Evidence is presented that multiple forms of cyclic nucleotide phosphodiesterase (PDE) activity chromatographically separated from the soluble fraction of bovine hypothalamus are co-eluted with multiple forms of 5'-nucleotidase (5'N) activity. The enzymes could not be resolved from each other by anion-exchange chromatography on DEAE-TSK; by affinity chromatography on phenyl-, blue-, concanavalin A-, 5'AMP-sepharose, cAMP-silica gel; or by gel filtration on sephacryl S-200. The catalytic activities were found to be associated with the tetrameric, dimeric, and monomeric forms of the enzymes. The molecular weights determined by gel filtration or by SDS-gel electrophoresis were 220, 114, and 57 kDa, respectively. Kinetic analysis revealed that the first-order rate constant for 5'AMP hydrolysis measured in the reactions: cAMP → 5'AMP → adenosine was 100 times higher than that in the reaction: 5'AMP → adenosine. Thus, functional interrelation between PDE and 5'N was expressed in drastic acceleration of the consecutive reactions: cAMP → 5'AMP → adenosine. The results confirm the conclusion about the existence of a multienzyme system involving PDE and 5'N or of a single bifunctional enzyme in brain tissue.

KEY WORDS: Coupled enzymes, cyclic nucleotides; phosphodiesterase; 5'-nucleotidase; hypothalamus.

INTRODUCTION

At the present time, the attention of enzymologists is turned to a poorly studied field of biochemistry that concerns multifunctional enzymes, protein-protein interactions, and enzyme cascades.

Enzymology, which developed earlier in the direction of isolation of individual enzymes and determination of their primary, secondary, and tertiary structure, is focused today on investigating quaternary structure and supramolecular enzyme organization (1).

Most enzymes have an oligomer structure. If the subunits are not identical and they catalyze different reactions, they form a multienzyme complex (2,3).

The active enzyme sites are assumed to be spatially close to each other. Protein-protein interactions can occur through areas of complementary contact surfaces exposed to each other upon conformational molecular changes (4). This allows the product of the first enzyme to easily reach the second enzyme. A detailed analysis of the kinetic and thermodynamic aspects of such a facilitated catalysis in a multienzyme system has been described (5).

Binding of two different catalytic proteins resulting in an acceleration of their total catalytic reaction represents a special level of supramolecular enzyme organization. The formation and stabilization of protein complexes appear to be dependent on the interactions involving hydrophobic or hydrogen bonds and Van der Waals forces (6, 7).

Many multienzyme complexes are quite stable. They are likely to exist in an associated state in a living cell. These complexes may exist, in an at least partially associated state, in the process of protein purification and may not dissociate into the constituents. Typical examples of multienzyme systems are tryptophan synthetase, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase complexes (3, 8).

Conjugates, in which two or more interconnected enzymes are parts of the same polypeptide chain, might represent another example of multienzyme systems. In
some respects, the structure of multienzyme conjugates is similar to that of monomeric enzymes containing several domains.

Conjugates contain various sites catalyzing individual enzymatic reactions. These multifunctional proteins are recognized now as regulators of many functions of the organism. Sometimes, such structures can be separated into individual enzymes as a result of limited proteolysis. This often occurs without disturbing the structure and catalytic activity of the components. The generation of conjugates may be attributed to fusion of adjacent genes (9). Examples of multienzyme conjugates are fatty acid synthetase, DNA-polymerase, and \( \beta \)-galactosidase (9, 10). In higher organisms, including mammals, the first stages of pyrimidine biosynthesis are catalyzed by glutamine-dependent carbamoyl-phosphate synthetase, aspartate carbamoyltransferase, and dehydroorotase. Data are available that these enzymes are interconnected, and are probably located in a single polypeptide chain (11).

It is known that various intracellular structures are involved in the formation of multienzyme complexes. Enzyme ensembles can occur, in which enzyme-enzyme interaction is realized via attachment to membrane surfaces or insertion into a membrane lipid bilayer, or there can be interaction between mitochondrial matrix enzymes. For example, malate dehydrogenase was found to form a complex with glutamate dehydrogenase, and with aminotransferase (12). This complex can accomplish dehydrogenation of amino acids, for example, aspartic acid and tyrosine, whereas individual enzymes are unable to do this. Beeckmans and Kanarek have demonstrated the existence of complexes between fumarase and malate dehydrogenase, between malate dehydrogenase and aspartate aminotransferase, and between malate dehydrogenase and citrate synthetase (13). However, little is known about details of these interactions.

The protein-protein interactions that can occur not only between the components of multienzyme complexes, but also between soluble individual enzymes, remain a central problem in the field of enzymology today.

We recently discovered that multiple forms of cyclic nucleotide PDE* (EC 3.1.4.17) and 5’N (EC 3.1.3.5) of the soluble fraction of bovine hypothalamus are co-eluted at all stages of purification of these enzymes. A functional relationship between PDE and 5’N was demonstrated, which was expressed in acceleration of the overall reaction catalyzed by these enzymes: cAMP--\( \rightarrow \)5’AMP--\( \rightarrow \)Ado (14). The interrelationship between PDE and 5’N was suggested to represent a new example of supramolecular enzyme organization.

The basic objective of this research was to improve our understanding of the structure and function of the PDE-5’N enzyme system, to elucidate its role in the regulation of cAMP catabolism. Some of our own data, and those of others as well, are summarized in this article. The results of investigation of certain physical, chemical, kinetic, and regulatory properties of bovine hypothalamic PDE and 5’N are presented.

**EXPERIMENTAL PROCEDURE**

**Materials.** Cyclic AMP was purchased from Calbiochem (U.S.A.). \( N^\alpha, O^\beta\)-Dibutyl-cAMP, cGMP, SDS-Na, EGTA, phenylmethylsulfonylfluoride, diisopropylfluorophosphate were from Serva (F.R.G.). DTT, Na\( _2 \)HPO\(_4 \), 5’AMP, 5’GMP were from Reanal (Hungary). 2-Mercaptoethanol was obtained from Merck (F.R.G.). \( \alpha \)-Methylmannose was kindly provided by Dr. Ya. V. Vozny (Institute of Biochemistry, Armenian SSR Academy of Sciences) or was purchased from Sigma (U.S.A.). \( [\beta ^3 H]5' \) AMP and \( [\gamma ^3 H]5' \) GMP were from Chemapol (Czechoslovakia). Thin-layer sheets Silufol UV-254 were from Kavalier (Czechoslovakia). Chromatographic sorbents: TSK DEAE-Toyopearl 650 M was from Toyopearl (Japan); phenyl-sepharose, blue dextran-sepharose, 5’AMP-sepharose, Con A-sepharose, sephacryl S-200 were from Pharmacia (Sweden); anion-exchange resin amberlite CG-400 and acrylamideagarose 202, from Serva (F.R.G.). All other chemicals, from Reachim (U.S.S.R.), were of analytical-reagent grade.

**Enzyme Purification.** Enzymes were isolated from bovine hypothalamus by the method used by us earlier for purification of PDE (15), modified according to methods including affinity chromatography on phenylsepharose (16) and blue dextran-sepharose (17). We used chromatography on 5’AMP-sepharose and on Con A-sepharose, as well as on the original affinity sorbent based on cAMP that was immobilized on epoxy-activated silica gel (18) by the technique described previously (19).

The frozen bovine hypothalamic tissue was homogenized in 2.5 volumes of 25 mM Tris-HCl buffer, pH 7.0, containing 0.1 mM NaN\(_3\), 1 mM DTT or 2-mercaptoethanol, 1 mM MgCl\(_2\), 5 mM phenylmethylsulfonyl fluoride, and 5 mM diisopropylfluorophosphate (buffer A). In certain cases, 0.1 mM EGTA was introduced into buffer A. The homogenate was centrifuged at 22000 g for 60 min.

The supernatant fluid was applied to a DEAE TSK column (4 \times 6 cm). The column was washed with buffer A containing 0.05 M NaCl. The column was developed with a linear gradient from 0.05 to 0.35 M NaCl in buffer A. However, due to the large volumes involved, it was found convenient to elute the enzyme from the DEAE resin stepwise with 0.35 M NaCl in buffer A at a flow rate of 300 ml/h.

The pooled enzyme of each peak eluted from the DEAE TSK column was applied to a phenyl-sepharose column (4 \times 6 cm) previously equilibrated with buffer A containing 1 mM CaCl\(_2\). The column was then washed with buffer A containing 0.1 mM CaCl\(_2\) and the enzyme was eluted by 0.2 mM EGTA in buffer A containing 0.15 M NaCl, followed by buffer A alone. The flow rate was 300 ml/h. Fractions of 7 ml were collected.

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* Abbreviations: PDE, phosphodiesterase; 5’N, 5’-nucleotidase; CaM, calmodulin; Ado, adenosine; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(\( \beta \)-amino-ethyl ether)-\( \gamma \),\( \delta \)-triacetic acid; SDS-Na, sodium dodecyl sulphate; Con A, concanavalin A; \( \alpha \)-MM, \( \alpha \)-methylmannose; DTT, dithiothreitol.