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AMP DEAMINASE FROM RAT BRAIN: PURIFICATION AND CHARACTERIZATION OF MULTIPLE FORMS

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SUMMARY

1. When brain extracts were chromatographed on a phosphocellulose column, using a NaCl gradient for elution, three peaks and a shoulder with AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) activity were obtained. They were designated as Peaks I, II, III and IV in the order of elution from the column. When Peaks I, II and IV were individually applied to the phosphocellulose column, each was chromatographed as a single peak at the same position as that for the original peak and the mixtures of them yielded only the peaks corresponding to the parental peaks.

2. Deaminase Peaks I, II and IV were purified by column chromatography using phosphocellulose and DEAE-cellulose. About 130-fold purification was obtained for Peak I, 280-fold for Peak II and 770-fold for Peak IV.

3. A broad optimum pH was observed in Peak I and a sharp optimum pH, at pH 6, in Peak IV. Peak I was rapidly inactivated when heated at 50 °C. Under the same condition, Peaks II and IV were inactivated more slowly. The rates of deamination of dAMP, relative to AMP, in three deaminase preparations are different from one another.

4. The molecular sizes of three deaminases are similar.

5. Deaminase Peak I has apparently a higher affinity for AMP than other types of deaminase both in the absence and presence of activators, while Peak IV has the lowest affinity of all.

6. The regulatory properties of these deaminases are similar. The velocity vs AMP concentration curves are sigmoidal in the absence of activator such as ATP, KCl or NaCl and the curves become nearly hyperbolic in the presence of activator. The maximum velocities were the same both in the absence and presence of activator, while the enzymes showed a higher affinity for AMP in the presence of activator.

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INTRODUCTION

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) catalyzes the deamination of AMP to form IMP and ammonia. This enzyme was found to be widely distributed in animals [1] and was purified from various tissues. The kinetic properties vary widely depending on the source and purity of the enzyme. The enzymes from muscle [2–14], erythrocytes [15–22], and brain [23–28] have been extensively studied. Although AMP deaminases from different sources vary quantitatively as regards their interaction with modifiers, they behave as allosteric enzymes.

Rao et al. [18] found that human erythrocytes contained two kinds of AMP deaminase, soluble and membrane bound, and that they differed from each other with respect to their activation by ATP. Birnbaum et al. [29] also reported that AMP deaminase from calf muscle resolved into two forms by chromatography on phosphocellulose, and that the forms differed from each other in their activation by alkali metal ions.

The biological function of AMP deaminase is unknown, but it may be important in regulating the interconversion and relative amounts of adenine, inosine, and guanine nucleotides in tissues [17, 23–25].

This paper deals with the purification and properties of multiple AMP deaminase forms in rat brain. The clearly separable forms of deaminase have a number of different properties. Part of this work has been presented in a preliminary form [28].

MATERIALS AND METHODS

Nucleotides were obtained from Boehringer and Soehne. DEAE-cellulose and phosphocellulose were purchased from Brown Co. Sepharose 6B was obtained from Pharmacia. All other reagents were of the best quality commercially available.

Enzyme activity was measured colorimetrically by estimating production of ammonia by the following two methods. Assay method 1. A typical reaction mixture contained 10 mM AMP, 5 mM ATP, 20 mM potassium phosphate buffer (pH 7.0), 50 mM NaCl, 0.02% 2-mercaptoethanol, 0.05% bovine serum albumin, and enzyme in a final volume of 0.25 ml. The amount of ammonia was determined directly by the phenol–hypochlorite reagent [30]. Assay method 2. This assay was used in the kinetic experiments. The reaction mixture contained 10 mM Tris–HCl buffer (pH 7.0), 0.05% bovine serum albumin, various concentrations of AMP and effectors, and enzyme free from 2-mercaptoethanol in a final volume of 0.25 ml. The amount of ammonia was estimated by Nessler's reagent. The reaction was carried out at 37 °C for 10 min. The zero time concentration of ammonia was used as a control since ammonia was present both in the reagents and in enzyme solution. One unit of enzyme activity is defined as the amount of enzyme that yields 1 μmole of ammonia per min under the assay conditions described above. Protein concentrations were measured by the method of Lowry et al. [31] using bovine serum albumin as a reference protein. All absorbance measurements were carried out with a Hitachi 181 spectrophotometer.

The cerebra (cerebral hemispheres dissected free of cerebellum and brain stem) of adult rats weighing 200–300 g were used throughout.

RESULTS

Existence of multiple forms

When the brain extracts were chromatographed on a small phosphocellulose column, using a NaCl gradient for elution, three peaks and a shoulder with AMP deaminase activity were obtained. They were designated as Peaks I, II, III and IV in the order of their elution from the column (Fig. 1A). In a series of these experiments, Peak I was eluted at 0.25–0.3 M NaCl, Peak II at 0.45–0.5 M NaCl, Peak III at 0.55–0.6 M NaCl and Peak IV at 0.75–0.8 M NaCl. Further elution with 2.0 M NaCl

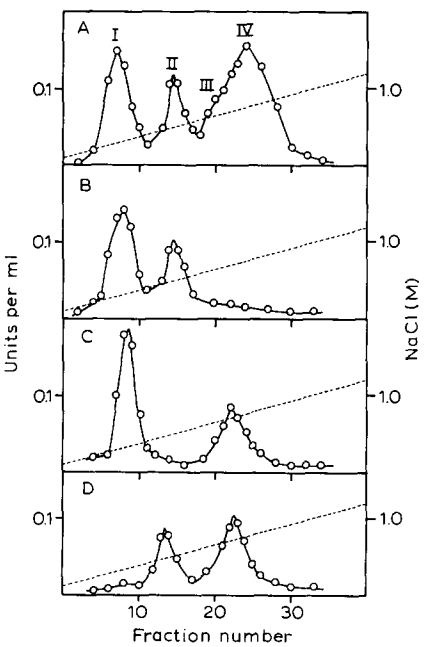

Fig. 1. Analytical phosphocellulose chromatography of AMP deaminase from an extract of rat brain and of the mixtures of different types of enzyme. A, a frozen preparation (12 g) of rat brain was blended for 5 min with 60 ml of cold 0.02 M potassium phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol (Buffer A), in a Waring blender at 18 000 rev./min. After centrifugation at 20 000 × *g* for 20 min, the supernatant solution was saved and solid NaCl was added to bring its concentration to 0.1 M. The solution was then placed on a phosphocellulose column (0.9 cm × 10 cm) which was previously equilibrated with 0.1 M NaCl in Buffer A. The column was washed with 30 ml of 0.1 M NaCl in Buffer A, and then, elution was carried out with a linear NaCl gradient from 0.1 to 1.2 M NaCl, in Buffer A. The reservoir and mixing bottles each contained 200 ml of the appropriate solution. Fractions of about 10 ml each were collected and assayed for enzyme activity (Assay 1). B, C and D, fractions containing Peak I (Tube number 5–10) were combined, as were fractions containing Peak II (Tube number 13–17) and Peak IV (Tube number 23–29). The fractions were kept in the cold and the activities concentrated to approx. 5 ml by ultrafiltration using a Collodion bag. Each concentrated enzyme solution was divided into two equal portions and the mixture of Peaks I and II (B) was dialyzed overnight against Buffer A, as were the mixtures of Peaks I and IV (C), and Peaks II and IV (D). After centrifugation to remove the precipitate formed during dialysis, each solution was chromatographed on a phosphocellulose column (0.9 cm × 5 cm) as described above, except that the reservoir and mixing bottle each contained 100 ml of the appropriate solution and the volume of each fraction collected was about 5 ml.

in phosphate buffer revealed no other activity peaks. The amount of Peak III is small, however its existence is undoubted from its reproducible appearance on phosphocellulose chromatography and its elution as an activity peak on rechromatography of Peak III fractions [28].

When Peak I was separated from the other peaks by phosphocellulose chromatography (Fig. 1A), followed by rechromatography after overnight dialysis in potassium phosphate buffer, it was observed that all of the total activity was eluted as Peak I. Likewise, Peak II or IV was chromatographed as a single peak; Peak II was eluted at 0.5 M NaCl, and Peak IV at 0.8 M NaCl.

When Peaks I and II were mixed and dialyzed overnight against 0.02 M potassium phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol, the mixture yielded only the peaks corresponding to Peaks I and II (Fig. 1B). Also, when Peaks I and IV were mixed and dialyzed, the mixture yielded Peaks I and IV (Fig. 1C). Likewise, the mixture of Peaks II and IV yielded Peaks II and IV (Fig. 1D). The overall recovery of activity in these experiments was about 100%. All of these results indicated clearly that in rat brain multiple forms of AMP deaminase exist and that they do not readily mix to form hybrids in the mild hybridizing conditions such as overnight dialysis employed in the experiments.

Purification of AMP deaminases

Unless otherwise specified, all manipulations were carried out at 0–4 °C. The enzymes obtained in various stages of purification were stored at 4 °C. Centrifugations were performed in a Hitachi 18 PR centrifuge.

Step 1: Preparation of the crude extract. Frozen rat brains (150 g) were homogenized for 5 min with a Waring blender in 5 volumes of Buffer A, which consisted of 0.02 M potassium phosphate (pH 7.0) and 0.1% 2-mercaptoethanol. After centrifugation at 20 000 × *g* for 20 min, the supernatant was saved, and the precipitate was again homogenized in 3 volumes of the same buffer solution. The supernatants so obtained were combined, and solid NaCl was added to bring its final concentration to 0.1 M.

Step 2: First phosphocellulose column chromatography. The enzyme solution (915 ml) was applied to a phosphocellulose column (5 cm × 15 cm) previously equilibrated with Buffer B, which was composed of 0.1 M NaCl and Buffer A. After washing with the same buffer to remove unadsorbed protein, the enzymes were eluted with Buffer A containing 1.5 M NaCl.

Step 3: DEAE-cellulose chromatography. Pooled fractions from step 2 (160 ml) were concentrated by precipitation with 60% saturated ammonium sulfate, dissolved in Buffer A and exhaustively dialyzed against this buffer. The precipitate formed during dialysis was removed by centrifugation, and the enzyme solution was applied to a DEAE-cellulose column (2 cm × 20 cm) equilibrated with Buffer A. After washing with the same buffer, the column was eluted with Buffer A containing 0.5 M NaCl.

Step 4: Second phosphocellulose chromatography. Pooled fractions containing AMP deaminase from the previous step were diluted with Buffer A to bring the NaCl concentration to 0.1 M and applied onto a phosphocellulose column (2 cm × 15 cm) equilibrated with Buffer B. The column was washed with 3 volumes of Buffer B to remove unadsorbed proteins, and a linear salt gradient from 0.1 to 1.2 M NaCl in Buffer A (500 ml per chamber) was then applied to the column. Fractions of 15 ml

each were collected and assayed for AMP deaminase activity. A typical elution profile of step 4 (Fig. 2) shows that the enzymes were separated effectively from each other by this technique. As is clear from the figure, three distinct peaks and a shoulder containing enzyme activity were obtained. The shoulder (designated as Peak III) was not investigated further because of its lower activity. Fractions comprising each peak (Peaks I, II and IV, for fractions 12–20, 21–29 and 37–46, respectively, in Fig. 2) were pooled and concentrated to approx. 5 ml by ultrafiltration using a Collodion bag.

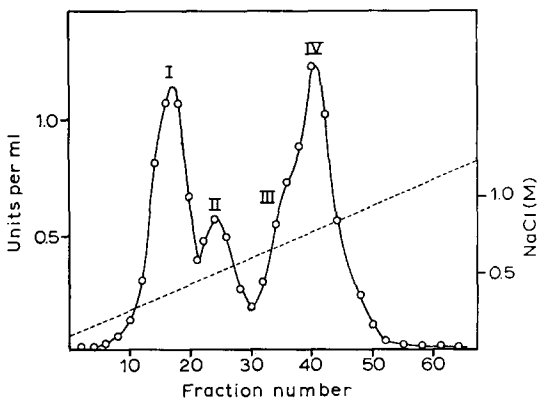

Fig. 2. Separation of AMP deaminase Peaks I, II and IV by phosphocellulose chromatography. Detailed experimental procedures were as described in the text. AMP deaminase activity was determined by Assay 1.

Step 5: Third phosphocellulose chromatography. Since each fraction was not completely separated from the other on the previous phosphocellulose chromatography, Peak I fraction as well as Peak II and IV fractions were again chromatographed on phosphocellulose for further separation. After overnight dialysis against 50 volumes of Buffer B, Peak I, II and IV fractions were adsorbed onto separate columns of phosphocellulose (0.9 cm × 10 cm) equilibrated with Buffer B. After washing with the same buffer, Peak I and II fractions were eluted with a linear gradient of 0.1 to 1.2 M NaCl in Buffer A (200 ml per chamber) at a flow rate of 45 ml per hour, and 10-ml fractions were collected (Fig. 3A). Peak IV fraction was also eluted with a linear gradient of 0.4 to 1.2 M NaCl in Buffer A (200 ml per chamber), and a typical elution profile is given in Fig. 3B. Fractions 6–12 and 13–19 in Fig. 3A and fractions 11–17 in Fig. 3B, were pooled, concentrated by ultrafiltration and tentatively identified as deaminases Peaks I, II and IV, respectively.

The overall purifications of Peaks I, II and IV are summarized in Table I. About 130-fold purification was obtained for Peak I, 280-fold for Peak II, and 770-fold for Peak IV. These purified preparations were used for further studies.

Properties of enzymes

pH optimum. The activities of AMP deaminases, Peaks I, II and IV, were compared over the pH range of 4.5–9.3 in the presence of 50 mM NaCl and 10 mM AMP (Fig. 4). The pH activity curves for Peaks I, II and IV were apparently different. The optimum activity was observed at pH 6–7 in Peak I, which fell off gradually to

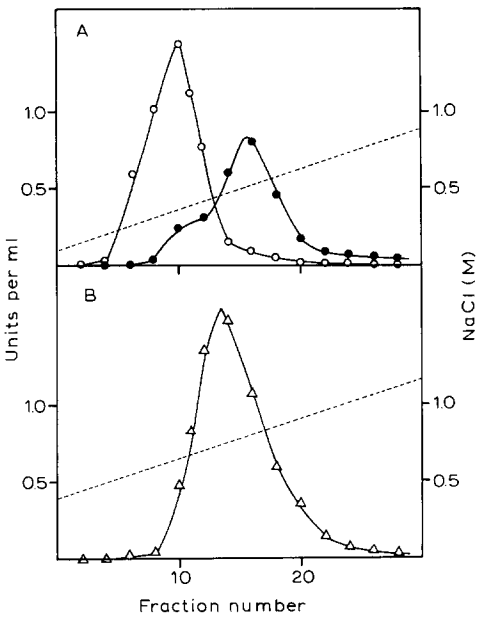

Fig. 3. Further resolution of AMP deaminase Peaks I, II and IV on phosphocellulose column. Peak I, II and IV fractions, initially resolved by second phosphocellulose chromatography were further purified using a small phosphocellulose column (0.9 cm × 10 cm). Each fraction contained 10 ml. The AMP deaminase activity was measured by Assay 1. A, deaminase Peak I (○) and Peak II (●); B, deaminase Peak IV.

TABLE I

PURIFICATION OF RAT BRAIN AMP DEAMINASE

Deaminase Peaks I, II and IV were purified as described in the text. Activities were determined by Assay 1.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification
Crude extract	652.4	19 929	0.033	1
1st phosphocellulose	474.4	486	0.976	29.6
DEAE-cellulose	421.1	159	2.648	80.2
2nd phosphocellulose				
I	110.6	37.2	2.973	90.1
II	71.0	13.0	5.462	165.5
IV	99.4	4.96	20.040	607.3
3rd phosphocellulose				
I	81.5	19.6	4.158	126.0
II	39.6	4.3	9.209	279.1
IV	49.8	1.97	25.279	766.0

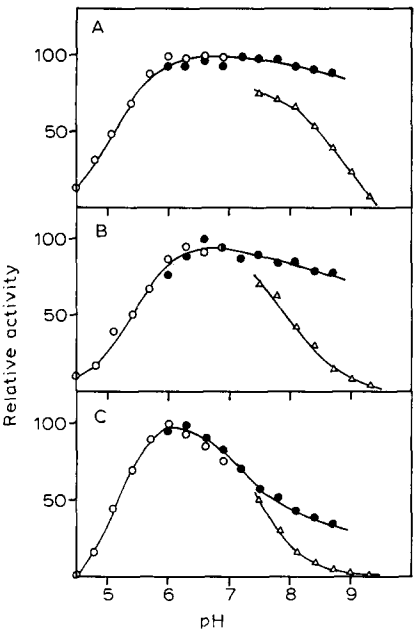

Fig. 4. Effect of pH on AMP deaminase activity. Assay 1 was used throughout. The following buffers were used, at a concentration of 20 mM; citrate–phosphate (○), potassium phosphate (●), and borate (△). A, Peak I; B, Peak II; C, Peak IV.

50% of the maximum at pH 5 and pH 8.5. Furthermore, activities measured in phosphate buffer were clearly different from those in borate buffer. Reasons for the difference were not investigated. Unlike Peak I, the optimum activity for Peak IV was at pH 6, and fell off sharply to 50% of the maximum at pH 5.2 and 7.5. The activities of Peak IV in phosphate and borate buffers were not too different from each other. Peak II showed an intermediate profile, showing the optimum at pH 6.5 and 50% of the maximum at pH 5.4 and pH 8.

Heat stability. Differences in the rate of inactivation of the deaminases at elevated temperatures were discernible. As shown in Fig. 5, Peak I was rapidly inactivated when heated at 50 °C. Under the same conditions, Peak IV was inactivated more slowly than Peak I. Also, Peak II was more heat stable than Peak I and more heat sensitive than Peak IV. The experiments were carried out also at 45 °C, where the same relationship was observed.

Substrate specificity. Zielke and Suelter [10] have previously drawn a conclusion, from their studies of the deaminase from rabbit muscle, that the same molecular species catalyzes deamination for both AMP and dAMP. The velocities obtained with dAMP as substrate catalyzed by our deaminase preparations, i.e. partially purified Peak I, II and IV fractions, expressed as a fraction of the rate obtained with AMP as substrate, are presented in Table II. Apparently, the dAMP:AMP ratios from three deaminase preparations are different from one another.

Molecular size. The molecular sizes of three types of AMP deaminase were determined at 4 °C according to the method of Andrews [32] by gel filtration on Sepharose 6 B using blue dextran, bovine serum albumin, aldolase, catalase, and

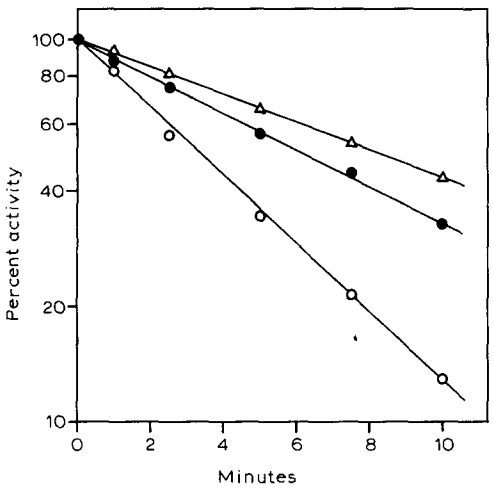

Fig. 5. Time course of heat inactivation of rat brain AMP deaminases. Peaks I, II and IV were heated at 50 °C in 0.1 ml of a 0.02 M potassium phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol and 0.05% bovine serum albumin. After the time intervals indicated, samples were removed, chilled, and assayed for deaminase activity by Assay 1. Peak I (○), Peak II (●), Peak IV (△).

ferritin as standards. One 0.5 ml aliquot of each type of the deaminase fraction was layered on a calibrated Sepharose column (60 cm × 0.9 cm) equilibrated with 0.2 M potassium phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol, and the same solution was used to collect 30 drops per fraction. In these experiments, the enzyme activity was eluted as one distinct peak in all three types of AMP deaminase and at the same point consistent with its having a molecular weight of 300 000 assuming it be spherical and of normal density. These results suggest that Peaks I, II and IV are of similar size but have significant characteristic differences.

TABLE II

SUBSTRATE SPECIFICITY OF RAT BRAIN AMP DEAMINASES

Enzyme activities were determined by Assay 1 as described in the text except that substrate concentrations were 100 mM in the reaction mixture. The value given is the ratio of activity observed with dAMP to that observed with AMP.

Peaks	dAMP/AMP
I	0.15
II	0.11
IV	0.02

Substrate affinity. Studies of AMP deaminase from various tissues have shown that an allosteric interaction is involved. Both in the absence and in the presence of activators, Peak I, II and IV AMP deaminases had very different kinetic parameters. When velocities were determined in the absence of activator with varying levels of AMP, a sigmoid response curve typical of an allosteric enzyme was observed for all types of AMP deaminase (Figs 6–8). Hill plots of such data gave straight lines from

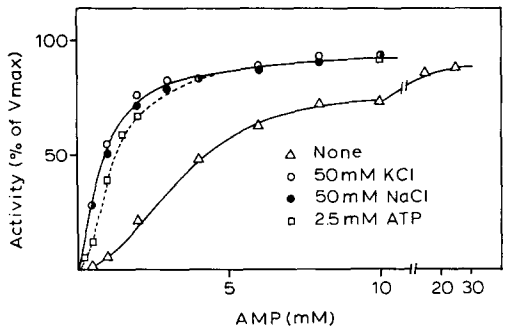

Fig. 6. Effect of AMP concentration on velocity of AMP deaminase Peak I from rat brain. The incubation mixtures contained the various concentrations of Tris–AMP and either 50 mM KCl (○), 50 mM NaCl (●), 2.5 mM Tris–ATP (□) or no effector (△). All other assay conditions were as described in Assay 2.

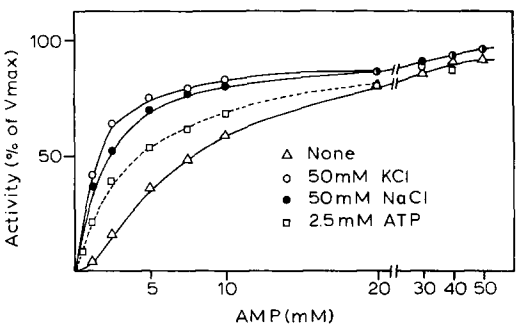

Fig. 7. Effect of AMP concentration on velocity of deaminase Peak II from rat brain. The incubation mixtures contained the various concentrations of Tris–AMP and either 50 mM KCl (○), 50 mM NaCl (●), 2.5 mM Tris–ATP (□) or no effector (△). All other assay conditions were as described in Assay 2.

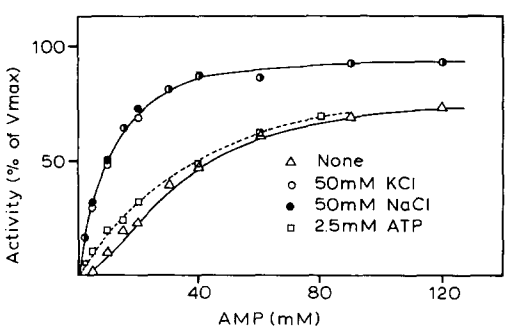

Fig. 8. Effect of AMP concentration on velocity of deaminase Peak IV from rat brain. The incubation mixtures contained the various concentrations of Tris–AMP and either 50 mM KCl (○), 50 mM NaCl (●), 2.5 mM Tris–ATP (□) or no effector (△). All other assay conditions were as described in Assay 2.

which one could calculate the concentration of substrate required to give half-maximum velocity, and estimate the number of interaction sites for AMP and the strength of interaction among them. In these experiments, the apparent K_m values of 4.4, 6.9 and 43.8 mM were obtained for Peaks I, II and IV, respectively. In the presence of activators such as ATP, NaCl, or KCl, the velocity vs AMP concentration curves were nearly hyperbolic, and the apparent K_m values for AMP were lowered; however, the maximum velocities were not changed in all types of deaminase (Figs 6–8) indicating that all of them belong to the K system according to Monod et al. [33]. Peak I was strongly activated by ATP (Fig. 6), whereas the activation was not so apparent in Peak IV, which was activated only at low concentration of AMP (Fig. 8). In the presence of 2.5 mM ATP, the K_m values for AMP were calculated to be 1.25, 5.5 and 44 mM for Peaks I, II and IV, respectively. In the presence of NaCl and KCl all three types of deaminase were strongly activated (Figs 6–8). The K_m values for AMP of 1, 2.5 and 10 mM in the presence of 50 mM NaCl and those of 1, 2 and 10 mM in the presence of 50 mM KCl were obtained for Peaks I, II and IV, respectively. Thus, Peak I enzyme has apparently a higher affinity for AMP than other types of deaminase both in the absence and presence of activators, while Peak IV has the lowest affinity of all.

DISCUSSION

The data presented in this report provide evidence for the existence of multiple forms of AMP deaminase in rat brain. These enzymes are clearly separable by employing conventional enzyme fractionation technology. Purification and characterization of Peak III were not carried out in the present experiment because of its lower activity and poor resolution from Peak IV, however, its existence is unquestionable [28]. Work is now in progress on the complete resolution of Peak III from other peaks.

Several batches of frozen rat brain were analyzed for the presence of the multiple forms of the enzyme using extraction and chromatography procedures described in the legend to Fig. 1A. All the batches of frozen brains yielded a very similar chromatographic pattern. To exclude the possibility that the multiple forms of AMP deaminase extracted from frozen brains were a result of changes occurring during the freezing of the brain, fresh brains were extracted and the extracts analyzed. In these experiments, a similar activity pattern of AMP deaminase was obtained. It may, therefore, be concluded that the multiple forms observed in extracts from frozen brains are not formed as a result of the freezing process, since they are also present in fresh brains.

The purified fractions did not contain either adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) or 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5), indicating that phosphohydrolysis of AMP, followed by deamination of adenosine, could be ruled out. Indeed, the product formed during or at the end of catalysis was only IMP. Neither adenosine nor inosine was detectable by means of cellulose thin layer chromatography.

When purified AMP deaminases were individually applied to a phosphocellulose column, each was chromatographed as a single peak at the same position as that for the original peak, and furthermore, the mixtures of them yielded only the

peaks corresponding to the parental peaks. These results indicate that hybrid forms are not readily formed under the condition such as overnight dialysis against Buffer A, and that none of the multiple forms is a hybrid. Our hybridization condition, however, is mild, and therefore the possibility cannot be ruled out that some of forms (e.g., Peak II) are indeed a hybrid of others.

The molecular weight of three types of AMP deaminase was determined by Sepharose 6B gel filtration; the same value of M_r 300 000 was obtained for all three forms. These results suggested that Peaks I, II and IV are of a similar size, but have significant characteristic differences as evidenced by their elution profiles from phosphocellulose column, differences in optimum pH, and kinetic properties. It is of interest to note that these molecular weights are approximately the same as that for muscle AMP deaminase, 270 000 [34]. The latter enzyme contains four apparently identical subunits [9, 14] suggesting that the brain enzymes may also consist of four subunits. Resolution of such questions must wait for the preparation of a substantially homogeneous form of the AMP deaminase. Such preparation methods are being developed. If multiple forms of AMP deaminase are homogeneously prepared, comparative biochemical studies of subunit structure and regulatory mechanism will become possible.

The regulatory properties of these deaminases are remarkably similar. The velocity vs AMP concentration curves are sigmoidal in the absence of activators such as ATP, NaCl, or KCl and the curves become nearly hyperbolic in the presence of activator. The maximum velocities were the same both in the absence and presence of activator, while the enzymes showed a higher affinity for AMP in its presence.

The regulatory properties are similar in all three types of deaminase, but discernible differences do exist in these enzymes. They vary from one another in their optimum pH, heat stability, substrate specificity, and apparent K_m values for AMP in the absence and presence of activator.

The physiological role and the nature of regulation of AMP deaminase in vivo have not been elucidated, although possible functions for the enzyme in brain [23–25], muscle [35, 36], and erythrocytes [17] have been speculated. A recent report of Chapman and Atkinson [37] provides very interesting observations in considering the role of AMP deaminase in vivo. According to them, the rate of deamination of AMP catalyzed by liver AMP deaminase increases with decreasing energy charge in the physiological condition, and the activity of enzyme decreases sharply as the adenine nucleotide pool decreases in and below the physiological range. From these results, they suggested that the deaminase functions to protect against sharp decreases in energy charge and to prevent excessive depletion of the adenine nucleotide pool in vivo. Reasons for the existence of multiple forms of deaminase in brain and for structural as well as functional differences of the enzymes remain to be answered.

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