. The resin was stirred occasionally during the 1 h. The eluate was collected from the column and the resin was washed again with 5 ml of buffer A containing 8 mM urea and 1 mg/ml of lysozyme.

**Method 2**—Alternatively, cyclic AMP-binding protein was eluted from the cyclic AMP-agarose resin by incubation in 5 ml of buffer A containing 1 mM cyclic AMP for 1 h at 30 °C. The eluate was collected from the column and the resin was rewashed with 5 ml of buffer A plus 1 mM cyclic AMP.

For both methods 1 and 2, the eluates were pooled and concentrated by dialysis against 70% sucrose in buffer A. The concentrated protein solution was exhaustively dialyzed against buffer A lacking sucrose. If the protein had been eluted with 1 mM cyclic AMP, activated charcoal was included in the dialysis buffer.

**Assays**

Cyclic nucleotide-dependent protein kinase activity was measured, essentially by the method of Gill and Walton (26), in a final volume of 100 ml containing 30 mM potassium phosphate, pH 6.8, 2 mM dithiothreitol, 5 mM magnesium chloride, 10 $\mu$m cyclic AMP (when used), 80 $\mu$m [y-32P]ATP at a specific activity of 50 to 100 cpm/pmol, 50 $\mu$g of histone H2B, and varying amounts of enzyme solution, incubated for 10 min at 30 °C. Reactions were stopped by pipetting 8 ml of the reaction mixture onto 2.3 cm filter disks. The filters were washed immediately in 50 ml of trichloroacetic acid for 15 min. Three additional 15-min washes were repeated in 5% trichloroacetic acid, the second of these performed at 90 °C and the others at 0–5 °C. The filters were then washed in 95% ethanol, ethanol/ether (1:1 v/v), and ether. The filters were dried at room temperature and counted in a toluene-based scintillant.

Cyclic nucleotide binding was measured by the Millipore filter technique as described by Gill and Walton (26) in a final volume of 200 ml containing 30 mM potassium phosphate, pH 6.8, 2 mM dithiothreitol, 10 mM magnesium chloride, and cyclic [3H]AMP. When purified Neurospora cyclic AMP-binding protein was assayed, 100 $\mu$g of bovine serum albumin was included in the reaction mixture. Reactions were initiated by addition of cyclic AMP-binding protein and were incubated for 60 min at either 0 °C or 23 °C as noted. The reaction mixture was diluted with 3 ml of ice-cold 30 mM potassium phosphate, 10 mM magnesium chloride buffer, pH 6.8, passed through a HAWP Millipore filter (0.45 $\mu$m pore size) and washed three times with 5 ml of the same buffer. Filters were dissolved and counted in scintillation mixture as described by Murad (27).

**Photoaffinity Labeling**

Photoaffinity-labeling experiments with 8-N3-cyclic [32P]AMP were performed essentially by the method of Walter et al. (33) in a final volume of 100 ml containing 50 mM MES buffer, pH 6.2, 10 mM magnesium chloride, 500 $\mu$m 2-mercaptoethanol, 1 mM N-ethyl-$\beta$-cycl AMP, specific activity 7,000 cpm to 20,000 cpm/pmol, various amounts of protein up to 140 $\mu$g, and various concentrations of other nucleotides where indicated. Isoelectric focusing and two-dimensional electrophoresis were performed according to the method of O’Farrell (28). For SDS-polyacrylamide slab electrophoresis, 10% gels were used as described for the second dimension of two-dimensional gel electrophoresis (28).

**Preparative Isoelectric Focusing**

Preparative isoelectric focusing was performed in a 110-ml LKB 8100 Ampholine electrophoresing column cooled to -2 °C with a Fisher refrigerated bath (model 90) containing methanol/H$_2$O (1:1 v/v). The column was filled with a 5 to 70% glycerol gradient containing 1% glycine (w/v), 100 mM EDTA, 100 $\mu$m dithiothreitol, and 2.5% pH 3 to 10 Biolyte Ampholines. The 5% glyceral solution contained up to 100 mg of Neurospora protein from 100,000 $\times$ g supernatant in which the lipid layer had been removed and which had been dialyzed for 2 h against 1% (w/v) glycine, 100 $\mu$m EDTA, and 100 $\mu$m dithiothreitol, pH 7.5 at 4 °C. The cathode electrode solution was 0.02 $\times$ NaOH. The anode electrode solution was 10 mM H$_2$PO$_4$. Electrophoresis using an ISCO electrophoresis power supply model 494 was performed at 3 watts until 1600 V was achieved (approximately 14 h) and then was focused at 1600 V for an additional 4 to 6 h. Upon completion of the electrophoresing, 2-ml fractions were collected from the bottom of the column using a peristaltic pump.

*The abbreviations used are: EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; MES, 4-morpholinoethanesulfonic acid.*
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Sucrose Density Centrifugation

Linear gradients of 4.7 ml of 5 to 20% sucrose in 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM NaF, and 100 mM NaCl, 20 mM β-mercaptoethanol were prepared. Protein samples of 100 μl were layered on top of the gradient and spun at 30,000 rpm in a Beckman SW 50.1 rotor for 22 h at 4°C in a Beckman model L2-65B ultracentrifuge. Eight-drop fractions were collected from the bottom of the tube. Bovine liver catalase and horse hemoglobin were used as markers. Collected fractions were assayed for protein by the method of Lowry et al. (29).

Stokes Radius Determination

Neurospora cyclic AMP-binding protein was gel-filtered on Sephadryl 200 (2.5 × 51 cm) equilibrated with 100 mM NaCl in buffer A. The column was calibrated (Stokes radius) with catalase (32 Å) and cytochrome c (17 Å). The data were calculated by the method of Seigel and Monty (30).

RESULTS

8-N3-cyclic [32P]AMP-labeled Proteins in Crude Extracts of N. crassa—The photoaffinity label 8-N3-cyclic [32P]AMP has been demonstrated successfully by several laboratories (31–34) to identify cyclic AMP-binding proteins. Photolysis of 8-N3-cyclic [32P]AMP leads to the formation of a covalent bond between the cyclic [32P]AMP ligand and its respective binding protein(s), and thus allows identification of multiplicity of cyclic AMP-binding proteins in cell extracts.

A survey of cyclic AMP-binding proteins in crude extracts of Neurospora using the 8-N3-cyclic [32P]AMP photoaffinity probe showed virtually all of the radioactively labeled protein to be in the soluble (100,000 × g supernatant) fraction (data not shown). Fig. 1 (lanes A and H) shows two major labeled soluble proteins with subunit molecular weights of 47,000 and 40,000. The binding of 8-N3-cyclic [32P]AMP to the 40,000-dalton protein is effectively inhibited by 5'-AMP, 5'-ADP, 5'-ATP, adenosine, cyclic AMP, and cyclic GMP (Fig. 1, lanes B to G), showing the binding of 8-N3-cyclic AMP to this protein to be nonspecific. The binding of 8-N3-cyclic [32P]AMP to the 47,000-dalton protein, on the other hand, is effectively inhibited only by cyclic AMP and cyclic GMP (Fig. 1, lanes F to G); there is no observable inhibition with the other noncyclic nucleotides (Fig. 1, lanes B to E).

Partial Purification of the 47,000-dalton Cyclic AMP-binding Protein from Neurospora—The 47,000 estimated subunit molecular weight cyclic AMP-binding protein from Neurospora was purified as described under “Experimental Procedures.” Table I presents the yield and extent of purification of the binding protein at each step of the purification. A 2000-fold purification with a 30% yield was achieved when 1 mM cyclic AMP was used to elute the binding protein from the cyclic AMP affinity resin (described as Method 2 under “Experimental Procedures”). Over 90% of the binding activity applied to the affinity column was adsorbed to the resin. Only 45% of these units are recovered upon elution of the affinity resin with 1 mM cyclic AMP (see Table I). The loss of units may be due to the dilute nature of the eluted protein solution (0.1 mg/ml). Factors such as surface denaturation or nonspecific adsorption of the protein to glassware might account for the loss of units. Photoaffinity labeling with 8-N3-cyclic [32P]AMP of the protein eluted from the affinity resin resulted in the labeling of a single protein which migrated with a subunit molecular weight of 47,000 upon electrophoresis in a 10% SDS-polyacrylamide gel (see Fig. 3, lane 3). This molecular weight agrees perfectly with that of the cyclic AMP-binding protein isolated in the crude extract of Neurospora (Fig. 1). The fact that lower molecular weight cyclic AMP-binding proteins were not found indicates that this purification protocol avoids substantial proteolytic degradation of the cyclic AMP-binding protein. The degradation of cyclic AMP-binding proteins by limited proteolysis during the purification of both the mammalian (16) and yeast (9) cyclic AMP-binding proteins has been reported.

An alternative method of eluting the Neurospora cyclic AMP-binding protein from the affinity resin was to use 8 M urea (described in Method 1 under “Experimental Procedures”). This method generates cyclic nucleotide-free cyclic AMP-binding protein, but renaturation of the protein on urea removal by dialysis results in only a 10% total yield of cyclic AMP-binding units. Fig. 2A shows the protein profile upon 10% SDS-polyacrylamide gel electrophoresis of the 8-N3-cyclic [32P]AMP-labeled protein extracts at various stages of the purification in which urea was used to elute the affinity resin. Fig. 2B is the corresponding autoradiogram. Lanes 1 and 2 show the protein which did not adsorb to DEAE-cellulose. As seen in the corresponding lanes in Fig. 2B, very little 8-N3-cyclic AMP-binding protein could be detected in this fraction. Lane 3 is the protein which was eluted from the DEAE-cellulose column. Among the proteins eluted was the 47,000-dalton cyclic AMP-binding protein as seen in Fig. 2B. Lane 4 is the protein which was not immobilized on the affinity column (affinity column flowthrough) and is indistin-

Table I

<p>| Purification of the Neurospora 47,000-dalton cyclic AMP-binding protein |
|-----------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
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<th>Purification step</th>
<th>Protein</th>
<th>Units</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude (4,920 × g supernatant)</td>
<td>6400</td>
<td>8000</td>
<td>1.2</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>2200</td>
<td>5900</td>
<td>2.7</td>
</tr>
<tr>
<td>Cyclic AMP-agarose affinity chromatography</td>
<td>1</td>
<td>2400</td>
<td>2400</td>
</tr>
</tbody>
</table>

a 1 unit is equivalent to 1 pmol of cyclic [3H]AMP bound under the reaction conditions described under “Experimental Procedures.” To minimize spurious nonspecific binding of cyclic [3H]AMP, 100 μM 5′-AMP was included in the cyclic AMP-binding reaction mixture.

b 1 mM cyclic AMP was used to elute the cyclic AMP-binding protein from the affinity resin (Method 2 under “Experimental Procedures”).
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Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Neurospora cyclic AMP-binding protein at various stages of purification. A, electrophoresis of Neurospora protein in 10% SDS-polyacrylamide gel at various stages of the purification protocol (see "Methods"). Lanes 1 and 2, the protein fraction which did not adsorb to DEAE-cellulose; lane 3, the 150 mM NaCl eluate from the DEAE-cellulose column; lane 4, cyclic AMP-agarose affinity column flowthrough fraction; lanes 5 and 6, protein eluted from the affinity column with 8 M urea. Photograph was taken after staining with Coomassie brilliant blue. B, autoradiogram of gel in A showing the photoactivated incorporation of 8-32P-cyclic [32P]AMP into Neurospora protein at various stages of the purification protocol. Protein in lane 6 was photoaffinity-labeled in the presence of 10 mM nonradioactive cyclic AMP. There appears to be some label bound to the lysozyme used as carrier (lanes 5 and 6, lower part of B).

guishable from the protein profile on the DEAE-cellulose eluate. However, the absence of the labeled 47,000-dalton protein band in Fig. 2B, lane 4, indicates that this cyclic AMP-binding protein has been immobilized on the affinity column. Lanes 5 and 6 (Fig. 2A) show the protein which was eluted from the affinity resin with 8 M urea. As shown in the autoradiogram (Fig. 2B, lanes 5 and 6), this results in the elution of the 47,000-dalton binding protein. The 47,000-dalton protein band is one of five protein bands observed on the SDS-polyacrylamide gel and thus has not been purified to homogeneity by affinity chromatography. It may be that further washing of the affinity resin at 30 °C is required to achieve greater purification of cyclic AMP-binding protein.

Physical Characteristics of Cyclic AMP-binding Protein from Neurospora—As reported above, the Neurospora cyclic AMP-binding protein has a subunit molecular weight of 47,000. This is very similar to the subunit molecular weights reported for both the yeast (9) and mammalian type I (15, 35) cyclic AMP-binding regulatory subunits of the cyclic AMP-dependent protein kinases. A comparison of the mobilities of the mammalian type I and type II regulatory subunits and the Neurospora cyclic AMP-binding protein in 10% SDS-polyacrylamide gels is shown in Fig. 3. Two-dimensional gel electrophoresis of the photoaffinity-labeled Neurospora cyclic AMP-binding protein resulted in two labeled protein spots of pI equal to 5.5 and 5.4 (data not shown). These isoelectric points are similar to the pI values reported for both bovine skeletal muscle and rat soleus muscle, type I regulatory subunits (36). The charge heterogeneity of type I regulatory subunits has been shown to be due to different degrees of phosphorylation of the subunit. It has not been determined whether this type of protein modification is responsible for the observed charge heterogeneity of the Neurospora cyclic AMP-binding protein.

To determine the native molecular weight of the Neurospora cyclic AMP-binding protein, the sedimentation coefficient and Stokes radius were measured. Sucrose density centrifugation of the native Neurospora cyclic AMP-binding protein showed it to have a $s_{20,w} = 4.1$. The Stokes radius was estimated to be 46 Å by gel filtration on Sephacryl 200. Using these values for Stokes radius and sedimentation coefficient and assuming a partial specific volume of 0.74 cm$^3$/g, the native molecular weight of the cyclic AMP-binding protein was calculated to be 82,300 by the method of Seigel and Monty (30). The cyclic AMP-binding protein, as stated above, was found to have a single subunit molecular weight of 47,000. Thus, the calculated native molecular weight of 82,300 shows the native protein may exist as a dimer of two identically sized subunits. The frictional coefficient was calculated to be 1.59, showing the cyclic AMP-binding protein to have an asymmetric shape. Assuming a prolate ellipsoid, the axial ratio was determined to be 11.1 (37). The physical properties found for the Neurospora cyclic AMP-binding protein are very similar to those properties previously described for the yeast and mammalian cyclic AMP-dependent protein kinase regulatory subunits, and are shown in Table II.

Specificity of Binding for Various Nucleotides to the Neurospora cyclic AMP-binding Protein—The specificity of cyclic [3H]AMP binding to the purified Neurospora cyclic AMP-binding protein was investigated (Table III). At concentrations of 1 μM, only cyclic nucleotides were effective in competing for binding with 0.1 μM cyclic [3H]AMP. At the concentration of 1 μM, cyclic AMP was the most effective competitor; inhibiting 89% of the cyclic [3H]AMP binding. At

Fig. 3. Autoradiogram of photoactivated incorporation of 8-32P-cyclic [32P]AMP into Neurospora and mammalian cyclic AMP-binding proteins. Lane 1, partially purified type I regulatory subunit from rabbit muscle (estimated subunit molecular weight is 47,000 to 48,000); Lane 2, partially purified type II regulatory subunit from bovine cardiac muscle (estimated subunit molecular weight is 54,000 to 55,000). The bovine cardiac muscle preparation also contained some type I regulatory subunit. Lane 3, partially purified Neurospora cyclic AMP-binding protein eluted from affinity column with cyclic AMP (see "Methods"). Photoaffinity-labeled proteins were electrophoresed in a SDS-polyacrylamide gel and autoradiographed as described under "Methods." Type I and type III regulatory subunits were obtained from Sigma.
Neurospora Cyclic AMP-binding Protein

Comparison of some properties of the Neurospora, yeast, and mammalian regulatory subunits

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Regulatory subunit type</th>
<th>Neurospora*a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native molecular weight</td>
<td>Skeletal muscle type I</td>
<td>92,500</td>
</tr>
<tr>
<td>Subunit molecular weight</td>
<td>Cardiac muscle type II</td>
<td>108,600</td>
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<tr>
<td>Aggregation state</td>
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<tr>
<td>Stokes radius</td>
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<tr>
<td>f/0</td>
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<td>Axial ratio</td>
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<td>11.1</td>
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<td>pI regulatory subunit</td>
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<td>5.4-5.5</td>
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<tr>
<td>Molar cyclic AMP bound/monomer</td>
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<tr>
<td>$K_d$ value for cyclic AMP binding</td>
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<td>10-100 nM</td>
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<tr>
<td>Specificity for cyclic AMP binding</td>
<td>60-150 nM</td>
<td>300 nM*</td>
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</table>

*a Data obtained from Refs. 15, 35, 36, 38, 39, 40, 41.
*b Data obtained from Refs. 13, 15, 16, 35.
*c Data obtained from Refs. 8 and 9.
*d Data obtained from this paper.
*e Neurospora protein data presented below.
*f $K_d$ values are greatly influenced by protein concentration and reaction conditions which accounts for the variable $K_d$ values reported.

Comparison of specificity of cyclic [3H]AMP binding to isolated N. crassa cyclic AMP-binding protein and to crude N. crassa extract

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<th>Competing compound</th>
<th>Conc.</th>
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<tbody>
<tr>
<td></td>
<td>Purified binding protein</td>
<td>Crude extract</td>
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<tr>
<td>None</td>
<td>(100)</td>
<td>(100)</td>
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<tr>
<td>Cyclic AMP</td>
<td>$10^{-6}$</td>
<td>11</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>$10^{-4}$</td>
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<td>8-Bromo cyclic AMP</td>
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<td></td>
<td>$10^{-3}$</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>$10^{-2}$</td>
<td>90</td>
</tr>
<tr>
<td>ATP</td>
<td>$10^{-2}$</td>
<td>110</td>
</tr>
<tr>
<td>AMP</td>
<td>$10^{-3}$</td>
<td>98</td>
</tr>
<tr>
<td>GTP</td>
<td>$10^{-3}$</td>
<td>118</td>
</tr>
</tbody>
</table>

Neurospora Cyclic AMP-binding protein at concentrations in 100-fold molar excess of 0.1 μM cyclic [3H]AMP (Table III). At 1 μM, 8-bromo cyclic AMP inhibits approximately 42% of the cyclic [3H]AMP binding (Table III). If 8-bromo cyclic AMP were competing cyclic [3H]AMP binding at a single site showing no cooperativity, 10 μM 8-bromo cyclic AMP should inhibit about 88% of the cyclic [3H]AMP binding (calculated from the 42% inhibition found at 1 μM as compared with the inhibition actually found from 10 μM 8-bromo cyclic AMP (Table III). Fig. 4 shows the inhibition of saturating (1 μM) cyclic [3H]AMP binding to the Neurospora binding protein by increasing concentrations of 8-bromo cyclic AMP. Equimolar 8-bromo cyclic AMP inhibits cyclic [3H]AMP binding by about 40%. Increasing the 8-bromo cyclic AMP concentration to 3- to 100-fold molar excess over cyclic [3H]AMP inhibits half of the cyclic AMP binding, the other half being quite resistant to inhibition by 8-bromo cyclic AMP. It is only at tremendously high 8-bromo cyclic AMP concentrations (i.e. at 300 to 1,000 molar excess) that a further increase to the inhibition of cyclic [3H]AMP is observed. These results show the Neurospora cyclic AMP-binding protein has two classes of cyclic AMP binding sites present in equal number. 8-Bromo cyclic AMP is effective at competing cyclic [3H]AMP binding at one site but not the other.

Evidence for Two Classes of Binding Sites on the Neurospora Cyclic AMP-binding Protein—An interesting observation arising from the specificity studies described above was the inability of 8-bromo cyclic AMP to substantially inhibit over 50% of the cyclic [3H]AMP binding to the purified

a 10,000-fold molar excess over cyclic [3H]AMP, 5'-ATP, 5'-GTP, and 5'-AMP were ineffective competitors suggesting a strict requirement of the 3':5'-phosphodiester configuration. Adenosine at a 100-fold excess only weakly competed for cyclic [3H]AMP binding. There does not appear to be stringent specificity of the binding protein for the nucleotide base. At a 10-fold excess, 8-bromo cyclic AMP, cyclic IMP, cyclic GMP, 8-azido cyclic AMP, and N6-monobutyryl cyclic AMP are all effective inhibitors of 0.1 μM cyclic [3H]AMP binding. Bulky modifications of the 2' position of the ribose ring appear to render cyclic nucleotides incapable of binding to the Neurospora-binding protein, since cyclic [3H]AMP binding is not effectively inhibited by N6',O2'-dibutyryl or O2'-monobutyryl cyclic AMP. The inhibition observed with these cyclic nucleotide analogs at 100 μM may be due to the 1 to 3% contamination by cyclic AMP or N6'-monobutyryl cyclic AMP in the N6',O2'-dibutyryl and O2'-monobutyryl cyclic AMP preparations obtained from Sigma. Similar nucleotide specificities have been reported for both the yeast and mammalian regulatory proteins (9, 38, 42). The specificity of cyclic [3H]AMP binding was also investigated in crude extracts of Neurospora. The relative effectiveness with which the various cyclic AMP analogs competed for cyclic [3H]AMP binding in the crude extract was similar to that found for the Neurospora cyclic AMP-binding protein. However, each analog was less effective in the inhibiting cyclic [3H]AMP binding in the crude extract compared to the purified binding protein. Cyclic AMP inhibited, at a 10-fold molar excess over cyclic [3H]AMP, 70% of the cyclic [3H]AMP binding in the crude extract as compared to inhibiting 85% of the cyclic [3H]AMP binding to the purified cyclic AMP-binding protein. The differences in competition observed between the crude extract and the purified binding protein may be due to interferences by other nucleotide-binding proteins in the crude extract or the influence of low molecular weight constituents in the extract.

Comparison of some properties of the Neurospora, yeast, and mammalian regulatory subunits

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Regulatory subunit type</th>
<th>Neurospora*a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native molecular weight</td>
<td>Skeletal muscle type I</td>
<td>92,500</td>
</tr>
<tr>
<td>Subunit molecular weight</td>
<td>Cardiac muscle type II</td>
<td>108,600</td>
</tr>
<tr>
<td>Aggregation state</td>
<td>Yeast</td>
<td>95,000</td>
</tr>
<tr>
<td>Stokes radius</td>
<td>52.8 Å</td>
<td>50 Å</td>
</tr>
<tr>
<td>f/0</td>
<td>1.67</td>
<td>1.66</td>
</tr>
<tr>
<td>Axial ratio</td>
<td>12.1</td>
<td>11.1</td>
</tr>
<tr>
<td>pI regulatory subunit</td>
<td>5.5-5.5</td>
<td>5.4-5.5</td>
</tr>
<tr>
<td>Molar cyclic AMP bound/monomer</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>$K_d$ value for cyclic AMP binding</td>
<td>15-20 nM</td>
<td>10-100 nM</td>
</tr>
<tr>
<td>Specificity for cyclic AMP binding</td>
<td>60-150 nM</td>
<td>300 nM*</td>
</tr>
</tbody>
</table>

*a Data obtained from Refs. 15, 35, 36, 38, 39, 40, 41.
*b Data obtained from Refs. 13, 15, 16, 35.
*c Data obtained from Refs. 8 and 9.
*d Data obtained from this paper.
*e Neurospora protein data presented below.
*f $K_d$ values are greatly influenced by protein concentration and reaction conditions which accounts for the variable $K_d$ values reported.
AMP; the results are in excellent agreement with the prediction. The cyclic AMP-dependent protein kinase binds with AMP-eluted Neurospora cyclic AMP-binding protein was incubated with 1 μM cyclic [3H]AMP and various concentrations of 8-bromo cyclic AMP. The reaction mixture was incubated 60 min at 23 °C and then filtered onto a Millipore filter and washed as described under "Methods." The data are expressed as the per cent of cyclic [3H]AMP bound in the absence of 8-bromo cyclic AMP. Further evidence for the existence of two different cyclic AMP binding sites in the Neurospora binding protein was obtained from studies of dissociation of cyclic [3H]AMP from the protein (Fig. 5). In these studies, saturating (1 μM) cyclic [3H]AMP was incubated with the binding protein with or without 100 μM 8-bromo cyclic AMP for 60 min to allow maximum binding. Then a great excess (500 μM) of unlabeled cyclic AMP was added and dissociation of the [3H]-labeled material from the binding protein was measured (Fig. 5). While the cyclic [3H]AMP binding during preincubation with 8-bromo cyclic AMP follows a simple exponential decay consistent with dissociation from a single binding site, the binding without 8-bromo cyclic AMP does not follow such single site kinetics (Fig. 5). The simplest explanation is that there are two binding sites, each with its own dissociation constant according to:

$$y = A_1 e^{-kt_1} + A_2 e^{-kt_2}$$

where $t = \text{time after addition of excess cold cyclic AMP}$; $y = \text{cyclic [3H]AMP bound}$; $k_1$ and $k_2$ are the reciprocals of the time constants for the two sites; $A_1$ and $A_2$ represent the relative initial binding to each of the two sites at $t = 0$.

The data in Fig. 5 (without 8-bromo cyclic AMP) were used to calculate values for the parameters in Equation 1 ($A_1$, $A_2$, $k_1$, $k_2$) by regression using two different computer programs based on Marquardt's (nonlinear least squares iterative) method (43). The results are shown in Table IV.

Both programs gave parameters with excellent fit to the data ($R^2$ very close to 1). They both gave ratios of $A_1/A_2$ of 1 showing equal binding to each of the two sites. Finally, the reciprocal of the time constant of the 8-bromo cyclic AMP-resistant binding (Fig. 6) which was 0.103 was in excellent agreement with the reciprocal calculated for one of the two sites ($k_1$). The predictions in Table IV are based on the assumption of two binding sites in equal numbers, one of which is identical with the 8-bromo cyclic AMP-resistant site and the other being sensitive to competition by 8-bromo cyclic AMP; the results are in excellent agreement with the prediction.

The regulatory subunits of both isozymes of mammalian cyclic AMP-dependent protein kinase bind 2 mol of cyclic AMP/mol of regulatory monomer (16, 44). The two intrachain cyclic AMP binding sites of the mammalian regulatory protein appear to be different in their rates of dissociation of cyclic AMP and in their relative selectivity for binding various cyclic AMP analogs (45). The above data show that the Neurospora cyclic AMP-binding protein resembles the animal regulatory proteins in having two classes of cyclic AMP binding sites which differ from each other in dissociation rates and sensitivity to the cyclic AMP analog 8-bromo cyclic AMP. As in the mammalian studies, the Neurospora sites are present in equal numbers.

**Association of 47,000-dalton Neurospora Cyclic AMP-binding Protein with Cyclic AMP-dependent Protein Kinase Catalytic Subunit**—Several lines of evidence show the 47,000-dalton cyclic AMP-binding protein to be the regulatory subunit of the Neurospora cyclic AMP-dependent protein kinase. First, the binding protein was purified from the 150 mM salt wash of the DEAE-cellulose column containing the cyclic AMP-dependent protein kinase activity reported by Powers and Pall (10). Second, soluble (100,000 × g supernatant) protein from Neurospora was subjected to isoelectric focusing. As shown in Fig. 6, two peaks of cyclic AMP-binding activity occurred at pH 5.5 and 6.7. The peak of cyclic AMP binding at pH 6.7 was coincident with cyclic AMP-dependent protein kinase activity. The cyclic AMP binding peak at pH 5.5 was free of cyclic AMP-dependent protein kinase and most likely represents the free cyclic AMP-binding regulatory subunit.
Photoaffinity labeling was used to identify the Neurospora cyclic AMP-binding protein. A preparative isoelectric focusing experiment was performed, and fractions were assayed for cyclic AMP-binding activity. The PI of the cyclic AMP-binding peak was determined to be 6.7, which is consistent with the PI of the purified protein. The cyclic AMP-binding protein was isolated and purified by affinity chromatography using cyclic AMP-agarose resin. The resulting protein was subjected to SDS-PAGE and autoradiography, and the 47,000-dalton band was identified as the cyclic AMP-binding protein.

The association of the cyclic AMP-binding protein with the catalytic subunit of cyclic AMP-dependent protein kinase was studied. The 47,000-dalton cyclic AMP-binding protein was isolated and assayed for its ability to dissociate the regulatory and catalytic subunits of the enzyme. The dissociation constant was calculated to be 0.3 μM, which suggests that the cyclic AMP-binding protein serves as a regulatory subunit of the enzyme.

The cyclic AMP-binding protein was also used as a source of enzyme in experiments on nucleotide-free cyclic AMP-binding protein. The results showed that the 47,000-dalton protein is the regulatory subunit of the enzyme, and that it is associated with the catalytic subunit to form the complete enzyme.

**Table V**

<table>
<thead>
<tr>
<th>Cyclic AMP-binding protein</th>
<th>Phosphotransferase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic AMP</td>
<td>14,047</td>
</tr>
<tr>
<td>Cyclic AMP-free (M₉)</td>
<td>24,653</td>
</tr>
</tbody>
</table>

**Fig. 6. Isoelectric focusing studies.** A, preparative isoelectric focusing of *Neurospora* cyclic AMP-binding protein and cyclic AMP-dependent protein kinase. *Neurospora* cytosolic protein was electrophoresed in a pH 3 to 10 gradient as described under "Methods." 30-μl aliquots of fractions were assayed for cyclic AMP-binding activity by the Millipore filter binding assay (26). Additional 30-μl aliquots were assayed for protein kinase activity in the presence of [γ-32P]ATP, utilizing histone H2B as substrate (16). (C--C) pH B, autoradiogram showing photoaffinity incorporation of 8-N-cyclic [32P]AMP into cyclic AMP-binding fractions obtained from isoelectric focusing electrophoresis. 30-μl aliquots of cyclic AMP-binding fractions obtained from the isoelectric focusing experiment in A were photoaffinity-labeled with 8-N-cyclic [32P]AMP as described under "Methods." Photoaffinity-labeled protein was subjected to SDS-polyacrylamide electrophoresis and subsequent autoradiography. Label at bottom of the autoradiogram migrated ahead of the proteins and represents traces of unincorporated material at the tracking dye front. Fraction numbers correspond to those shown in A.

**Fig. 7. Binding of cyclic [3H]AMP at various concentrations by the *Neurospora* nucleotide-free cyclic AMP-binding protein.** Cyclic AMP binding was determined in 200 μl of 30 mM potassium phosphate, 2 mM dithiothreitol, 10 mM magnesium chloride, 0.1 mg/ml of bovine serum albumin, cyclic [3H]AMP of known specific activity, pH 6.8. Binding reactions were initiated by addition of 9 μg of nucleotide-free cyclic AMP-binding protein. After 60 min at 23°C, reaction mixtures were filtered on Millipore filters, and the concentration of bound versus free cyclic AMP was calculated.

**Fig. 8.** The binding of cyclic [3H]AMP by *Neurospora* Regulatory Protein and Activation of *Neurospora* Cyclic AMP-dependent Protein Kinase—The binding of cyclic [3H]AMP at various concentrations by *Neurospora* nucleotide-free regulatory protein was measured (Fig. 7). Different concentrations of cyclic [3H]AMP of known specific activity were incubated with nucleotide-free regulatory protein for 60 min at 23°C under conditions described under "Experimental Procedures." After this time, the reaction solution was passed over a Millipore filter to separate free from bound cyclic [3H]AMP. The protein-bound cyclic [3H]AMP remained adsorbed to the filter and

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**Neurospora Cyclic AMP-binding Protein**

The PI of this cyclic AMP binding peak is essentially the same as the pl found for the purified 47,000-dalton binding protein. Labeling of both the pH 5.5 and pH 6.7 cyclic AMP binding peaks with 8-N-cyclic [32P]AMP showed the 47,000-dalton cyclic AMP-binding protein to be present in both peaks (Fig. 6). This is most easily explained as follows. The 47,000-dalton cyclic AMP-binding protein is the regulatory subunit of the *Neurospora* cyclic AMP-dependent protein kinase. It is associated with the catalytic subunit of the protein kinase as the holoenzyme with pl = 6.7, and is capable of dissociating from the catalytic subunit to give free regulatory subunit with a pl = 5.5.

A third type of supportive evidence that the *Neurospora* cyclic AMP-binding protein is the cyclic AMP-dependent protein kinase regulatory subunit comes from the fact that the purification of the cyclic AMP-binding protein also resulted in obtaining a cyclic AMP-dependent protein kinase which was stimulated 3.5- to 5-fold by 1 μM cyclic AMP (Table V). The cyclic AMP-dependent protein kinase was calculated to be purified approximately 15-fold and consequently represented only a small portion of the cyclic AMP-binding protein.

It should be noted that the 47,000-dalton cyclic AMP-binding protein is the only cyclic AMP-binding protein detected with 8-N-cyclic [32P]AMP in this fraction. Additionally, Moreno and Passeron (5) have reported that cyclic AMP concentrations as high as 0.3 mM fail to completely dissociate the cyclic AMP-dependent protein kinase of *Mucor rouxii*. It appears that immobilization of the *Neurospora* cyclic AMP-binding protein on cyclic AMP-agarose does not result in the complete dissociation of the regulatory and catalytic subunits of the cyclic AMP-dependent protein kinase, and resembles the *Mucor* enzyme in this property. The subsequent elution of the affinity resin with 1 mM cyclic AMP results in obtaining a small fraction of cyclic AMP-dependent protein kinase holoenzyme.

**Binding of Cyclic AMP by Neurospora Regulatory Protein and Activation of Neurospora Cyclic AMP-dependent Protein Kinase**—The binding of cyclic [3H]AMP at various concentrations by *Neurospora* nucleotide-free regulatory protein was measured (Fig. 7). Different concentrations of cyclic [3H]AMP of known specific activity were incubated with nucleotide-free regulatory protein for 60 min at 23°C under conditions described under "Experimental Procedures." After this time, the reaction solution was passed over a Millipore filter to separate free from bound cyclic [3H]AMP. The protein-bound cyclic [3H]AMP remained adsorbed to the filter and
was quantified. The apparent dissociation constant ($K_D$) for binding of cyclic AMP was 300 nM (Fig. 7). Binding of cyclic AMP to the *Neurospora* regulatory subunit was temperature-dependent. The same binding studies performed at 0 °C resulted in an apparent dissociation constant of 100 nM (data not shown).

The apparent activation constant ($K_a$) of the *Neurospora* cyclic AMP-dependent protein kinase was also measured (Fig. 8). The cyclic AMP-dependent protein kinase peak which focused at pH 6.7 during preparative isoelectric focusing was used as a source of enzyme for these studies. Different concentrations of cyclic AMP or 8-bromo cyclic AMP were included in the protein kinase reaction buffer. Protein kinase activity was measured as described under “Experimental Procedures.” The $K_a$ for cyclic AMP is 90 nM and is lower than the estimated $K_D$ of 300 nM for the *Neurospora* free cyclic AMP-binding protein. The $K_a$ and $K_D$ measured should not necessarily be the same because of differences in conditions used in the two measurements and proteins used (binding protein or cyclic AMP-dependent protein kinase holoenzyme). In addition, it is not known how many molecules of cyclic AMP must bind to activate the protein kinase. 8-Bromo cyclic AMP which was less effective in competing for cyclic [H]$3^\text{H}]AMP than cyclic AMP itself is also less effective in activating *Neurospora* cyclic AMP-dependent protein kinase (Fig. 8). The apparent activation constant for 8-bromo cyclic AMP is 0.5 m.

**DISCUSSION**

Photoaffinity labeling of *Neurospora* crude extracts with 8-$N_9$-cyclic [3$^\text{P}$$3^\text{P}$]AMP resulted in the identification of a cytosolic cyclic AMP-binding protein having an estimated subunit molecular weight of 47,000. Unlike mammalian cells, no evidence was found for multiple specific cytosolic cyclic AMP-binding proteins in *Neurospora*.

The *Neurospora* cyclic AMP-binding protein was purified 2,000-fold by affinity chromatography. The cyclic AMP-binding protein appears to be the regulatory subunit of *Neurospora* cyclic AMP-dependent protein kinase by the following criteria: (i) The 47,000-dalton cyclic AMP-binding protein eluted from DEAE-cellulose with the cyclic AMP-dependent protein kinase activity previously reported by Powers and Pall (10). (ii) Upon isoelectric focusing, the cyclic AMP-binding protein co-focused at pH 6.7 with the cyclic AMP-dependent protein kinase activity. The cyclic AMP-binding protein also focused at pH 5.5, presumably as the dissociated free regulatory subunit. (iii) The purification of the 47,000-dalton cyclic AMP-binding protein was accompanied by a lesser fold purification of the cyclic AMP-dependent protein kinase activity. (iv) The concentration of cyclic AMP necessary to attain half-maximal binding to the 47,000-dalton cyclic AMP-binding protein is 300 nM and is approximately equal to the concentration of cyclic AMP required to attain half-maximal activation of the cyclic AMP-dependent protein kinase.

Some properties of the *Neurospora* regulatory subunit are compared with both the yeast and the mammalian type I and type II regulatory subunits in Table II under “Results.” All of the regulatory proteins have similar monomer molecular weights within the range of 47,000 to 55,000. The yeast, mammalian, and *Neurospora* native regulatory subunits exist as dimers. Recent evidence suggests that the *Coprinus macro-rhizus* subunit has a molecular weight of about 46,000 (47).

There is some discrepancy between estimated subunit molecular weight of the *Neurospora* binding protein (47,000) which is more than half that of the purified protein which is probably a dimer (82,000). A similar discrepancy has been found in molecular weight studies of the mammalian type II regulatory subunit (see Refs. 35 and 39).

The stoichiometry of cyclic AMP binding has been reported to be 2 mol of cyclic AMP/mol of regulatory monomer for both type I and type II mammalian subunits (16, 44). The yeast regulatory protein differs from the mammalian proteins in this property, reportedly binding 1 mol of cyclic AMP/mol of yeast regulatory monomer (9). Evidence presented in this paper shows the *Neurospora* regulatory protein to have two types of cyclic AMP binding sites which are present in equal number. One type of cyclic AMP binding site selectively binds 8-bromo cyclic AMP. The two classes of cyclic AMP binding sites have different rates of dissociation for cyclic [H]$3^\text{H}]AMP and 8-bromo cyclic AMP appears to selectively bind the more slowly dissociating cyclic AMP binding site. Although we have not yet shown that the two *Neurospora* sites are present on each regulatory subunit, this appears quite likely from the animal protein precedent. Because 8-bromo cyclic AMP appears to selectively bind the slowly dissociating cyclic AMP binding site on the *Neurospora* regulatory protein it would be interesting to investigate whether there are cyclic AMP analogs which selectively bind the faster dissociating cyclic AMP binding site. By using site-selective analogs, it may be possible to determine which site or sites must bind cyclic AMP for activation of protein kinase. This was previously suggested by Rannels and Corbin (45) for the mammalian regulatory proteins.

The data reported here show that the *Neurospora* cyclic AMP-binding protein is strikingly similar to the mammalian protein kinase I regulatory subunit in apparent subunit molecular weight, isoelectric point, Stokes radius, number of different cyclic AMP binding sites, high affinity of 8-bromo cyclic AMP for one of the sites (the slowly dissociating one), apparent aggregation state, and sedimentation coefficient. They suggest that this protein has been highly conserved in evolution.

Data presented here strongly suggest that the 47,000-dalton cyclic AMP-binding protein is the regulatory subunit of the cyclic AMP-dependent protein kinase. Results previously reported (46) further support the idea that the 47,000-dalton cyclic AMP-binding protein mediates the physiological effects of cyclic AMP in vivo. It has been reported that cyclic AMP and various cyclic AMP analogs are effective in correcting the morphological defects of the cr-1 cyclic AMP-deficient mutant of *Neurospora* (20, 46). The cyclic nucleotides effective in correcting the abnormal cr-1 morphology in vivo include cyclic AMP, cyclic GMP, 8-bromo cyclic AMP, and $N^\text{6}$-mon-
obutyryl cyclic AMP. These same nucleotides are very effective in competing cyclic [3H]AMP binding to the 47,000-dalton cyclic AMP-binding protein. The cyclic nucleotides, N\textsuperscript{6},O\textsuperscript{2}-dibutyryl and O\textsuperscript{2}-monobutyryl cyclic AMP are inactive in correcting the morphology of cr-1 in vivo (46). Likewise, N\textsuperscript{6},O\textsuperscript{2}-dibutyryl and O\textsuperscript{2}-monobutyryl cyclic AMP are ineffective in competing cyclic [3H]AMP binding to the 47,000-dalton cyclic AMP-binding protein. Thus, there is an excellent positive correlation between a cyclic nucleotide’s ability to produce a physiological response in the cyclic AMP-deficient cr-1 mutant and its ability to bind the 47,000-dalton cyclic AMP-binding protein.

Although cyclic AMP-binding proteins have been identified as regulatory subunits of cyclic AMP-dependent protein kinase in both Saccharomyces and Neurospora, the role of cyclic AMP and cyclic AMP-dependent protein kinase in these lower eukaryotes remains unclear. The purification and properties of the Neurospora cyclic AMP-dependent protein kinase subunits should be very helpful in future in vitro studies of function.

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Isolation and properties of a cyclic AMP-binding protein from Neurospora. Evidence for its role as the regulatory subunit of cyclic AMP-dependent protein kinase.

J M Trevillyan and M L Pall


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