Structural Studies on a Family of cAMP-binding Proteins in the Nervous System of Aplysia

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Abstract. Five major cAMP-binding proteins that differ in size and charge have been identified in neurons of Aplysia californica by photoaffinity labeling with \[^{32}\text{P}]8-\text{N}_3\text{cAMP}. These proteins, which we believe are regulatory subunits of cAMP-dependent protein kinase, all differ from the major cAMP-binding protein of buccal muscle. We have compared the structures of these proteins by peptide mapping after chemical and proteolytic cleavage. These analyses indicate that the five binding proteins from nervous tissue and the major muscle protein are closely related to each other. For example, the three neuronal proteins that are most alike and the cAMP-binding protein from muscle have a similar, if not identical, Mr 20,000 domain that contains the 8-\text{N}_3\text{cAMP-binding site; beyond this domain they diverge. All six proteins appear to belong to a family in which homologous regions have been conserved to maintain common functions. We suggest that the regions of the molecules that differ mediate special functions such as ticketing to particular compartments of the cell. Evidence for regional assortment of the cAMP-dependent protein kinases according to structural type was afforded by subcellular fractionation of Aplysia nervous tissue; photoaffinity labeling of cytoplasm, cytoskeleton, and membrane fractions demonstrated a differential distribution of the five neuronal cAMP-binding proteins. Selective phosphorylation of specific substrates could be a consequence of the compartmentation of diverse cAMP-dependent kinases.

A cAMP-dependent kinase has been shown to regulate the function of ion channels in the central nervous system of the marine mollusc Aplysia (6, 7). We previously have used photoaffinity labeling with \[^{32}\text{P}]8-\text{N}_3\text{cAMP} followed by two-dimensional gel electrophoresis to identify five cAMP-binding proteins in Aplysia nervous tissue (16). Muscle contains a sixth binding protein, and a survey of the other parts of the body has revealed that each Aplysia tissue contains its own characteristic group of cAMP-binding proteins (36). Some heterogeneity also exists in vertebrate cAMP-dependent protein kinase. These enzymes are tetramers composed of two catalytic subunits and two cAMP-binding, regulatory subunits (19, 26, 34, 42). Two classes of regulatory subunits have been described (designated R\text{I} and R\text{II}). These differ in molecular weight (48), affinity for DEAE-cellulose (11), susceptibility to autophosphorylation (17, 39), and affinity for cAMP (5). Some also differ in their affinities for the catalytic subunit (21), and for calmodulin (23). Moreover, the two types of regulatory subunit can have different subcellular localizations (30). There also are tissue-specific forms: for example, the R\text{II} subunits of vertebrate heart, liver, brain, and adipose tissue differ in structure (22, 30, 41, 45) and in antigenicity (18, 44).

Because of the unexpected variety of cAMP-binding proteins that we found in Aplysia nervous tissue, we suggested that regulatory subunits with different molecular structures might be directed to specific sites within the cell where they can selectively phosphorylate local protein substrates (16). Heterogeneity of kinase subtypes would be especially adaptive in cells, like neurons, that carry out several complex functions and are regionally organized. Indeed, neurons have been shown to contain the greatest variety of cAMP-binding proteins of all the Aplysia somatic tissues (36). Their similar function implies that these proteins might share several structurally homologous regions (for example, regions for binding cAMP and for combining with catalytic subunit), while their molecular diversity predicts that the structurally different parts of the molecules might include heterologous domains that are responsible for directing the proteins to their proper subcellular locations. Here we present a study of the similarities and differences in the Aplysia cAMP-binding proteins, using peptide mapping, and further information about their subcellular distribution.

Materials and Methods

Tissue Homogenates

Neural components (consisting of cell bodies, clusters of axons and nerve endings, and associated glial cells) were obtained by dissection (20) from the cerebral, abdominal, and pleuropedal ganglia of 150–300 g Aplysia californica (Pacific Bio-Marine, Venice, CA) anesthetized by injection of isotonic MgCl\(_2\) (8). During the dissection we kept the isolated ganglia in a 1:1 mixture of supplemented artificial sea water (14) and isotonic MgCl\(_2\); to prevent synaptic transmission (8) and then transferred them at 0°C to buffer A: 350 mM sucrose, 170 mM NaCl, 3 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, and
Assays of cAMP-dependent Protein Kinase

dissection of neural components (2). Neural components were homogenized in buffer A by 11 gentle passes of a glass/Teflon tissue grinder with a clearance of ~0.05 mm driven by a motor at 300 rpm. The ratio of tissue to solution was 30–40 ganglia per ml. (Neural components from the four major central ganglia from one animal contain ~1 mg of protein.)

Buccal muscle masses from 20 animals were minced in 4 mM EDTA, 1 mM EGTA (pH 7.5), 5 mM 2-mercaptoethanol, Trasylol (2 trypsin-inhibiting units/ml, FBA Pharmaceuticals), and 5 mM benzamidine (Sigma Chemical Co., St. Louis, MO), homogenized with the PT-20 probe of a Brinkman Polytron (Brinkman Instruments Co., Westbury, NY), and centrifuged. The 10,000 g supernatant was filtered through Whatman 54 paper. The proteins in the filtrate were precipitated in 50% saturated ammonium sulfate, resuspended in 3 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, and 50 mM Tris-HCl (pH 7.5) (10 ml), and dialyzed extensively against the same solution.

Unless otherwise stated, experiments were conducted at 4°C and solutions are percent (wt/vol) for solid solutes and percent (vol/vol) for liquid solutes:

Chemical Co.), 0.1% Nonidet P-40, 50 mM morpholine propane sulfonic acid are stored at -20°C if not used immediately.

Protein was assayed according to Lowry et al. (3 l) using bovine serum albumin as the standard.

Photoaffinity Labeling

Proteins (0.1–0.4 mg/ml) were labeled at 4°C in solutions (50 µl) containing 0.6 µM [32p]8-N3cAMP (50–100 Ci/mmol; ICN), and 50 mM Hepes (pH 6.3) (49). After 15 min in the dark, the samples were photolyzed for 10 min at 254 nm (model UV G-11 Mineralight Lamp) from a distance of 5 cm. The labeled proteins were separated by one-dimensional (27) or two-dimensional (35) PAGE and were then detected by autoradiography of the dried gels with Kodak XAR-2 film at room temperature, or using DuPont Lightening Plus intensifier screens at ~70°C. Sections containing each labeled protein were cut out and stored at ~20°C if not used immediately.

Peptide Mapping

Exhaustive Treatment with Trypsin. Digestion with trypsin was carried out essentially as described by Elder et al. (15). Gel sections containing labeled protein were incubated for 18 h at 37°C with 0.13 mg/ml diphenylethamyl chloride–trypsin (Sigma Chemical Co.) in 50 mM NH4HCO3 (pH 8.0), and the digest filtered through glass wool and dried under vacuum. The peptides were oxidized with performic acid (24), dried, and washed to remove excess acid, and subjected to digestion with trypsin again. In a control experiment, we found that the radioactivity linked to the intact proteins labeled by the photoaffinity reagent was not released by the oxidation. The peptides were dissolved in acetic acid/formic acid/water (15:5:80) and separated by electrophoresis on cellulose-MN 300 plates (Brinkman) using a water-cooled chamber (Brinkman) at a field strength of 40 V/cm. After the plates had been air dried overnight, chromatography was performed in the second dimension in butanol/pyridine/acidic acid/water (32.5:25:5:20).

 Cleavage with Cyanogen Bromide. Each gel section was incubated for 24 h at 23°C in 0.4–0.8 ml of 1.3% cyanogen bromide (Sigma Chemical Co.) in 70% aqueous formic acid. Controls were incubated in the acid alone, which extracted intact proteins from the gels. The acid was removed from the extract by repeated evaporation to dryness from water. The residue was solubilized at 100°C in 50 µl of 2% SDS, 2% 2-mercaptoethanol, and 10% sucrose, and neutralized with 2.5 M Tris-HCl (pH 8.0). The peptides were separated on 15% SDS polyacrylamide gels.

Partial Digestions with Papain and S. aureus V-8 Proteases. The gel sections were then dried, and subjected to digestion with trypsin again. In a control experiment, we found that the radioactivity linked to the intact proteins labeled by the photoaffinity reagent was not released by the oxidation. The peptides were dissolved in acetic acid/formic acid/water (15:5:80) and separated by electrophoresis on cellulose-MN 300 plates (Brinkman) using a water-cooled chamber (Brinkman) at a field strength of 40 V/cm. After the plates had been air dried overnight, chromatography was performed in the second dimension in butanol/pyridine/acidic acid/water (32.5:25:5:20).

Results

Identification of Five Distinct cAMP-binding Proteins in Nervous Tissue

In previous preparations from nervous tissue we always found some cAMP-binding components with molecular weights of <45,000 that we assumed were produced by proteolysis during isolation (16). The regulatory subunits of cAMP-dependent protein kinases from vertebrates are also susceptible to proteolysis (12, 38). It is important to eliminate proteolytic activity because true lower molecular weight cAMP-binding proteins occur in Dictyostelium discoideum (the M, 41,000 regulatory subunit of a cAMP-dependent protein kinase [32]) and in Escherichia coli (CAP, the M, 22,000 catabolite gene activator protein [51]). To reduce proteolysis during tissue isolation and subsequent manipulations, Aplysia neural components were dissected from ganglia that had been rapidly frozen in 50% propylene glycol and quickly homogenized in the presence of several protease inhibitors. Under these conditions we detected only the five larger binding proteins after photoaffinity labeling with [32p]8-N3cAMP and two-dimensional gel electrophoresis (Fig. 1). This confirms our previous
Figure 1. cAMP-binding proteins photoaffinity labeled by [32P]8-N3cAMP in a homogenate of Aplysia neural components. (A) One-dimensional separation in a 8% SDS polyacrylamide gel (26). P indicates proteolytic degradation products. (B) Two-dimensional separation under stringent conditions to prevent proteolysis. An abdominal ganglion was dissected out and quickly frozen in 50% propylene glycol in 1 M NaCl at -20°C. Before homogenization the ganglion was transferred to buffer A (see Materials and Methods) containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.), Trasylol (20 trypsin inhibitory units/ml), and 5 mM benzamidine. Photoaffinity labeling of 30 µg protein samples was carried out at 0°C. The proteins, precipitated in 94% acetone at 0°C were separated by two-dimensional gel electrophoresis. The isoelectric focusing gels contained LKB ampholines (1.6% of pH 5-7 and 0.4% of pH 3.5-10). The second dimension consisted of a 3% polyacrylamide stacking gel and a 8% separating gel. In a previous paper from this laboratory on Aplysia cAMP-binding proteins (16), the two-dimensional gel electropherograms were mislabeled, with the acidic and basic ends reversed. We previously used another nomenclature for the binding proteins. The concordance between the present and earlier terminologies is: 1 = III; 2 = IId; 3 = IId; 4 = IIda--; 5 = IIda-.

assertion that the smaller species (previously observed and seen below most prominently in Fig. 7) are proteolytic fragments of the true cAMP-binding proteins of Aplysia. The possibility that some of the five remaining polypeptides are derived from others by proteolysis cannot be completely eliminated. Given the stringent precautions taken in the experiment described above, however, it is attractive to think that were such proteolysis to occur it would be before homogenization and of physiological significance (see Discussion).

We also observed isoelectric variants of four of the five Aplysia proteins (proteins 2, 3, 4, and 5), differing by single units of charge, which we do not recognize as distinct cAMP-binding proteins. Rather, we believe that they result from changes that occur within the cell (for example, phosphorylation [16] or other posttranslational modifications) or from experimental artifact (for example, elimination or introduction of charged groups by photoaffinity labeling).

A final consideration in defining the number of cAMP-binding proteins in Aplysia neurons was to rule out the possibility that protein 1 (Mr 105,000) is a dimer of one of the lower molecular weight species. We previously presented evidence that protein 1 is not a disulfide-linked dimer and that it is not generated by ultraviolet irradiation (16). We now find that no protein with a molecular weight greater than 60,000 is labeled by 8-N3cAMP in Aplysia muscle or in rat brain homogenates (data not shown). Therefore, artifactual dimerization does not occur in these tissues. Lastly, those proteins (4 and 5) which we find have peptide maps similar to that of protein 1 (see below) both differ greatly from it in charge. We might expect that the isoelectric point of a dimer formed from them would be the same as that of the monomer.

Tryptic Peptides from 8-N3cAMP-labeled Proteins
Analyses of tryptic peptides derived from each photoaffinity-labeled protein gave two distinct patterns. One, in digests from neuronal proteins 1 and 3-5 and the muscle cAMP-binding protein M (Fig. 2A), contained two labeled peptides (a and b in Fig. 2), with peptide a being more prominent. The proportion of peptide b in digests of the same protein varied.

Neuronal cAMP-binding protein 2 gave the other pattern of tryptic peptides (Fig. 2B). Its major peptide (c in Fig. 2B) moved further toward the negative electrode and was less soluble in the chromatographic solvent than were peptides a and b from the other cAMP-binding proteins. In all digests of protein 2 we also found a peptide with the same mobility as peptide a from the other cAMP-binding proteins. With longer exposures, peptide b also appeared.

The presence of peptides a and b in digests of protein 2 is not likely to be the result of contamination by another cAMP-binding protein, as shown by the following experiment. We prepared fractions by chromatography on DEAE-cellulose enriched in protein 2 (see below, Fig. 7B; fractions 32-40). The purified protein was photoaffinity labeled and separated
Autoradiographs of two-dimensional tryptic peptide maps of cAMP-binding proteins labeled with [\(^{32}\)P]8- \(N_3\)cAMP. Tryptic peptides derived from the designated cAMP-binding proteins were separated by electrophoresis (shown in the horizontal direction) and chromatography (vertical). Mix, mixture of tryptic digests (equalized for counts) of all the neuronal binding proteins. To facilitate presentation (see text), the autoradiographs have been divided into groups A, B, and C.

By analyzing a mixture of tryptic peptides from all of the neuronal cAMP-binding proteins, we showed that the mobilities of the peptides designated a in each sample were identical. The mobilities of the peptides designated b were also invariant (Fig. 2 C).

Cyanogen Bromide Peptides of 8- \(N_3\)cAMP-labeled Proteins

After cleavage with cyanogen bromide, neuronal cAMP-binding proteins 1 and 3-5 and protein M each gave a major labeled peptide migrating upon SDS PAGE with an apparent mass of 20,000 D, and a minor one of 28,000 D (Fig. 3). Protein 2 gave another pattern of peptides. Although we always detected traces of the M, 20,000 peptide, the two most prominently labeled peptides were smaller. The smallest peptide migrated to the dye front in a 15% gel (Fig. 3), but we have resolved it in 20% gels and estimate its mass to be ~12,000 D. The second prominent peptide had a mass of 15,000.

Although the major cyanogen bromide fragments are large, we believe that they are the major end products of chemical cleavage of these proteins. There is no evidence for any smaller peptides: very little radiolabeled material migrated at the dye front in 20% polyacrylamide gels. The radioactivity initially present in the protein before cleavage is almost all recovered in the large cyanogen bromide fragments (Fig. 3). The radioactive products are sensitive to digestion with papain (data not shown), and therefore are peptides and not derivatives of the photoaffinity reagent released by treatment with cyanogen bromide.
Limited Protease Digestion of 8-N3cAMP-labeled Proteins

We next compared the peptides resulting from incomplete proteolysis (10) of the photoaffinity-labeled cAMP-binding proteins. After digestion with V-8 protease, neuronal proteins 1, 4, and 5 gave similar peptide maps (Fig. 4A) in which peptides with molecular weights less than 20,000 were essentially identical. The same pattern also appears in V-8 digests of the major muscle cAMP-binding protein. In contrast, comparison of the larger peptides shows that each member of this group of proteins differs to some degree. The patterns of peptides obtained from proteins 1, 4, and 5 differed from that obtained from protein 2 over the entire molecular weight range. Protein 3 yielded a third pattern of peptides after digestion with V-8 protease.

Digestion with papain produced peptide patterns different from those produced by V-8 (Fig. 4B). From these patterns, the proteins could be divided into the same three groups. Thus, the peptide patterns from binding proteins 1, 4, 5, and M (data not shown) were similar to each other, while proteins 2 and 3 each yielded its own characteristic pattern. With papain no peptides of $M_r$ greater than 20,000 were observed.

Previously, we reported that proteins 2 and 3 could be phosphorylated by the catalytic subunit of the bovine kinase (16). In preliminary experiments, we have now examined the sequences in the vicinity of the phosphorylation sites of these proteins by limited proteolytic digestion. Proteins 2 and 3 were phosphorylated with $\gamma^{[32P]}$ATP and purified by electrophoresis. Peptides generated from the labeled proteins by treatment with V-8 protease were separated as described above revealing two distinct patterns with no peptides of identical mobility (data not shown). This result indicates that the phosphorylation sites of these two cAMP-binding proteins also differ from each other.

Association of cAMP-dependent Protein Kinase Activity and cAMP-binding Proteins with the Cytoskeleton

Using the extraction procedure of Pallas and Solomon (37), we separated Aplysia neural components into soluble and...
cytoskeleton fractions by extraction with Nonidet P-40 in 2 M glycerol. We found that 40–45% of the neuronal protein is associated with the detergent-insoluble fraction which is highly enriched in proteins with mobilities characteristic of the cytoskeletal components β-tubulin, actin, and neurofilament proteins (Fig. 5A). About half of the cAMP-dependent protein kinase activity was associated with the detergent-insoluble fraction (Table I). A substantial proportion of cAMP-binding proteins 1, 2, 3, and 4 were present in the cytoskeleton fraction; protein 5, on the other hand, appears to be completely unattached under these conditions of isolation (Fig. 5B).

**cAMP-dependent Kinase Activity and cAMP-binding Proteins in Membranes Prepared by Subcellular Fractionation**

cAMP-dependent protein kinase activity was distributed between the soluble fraction and several membrane fractions from homogenates of *Aplysia* neural components (Table I). Most of the particulate protein kinase activity was found in the 7,000 g membranes. This pellet was highly enriched in cAMP-binding proteins 1, 2, and 3 (Fig. 6A). These low speed membranes were further fractionated on sucrose density gradients, and the three proteins were found in the same proportions in all the fractions that contained them. The membranes that floated on 1.15 M sucrose were the most enriched in kinase activity (Table I) and, as expected, contained the highest proportion of cAMP-binding proteins. This fraction is enriched in synaptosomes (9, 16).

The membranes sedimenting at 120,000 g had a lower specific kinase activity than did the 7,000 g membranes and contained a different set of cAMP-binding proteins (Fig. 6B). This set, although similar to that of the low speed membranes in that it contains proteins 1 and 2, differs first because of the virtual absence of protein 3 and also because protein 4 is

**Table I. cAMP-dependent Kinase Activity in Subcellular Fractions**

|                     | +cAMP | −cAMP | % of total | % of total cAMP-dependent kinase activity |
|---------------------|-------|-------|------------|------------------------------------------|
| Cytoskeleton preparation | 1.19  | 0.35  | 60         | 48                                       |
| Supernatant         | 1.48  | 0.27  | 40         | 52                                       |
| Pellet              |       |       |            |                                          |
| Membrane preparation |       |       |            |                                          |
| Homogenate          | 1.68  | 0.20  | 100        | 100 (set)                                |
| 7,000 g pellet      | 2.14  | 0.13  | 20         | 29                                       |
| 15,000 g pellet     | 0.88  | 0.18  | 7          | 3                                        |
| 120,000 g pellet    | 1.32  | 0.32  | 16         | 10                                       |
| 1.15 M sucrose      | 3.10  | 0.12  | 8          | 16                                       |
| subfraction of 7,000 g pellet |      |       |            |                                          |

* Using the heptapeptide substrate (Materials and Methods).
* The data tabulated are from one of three independent experiments in which the values for the proportion of total protein in the pellet fraction range from 40 to 46%, and the proportion of total kinase activity, 50 to 53%.
* The data are from 1 of 11 experiments in which the proportion of kinase activity associated with membrane fractions was 33 ± 2.8% (SEM).

Figure 5. Association of cAMP-binding proteins with the cytoskeleton of *Aplysia* neural components. A cytoskeleton fraction was isolated by extracting neural components with 0.5% Nonidet P-40 (Materials and Methods). Proteins in the detergent-soluble and -insoluble fractions were separated by twodimensional gel electrophoresis. Coomassie Blue-stained gels of cytoskeletal (A1) and supernatant (A2) fractions. Proteins with sizes and isoelectric points of actin (A), tubulin (T), and *Aplysia* neurofilament proteins (N; see reference 27) are designated. The fractions were photoaffinity labeled with [*32P]8-N3cAMP and the labeled proteins separated by two-dimensional electrophoresis. Autoradiography of the dried gels was for 9 h with image intensifier screens. B1, cytoskeleton; B2, supernatant.
Figure 6. Subcellular distribution of cAMP-binding proteins from Aplysia neural components. Neural components were fractionated by differential and sucrose density gradient centrifugation; fractions containing 30 μg protein were photoaffinity labeled with [32P]8-N3cAMP and separated by two-dimensional gel electrophoresis. Autoradiograms, prepared at −70°C using an image intensifier screen, were obtained from the dried gels on which proteins from the following fractions had been separated. (A) Membranes collected at the 0.8:1.15 M sucrose interface after sucrose density gradient centrifugation of the 7,000 g pellet (6-h exposure). (B) The 120,000 g pellet obtained by centrifugation of the 15,000 g supernatant (14-h exposure). (C) The 120,000 g supernatant (soluble proteins; 14-h exposure).

Distribution of Lipids in the Subcellular Fractions

To estimate the purity of the particulate fractions, Aplysia ganglia were labeled overnight with [32P]8-N3cAMP and separated by two-dimensional gel electrophoresis. Autoradiograms, prepared at −70°C using an image intensifier screen, were obtained from the dried gels on which proteins from the following fractions had been separated. (A) Membranes collected at the 0.8:1.15 M sucrose interface after sucrose density gradient centrifugation of the 7,000 g pellet (6-h exposure). (B) The 120,000 g pellet obtained by centrifugation of the 15,000 g supernatant (14-h exposure). (C) The 120,000 g supernatant (soluble proteins; 14-h exposure).
Figure 7. Fractionation of *Aplysia* cAMP-binding and cAMP-dependent protein kinase activities by ion exchange chromatography on DEAE-cellulose. A 120,000 g supernatant and a 7,000 g pellet were obtained from the central ganglia of 10 animals. Solubilization of the membranes in Nonidet P-40, desalting of the soluble proteins, and ion exchange chromatography on DEAE-cellulose with a salt gradient from 0 to 0.4 M NaCl are described in Materials and Methods. Fractions were assayed for cAMP-dependent protein kinase activity and by photoaffinity labeling with [32p]8-N3cAMP: we plotted the difference between phosphorylation of the heptapeptide substrate in the presence and in the absence of cAMP. The photoaffinity-labeled proteins were separated in 8% SDS polyacrylamide gels. Autoradiograms of selected lanes from the dried gels are shown, labeled with the corresponding fraction numbers: (left) soluble proteins; (right) membrane proteins.

suggest a functional role for those regions of the proteins that are structurally variable.

**Are the cAMP-binding Proteins Regulatory Subunits?**

*Aplysia* muscle and nervous tissue both contain protein kinase activity. Essentially one form of 8-N3cAMP-binding protein (*M*) predominates in muscle. It seems logical to assume that this species functions as a regulatory subunit; protein *M* resembles the vertebrate subunits in size, and it purifies together with kinase activity on DEAE-cellulose chromatography. After treatment with cAMP, higher salt concentrations are required to elute *M* from the DEAE-cellulose suggesting that, like the vertebrate protein, it is associated with a basic catalytic subunit that dissociates in the presence of cAMP (unpublished data). In nervous tissue, where five distinct forms have been separated, the total amounts of the binding proteins (as estimated either by photoaffinity labeling or by [3H]cAMP-binding [1]) are of the same order as that of the catalytic subunit (as estimated from kinase activity). Each of the five cAMP-binding proteins is eluted with cAMP-dependent kinase activity during DEAE-cellulose column chromatography. Since here we demonstrate structural homology between the muscle cAMP-binding protein and all of the neuronal species, it is reasonable to think that the neuronal binding proteins also function as regulatory subunits. Because each one has not been clearly separated from the others in the form of the holoenzyme, the biochemical evidence that they all function as regulatory kinase subunits is not yet compelling. The evidence for the largest component, protein *J*, is least persuasive because it is not well resolved from protein *I* by DEAE-cellulose chromatography and is present in relatively small amounts (Fig. 7B).

**Structure of Aplysia cAMP-binding Proteins**

We have compared the structures of the five neuronal cAMP-binding proteins from *Aplysia* and the protein from muscle. In many respects, these proteins seem similar to vertebrate regulatory subunits. Despite the strong emphasis in the biochemical literature on there being only two forms, R1 and R2, within the same animal, evidence for considerable molecular heterogeneity in some vertebrate cells and tissues has accumulated over the past few years (for review see reference 30). Especially pertinent is the diversity exhibited by the R1 subunits of bovine brain (29). The structural relationships of the
various heterogeneous forms of vertebrate subunits have not yet been scrutinized, and it has been assumed that each variant is highly homologous to one of the two basic types from bovine muscle, R1 (47) or R1 (46).

Bovine R1 and R1 each consist of three domains (A, B, and C) of approximately equal size (47). Domains B and C, at the COOH-terminal ends of the subunits, each can bind a molecule of cAMP, and are highly homologous to their counterparts in the other subunit. Internal homology exists between domains B and C within each regulatory subunit but it is weaker than the interchain homology between analogous domains, suggesting an early internal gene duplication before a second duplication and divergence of the R1 and R1 genes. The NH2-terminal A domains show far weaker but still significant homology with each other suggesting that they did not arise from the splicing of heterologous segments of DNA to those encoding the two cAMP-binding domains.

After labeling with [32P]8-N3cAMP followed by exhaustive treatment with trypsin, Aplysia proteins 1, 3, 4, 5, and M gave the same two peptides (a and b; Fig. 2) suggesting that the five proteins are homologous in the vicinity of the 8-N3cAMP-binding site. In porcine R1, 8-N3cAMP had been found to bind selectively to the cAMP-binding site of domain C (25). After photolysis and exhaustive trypsin digestion, this vertebrate subunit yielded a single labeled peptide and sequence analysis revealed that a tyrosine residue (corresponding to Tyr38 in the fully sequenced bovine R1) had reacted. Recently, 8-NacAMP has been shown to label two amino acid residues on two distinct tryptic peptides of the porcine R1 subunit (which correspond to Pro27 and Tyr37 of bovine R1 [3]). With the Aplysia subunits, we cannot tell whether the two peptides are derived from two distinct cAMP-binding sites within a single protein (as with porcine R1 [4]), or whether two different peptides were produced after labeling of one site. Generation of two peptides from a single site might have occurred (a) if there were two residues labeled at the single site; (b) if, despite our efforts, cleavage by trypsin were incomplete; or (c) if chemical modification of the reagent-peptide adduct took place after the proteins had been extracted from the tissue (for example, by reaction with performic acid). In contrast with Aplysia, the 8-N3cAMP–labeled tryptic peptides that are generated from porcine R1 and R1 differ considerably and would not be expected to fingerprint together (4, 25).

Protein 2 also yielded peptides a and b but they were minor components compared with a third peptide, c. Again we cannot tell whether c is derived from still another cAMP-binding site or whether a subtle difference in the structure of protein 2 leads to labeling of a subsite (represented by peptide c) that is part of one of the sites that yields peptide a or peptide b. The R1 subunit from bovine brain also yielded three radioactive peptides upon photolabeling and digestion with trypsin (45).

In a second analysis, we examined peptides generated by treatment with cyanogen bromide from the labeled cAMP-binding proteins. Proteins 1, 3, 4, 5, and M again gave identical fragments: a major M, 20,000 peptide and a minor M, 28,000 peptide. One interpretation of this result is that the M, 20,000 fragment was derived from one cAMP-binding site and the M, 28,000 from another. A second interpretation, which we favor, is that the larger fragment resulted from incomplete cleavage. First, if there were two sites labeled, the two fragments together would make up almost the entire mass of each subunit: an unlikely possibility given the prevalence of methionine residues in proteins. Second, if the Aplysia proteins are homologues of the vertebrate proteins, a large peptide derived from photoaffinity labeling in domain C would span both known 8-N3cAMP–binding sites (4). Consequently, labeling in domain B would not be expected to yield a new peptide.

Protein 2 again gave a different result. The major cyanogen...
bromide fragment had a molecular weight of 12,000. Less intense bands corresponding to peptides of M, 20,000 and 15,000 were also seen. One interpretation, based once again on presumed homology with vertebrate R and R, is that methionine residues absent from 1, 3, 4, 5, and M are present in protein 2 in the vicinity of the two cAMP-binding sites. The M, 15,000 and 20,000 peptides would now be incomplete cleavage products and the M, 12,000 fragment a limit peptide. Again, alternative explanations based on two (or more) widely separated cAMP sites are conceivable. It is important to note that because of the likelihood of incomplete fragmentation and the strong possibility of large fragments spanning two sites, our results do not allow us to distinguish between the possibilities of one or several cAMP-binding sites. Again for comparison we note that bovine R contains seven methionine residues and that R contains nine. Only two of these are conserved, and their distribution is such that no cyanogen bromide fragments from the two vertebrate subunits are the same size.

In a third analysis, we carried out partial proteolytic digestions with V-8 protease or with papain on each Aplysia subunit and examined the resulting fragments by gel electrophoresis in one dimension. Peptides smaller than M, 20,000 generated from proteins 1, 4, 5, and M were identical, indicating strong and extended homology on both sides of the major 8-NcAMP labeling site. (Of course, the homology would extend in one direction only if the labeling site were close to the COOH-terminus as it is in bovine R.) The sets of peptides larger than M, 20,000 generated from 1, 4, 5, and M differed, providing evidence that these proteins diverge in sequence at their extremities (or towards the NH2-terminus only, if the analogy with bovine R is continued). The one-dimensional peptide maps of proteins 2 and 3 clearly differed from each other and from the common pattern of 1, 4, 5, and M. With protein 2, we cannot be certain if this is because the major labeling site is different (see above); with protein 3 (which was identical to 1, 4, and 5 by the other criteria) we cannot quantify the extent of sequence divergence because only a few substitutions could alter the peptide pattern completely. Comparison of partial V-8 protease digests of 32P-labeled phospho forms of proteins 2 and 3 indicate that these two binding proteins also differ in the vicinity of their auto-phosphorylation sites.

The three methods of peptide mapping that we used indicate that the cAMP-binding proteins in Aplysia neurons are closely related. Proteins 1, 4, and 5 appear identical, given the limitations of the methods, within 20,000 D of a major 8-NcAMP-labeling site. The muscle cAMP-binding protein, M, is a close relative of these three. Protein 3, and to a lesser degree 2, are also related to the others. To the extent that the Cleveland procedure (10) relies on sequence differences alone it should be a sensitive test of homology. Vertebrate R and R, which are ~50% homologous over much of their sequences (47), have completely different peptide maps (40, 52). It is not possible to quantitate our results but it does seem very likely that all six polypeptides are more closely related than is bovine R to R,1 Their structural relationship seems

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1 Phosphorylated 2 and 3 have identical electrophoretic mobilities when compared with the unmodified proteins (our unpublished results). This observation adds to the evidence against placing the Aplysia proteins in the category R or R (17, 39, 40, but see also reference 41). More analogous to the close relationship demonstrated between the bovine brain and heart R subunits (45). Until the structures of the genes encoding the family of cAMP-binding proteins in Aplysia have been analyzed, it will be impossible to choose among the several possible molecular mechanisms by which the six related proteins might have been generated (for example, from a multiple gene family, by differential RNA processing, or by posttranslational modification; see reference 13). For example, although we favor a gene family, it is possible that a subset of the cAMP-binding proteins in neurons is generated by proteolytic cleavage of another member of the group. Generation of diverse structures by this mechanism is well known for hormones and neuropeptides (13).

**Functional Significance of Molecular Heterogeneity of cAMP-binding Proteins**

We can now ask why Aplysia neurons should contain five cAMP-binding proteins, presumed to be subunits of cAMP-dependent protein kinases, that structurally are partly homologous and partly divergent. Regions of homology would be required to maintain the functions of common regions such as the sites for cAMP binding, catalytic subunit binding, dimerization, and autophosphorylation. Some heterogeneity in the cAMP-binding domain might also be expected because the cell contains subunits with different affinities for cAMP (1). Because the binding proteins can be distinguished both with respect to regional localization within the tissue (for example, in neurons to cell body, axon and nerve endings) and with respect to subcellular distribution (association with membrane or cytosol), the cell contains subunits with different affinities for cAMP (1). Because the binding proteins can be distinguished both with respect to regional localization within the tissue (for example, in neurons to cell body, axon and nerve endings) and with respect to subcellular distribution (association with membrane or cytosol), we speculated earlier (16) that the type of regulatory subunit might predispose the particular holoenzyme that contains it to a specialized function within the cell. Many physiological functions are altered by cAMP-dependent protein phosphorylation (19, 26, 34, 42), but since the cAMP-dependent protein kinase is thought to possess only a single kind of catalytic subunit, it is attractive to think that selective phosphorylation might be achieved by building holoenzymes of regulatory subunits with different properties.

Heterologous regions would contain modules for targeting the proteins to particular subcellular locations. Thus a hydrophobic domain could cause association with membranes or a signal sequence insertion into a particular organelle. We suggest that these modules lie in the regions outside the conserved domain in proteins 1, 4, 5, and M, four structurally similar proteins with quite different subcellular addresses. These heterologous regions might also confer other properties on the molecules. The M, 105,000 protein, protein 1, for example, conceivably could possess a fused catalytic domain like the vertebrate cyclic guanosine monophosphate-dependent protein kinase (47).
In vertebrates, others have also found different distributions of regulatory subunits within cells and among tissues (see introductory paragraphs), though none have the striking complexity of the distribution in Aplysia in which there are at least eight different cAMP-binding proteins in several combinations in various tissues (36) and where the five neuronal proteins are produced within a single nerve cell (16). The definitive answer to the question of whether more versions of proteins exist in definitive answer to the question of whether more versions of proteins are produced within a single nerve cell (16). We thank Tsunao Saitoh for advice on experimental design, Stephen Sturner for technical help, Alice Elste and Robin Tewes for preparing the figures, and Marlene Zurack for preparing the manuscript.

C. Mark Eppler was supported in part by National Institutes of Health grant NS 15834, and Steven Greenberg is supported by the National Institutes of Health training grant GM 07367.

Received for publication 12 June 1985, and in revised form 6 September 1985.

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