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ISOZYMES OF RAT AMP DEAMINASE

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Summary

Three AMP deaminase isozymes (EC 3.5.4.6 AMP aminohydrolase) were purified from rat heart, kidney and muscle. These enzyme preparations contained only the required isozyme. Antisera to individual isozymes were prepared and immunological relationships were tested. There was no cross-reactivity as tested by precipitation experiments. The antisera precipitated only the corresponding isozyme and there was no effect on other isozymes. These isozymes were also different in K_m values for AMP and in substrate specificity. From the present studies, combined with previous results, it seems clear that the heart, kidney and muscle enzymes are different basic types. It is proposed that the muscle enzyme be designated as AMP deaminase A; the enzyme in kidney and liver, AMP deaminase B; the enzyme in heart, AMP deaminase C. Brain extracts contained five isozymes; two parent isozymes (B and C) and presumably their three hybrids.

Introduction

The importance of AMP deaminase (AMP aminohydrolase EC 3.5.4.6) in the purine nucleotide cycle [1—3], interconversion of adenine, inosine and guanine nucleotides [4—7], and stabilization of energy charge [8] in different rat tissues makes this enzyme an interesting subject from the regulatory point of view.

Previously, we reported that in rat brain at least four types of AMP deaminase are present and that they can be distinguished from one another by ion-exchange chromatography and on the basis of kinetic properties [9,10]. We extended these studies to compare the chromatographic behavior of AMP deaminase from various rat tissues and the results indicated that there are multiple isozymes of AMP deaminase, with different patterns in almost every tissue studied [11].

In this paper, further studies on the interrelationships between the immunological properties of the heart, kidney and skeletal muscle isozymes are

reported. Combined with previous report [11], chromatographic, kinetic, immunological and other data strongly support the concept that there are three main types of AMP deaminase in adult rat tissues. In brain, there exist five isozymes; two parent enzymes (heart and kidney types) and presumably their three hybrids.

Materials and Methods

Materials

Adenosine monosulfate and adenosine phosphoramidate were obtained from Sigma. Other nucleotides were purchased from Boehringer. DEAE- and phosphocellulose were obtained from Brown Co. Other reagents were commercial preparations of the highest purity available.

Enzyme assay

Enzyme activity was measured colorimetrically by estimating production of ammonia. A typical reaction mixture contained 30 mM AMP, 20 mM potassium phosphate (pH 7.0), 150 mM NaCl, 0.02% 2-mercaptoethanol, 0.05% bovine serum albumin in a final volume of 0.25 ml. The amount of ammonia was determined by the phenol-hypochlorite reagents [12]. For the kinetic experiments, the reaction mixture of 0.25 ml contained 20 mM Tris/HCl (pH 7.0), 0.05% bovine serum albumin, various concentrations of AMP and effectors, and enzyme free from 2-mercaptoethanol. The amount of ammonia was estimated by Nessler's reagent. The reaction was usually carried out at 37°C for 10 min. Units of enzymatic activity are international units. Specific activity is defined as units per mg protein. Protein was determined by the method of Lowry et al. [13] using bovine serum albumin as the standard.

Enzyme preparation

In experiments in which individual isozyme from heart, kidney and skeletal muscle were used, they were prepared from 20 000 × *g* supernatant using phosphocellulose and DEAE-cellulose chromatography. In early studies [11], it was found that chromatography of crude extracts from these tissues gave only one peak of AMP deaminase activity unique for each tissue. Thus, these tissues seem to be the best material from which to prepare each isozyme because of the absence of other isozyme contamination.

For the preparation of the heart enzyme, the tissue was homogenized with 5 volumes of 0.05 M NaCl in 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 adsorbed onto phosphocellulose. A column (approximately 5 × 20 cm for 70 g tissue) was washed with the same buffer to remove unbound protein. AMP deaminase was eluted with 0.6 M NaCl in Buffer A. Fractions with the enzyme activity were pooled and dialyzed against 5 mM potassium phosphate (pH 7.4) containing 0.1% 2-mercaptoethanol. After centrifugation to remove the precipitate, the enzyme solution was applied on to a DEAE-cellulose column (0.9 × 10 cm) and a linear gradient of 5 to 200 mM potassium phosphate (pH 7.4) was then applied. Fractions containing the enzyme activity were pooled and adjusted pH to 7.0 by the addition of 0.1 N HCl. The enzyme solution was then adsorbed onto a phosphocellulose column (0.9 × 10 cm) and a linear salt gradient of 0.05 to 0.6 M NaCl in

Buffer A (200 ml each) was applied to the column. Fractions comprising peaks were pooled and concentrated by ultrafiltration. The specific activity of the heart enzyme at this point ranged between 40—50 units per mg protein, a purification of approximately 600-fold from the 20 000 × *g* supernatant. Of the activity present in the original supernatant, 20% was recovered in the final fraction.

Deaminase from adult rat kidney was purified approximately 400-fold from the 20 000 × *g* supernatant; the procedure involved adsorption of the enzyme on phosphocellulose, removal of unbound protein by washing with 0.3 M NaCl in Buffer A and elution with a linear gradient of 0.3 to 1.2 M NaCl in Buffer A. The fractions containing enzyme activity were pooled, concentrated by ultrafiltration and stored at 0 °C. The final specific activity was 20—25 units per mg protein and about 50 per cent of the original supernatant activity was recovered.

Muscle deaminase was prepared from leg muscle with the similar procedure used for the purification of the enzyme from kidney. The enzyme was purified about 400-fold from the supernatant with a recovery of 40 per cent. The specific activity of the muscle enzyme ranged between 800 and 850 units per mg protein.

Preparation of antisera

For the preparation of the antisera to AMP deaminase, the following procedure was employed. The purified enzyme preparations were concentrated by ultrafiltration and dialyzed overnight against 0.02 M potassium phosphate (pH 7.0) containing 0.15 M NaCl and 0.1% 2-mercaptoethanol. After dialysis, the enzyme solution was mixed with an equal volume of Freund's complete adjuvant. 1.0 ml of this suspension was injected into the hind foot pads of a series of adult rabbits. Similar injections were repeated 1 week later. Two weeks after the first injection the same amount of the enzyme solution was injected intravenously in the marginal ear vein. Total amounts of the heart, kidney and muscle enzymes were 34, 27 and 436 units, respectively. Ten days following the last injection, blood was collected from the marginal ear vein, allowed to clot, and centrifuged to obtain the clear serum. Control sera were prepared by collecting blood prior to immunization and removing the clot. The antisera preparations specifically precipitated the enzyme to which they were directed and did not cross-react with other isozymes. One ml of antisera against the heart, kidney and muscle enzymes precipitated 38.6, 4.6 and 1212 units of the corresponding enzymes, respectively.

Precipitation of enzyme activity with antisera

Purified enzyme was diluted with 0.02 M potassium phosphate (pH 7.0) containing 0.15 M NaCl, 0.1% 2-mercaptoethanol and 0.05% bovine serum albumin to a final concentration of approximately 1 unit per ml. The diluted enzyme in a volume of 0.1 ml was mixed with 0.3 ml of antiserum that had been diluted to the appropriate concentration with the buffer described above. The mixture of enzyme and serum was incubated for 18 h at 4 °C. The samples were then centrifuged for 15 min at 20 000 × *g*, and the activity remaining in the supernatant was assayed and compared with non-immunized serum.

Results and Discussion

To test whether the purified heart, kidney and muscle AMP deaminases are chromatographically distinct and further to test whether the enzyme preparations contain only the required isozyme, the purified enzymes were individually subjected to chromatography on phosphocellulose. The enzyme was eluted with a linear gradient from 0.05 to 1.2 M NaCl and the elution profile is shown in Fig. 1. This figure represents a composite of three different chromatographic separations, partially purified heart, kidney and muscle enzymes. In each case, an identical column of phosphocellulose (0.9 × 6 cm) was employed. The heart enzyme was bound less tightly to the cation exchange cellulose and it started to elute at NaCl concentration of 0.2 M. The muscle enzyme started to elute at 0.5 M NaCl concentration. The kidney enzyme was bound most tightly and it started to elute at 0.7 M NaCl. The figure also demonstrates the presence of only the corresponding AMP deaminase isozyme in the purified preparation. Other analytical techniques such as electric focusing, polyacrylamide disc electrophoresis or cellulose acetate electrophoresis, were also employed to identify these three isozymes. These attempts were, however, unsuccessful for the enzymes irreversibly lost catalytic activity during electrophoresis.

The antisera used in these experiments precipitated the corresponding AMP deaminase (Fig. 2), but the direct inhibitory effect was rather weak. Overnight incubation of antisera against muscle AMP deaminase (anti-M) with purified muscle enzyme resulted in about 70% inhibition of the activity, whereas the antisera against heart enzyme (anti-H) and kidney enzyme (anti-K) did not inhibit the corresponding enzyme at all. A much clearer demonstration of the formation of the antigen-antibody complex was obtained by centrifugation of antigen-antibody mixture.

Fig. 2A presents the precipitation curves obtained by mixing a constant amount of the heart, kidney or muscle enzyme with a serial dilution of anti-H.

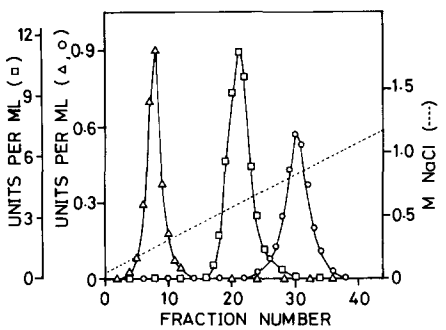

Fig. 1. Phosphocellulose chromatography of purified heart, kidney and muscle AMP deaminases. Figure is a composite of three separations with a column (0.9 × 6 cm) equilibrated with 0.05 M NaCl in Buffer A which consisted of 0.02 M potassium phosphate (pH 7.0) and 0.1% 2-mercaptoethanol. The column was eluted with a linear gradient obtained by employing 200 ml of 0.05 M NaCl in Buffer A in mixing chamber connected to a reservoir containing 200 ml of 1.2 M NaCl in Buffer A. Fractions, about 10 ml/40 min per tube, were collected and assayed for enzyme activity. △, heart enzyme, 32 units applied, 29 units recovered; ○, kidney enzyme, 38 units applied, 33 units recovered; □, muscle enzyme, 704 units applied, 611 units recovered.

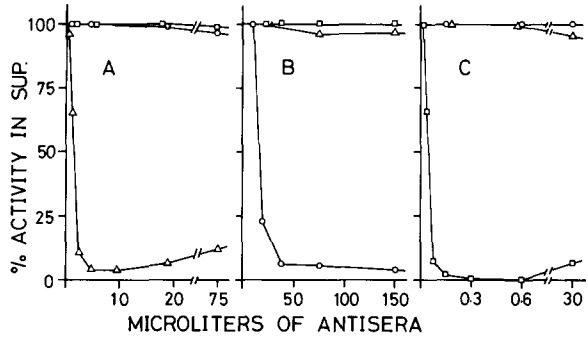

Fig. 2. Immunological distinction of the heart, kidney and skeletal muscle AMP deaminases. Precipitations were carried out as described under Materials and methods with the indicated amount of antisera against heart AMP deaminase (A), against kidney enzyme (B) and against muscle enzyme (C). The enzyme activity remaining in the supernatant was measured. △, heart enzyme; ○, kidney enzyme; □, muscle enzyme.

The antisera precipitated over 95% of heart AMP deaminase activity, while there was no significant effect on AMP deaminase activity of kidney and muscle. Anti-K precipitated the kidney enzyme, but there was no effect of the antisera on activity of the heart or skeletal muscle enzyme (Fig. 2B). Similarly, anti-M removed the muscle enzyme completely, but demonstrated no pronounced effect on the heart and kidney enzymes (Fig. 2C).

Fig. 3 shows plots of initial velocity of the heart, kidney and muscle AMP deaminases against AMP concentration. Both in the absence and presence of 50 mM NaCl, three isozymes had very different kinetic parameters. When velocities were determined in the absence of activator, a sigmoid response curve typical of an allosteric enzyme was observed, and the apparent K_m values of 6, 37 and 24 mM were obtained for the heart, kidney and muscle enzymes, respectively. In the presence of 50 mM NaCl, all three types of deaminase were strongly activated and the velocity vs AMP concentration curves were nearly hyperbolic. The K_m values of 1, 6 and 3 mM were obtained for the heart, kidney and

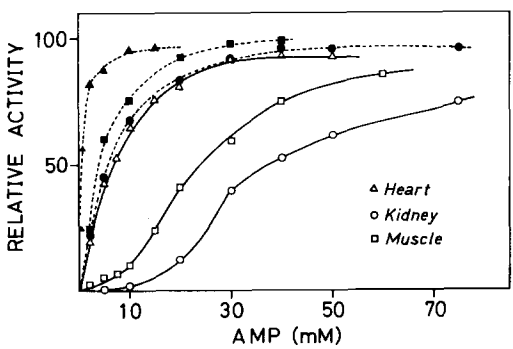

Fig. 3. Effect of AMP concentration on velocity of the heart, kidney and skeletal muscle enzymes. The incubation mixtures contained 20 mM Tris/HCl buffer (pH 7.0), 0.05% bovine serum albumin and various concentrations of Tris/AMP. The reaction was carried out in the absence (open symbols) or presence (closed symbols) of 50 mM NaCl. Reaction rates are expressed as percentages of the maximum velocity.

muscle enzymes, respectively, in the presence of 50 mM NaCl. Thus, the heart enzyme has apparently a higher affinity for AMP than other types of deaminase, while the kidney enzyme has the lowest affinity of all.

Zielke and Suelter [14] have shown that AMP deaminase from rabbit muscle deaminates AMP, dAMP, adenosine monosulfate and adenosine phosphoramidate. Therefore, it was thought that the comparison of the relative rates of deamination of these four substrates might allow further distinction between the enzymes from heart, kidney and muscle. As shown in Table I, the heart deaminase catalyzes the deamination of dAMP, adenosine monosulfate and adenosine phosphoramidate several times more efficiently than the enzyme from kidney.

From the present studies, along with previous results [11], it is obvious that the heart, kidney and muscle enzymes are distinct from one another, since they are different in elution profile from the phosphocellulose column, different in optimum pH, different in K_m value for AMP, different in substrate specificity, and are different in immunological properties.

Although heart, kidney and muscle extracts reveal the presence of a single chromatographic species that accounts for at least 90% of the total AMP deaminase activity [11], previous studies have shown that brain extracts have activities associated with several chromatographic species [9,10]. In a preliminary immunological experiment, it was observed that AMP deaminase activity of brain extracts was partially precipitated by antisera to the heart enzyme and also by those to the kidney enzyme, but not by antisera against the muscle enzyme. These results indicate the possibility that the multiple forms of AMP deaminase in rat brain extracts are composed of subunits of the heart and kidney enzymes, and also suggest the existence of five isozymes, if we assume the tetrameric structures for brain AMP deaminases as shown in the enzymes from rabbit and chicken muscle [15]. Under the conditions of chromatography employed in the present study, five peaks of AMP deaminase activity were clearly observed in brain extracts; the first and last peaks with elution positions corresponding to those of heart enzyme and kidney enzyme, and the intermediate three peaks (Fig. 4). As indicated in the figure, the peaks are tentatively designated as Peaks I, II, III, IV and V in the order of elution from the column.

TABLE I

SUBSTRATE SPECIFICITY OF THE HEART, KIDNEY AND MUSCLE ENZYMES

Assays were carried out in the presence of 30 mM substrate, 2 mM ATP and 4 mM $MgCl_2$ [8]. The value given is the ratio of activity observed with dAMP, adenosine monosulfate or adenosine phosphoramidate to that observed with AMP.

Ratio	Enzyme source		
	Heart	Kidney	Muscle
dAMP/AMP	0.175	0.033	0.077
Adenosine monosulfate/AMP	0.270	0.037	0.156
Adenosine phosphoramidate/AMP	0.235	0.091	0.117

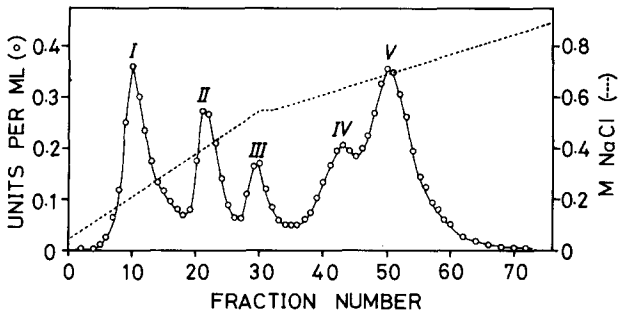

Fig. 4. Phosphocellulose column chromatography of AMP deaminase from rat brain extracts. A frozen preparation (13 g) of rat brain was homogenized with 5 volumes of cold 0.05 M NaCl in Buffer A. After centrifugation at 20 000 × *g* for 20 min, the supernatant was placed on a phosphocellulose column (0.9 × 10 cm) which was previously equilibrated with 0.05 M NaCl in Buffer A. After washing with 30 ml of the same buffer, a linear gradient, 0.05—0.55 M NaCl in Buffer A, 100 ml for each of the two vessels, was then applied. At the end of first gradient, the column was washed with 20 ml of 0.55 M NaCl in Buffer A. Then, a second gradient, 0.55—0.9 M NaCl in Buffer A, 150 ml per vessel, was applied. Fractions, about 7 ml/30 min per tube, were collected and assayed for enzyme activity.

Most of the accumulated evidence for AMP deaminase in muscle is consistent with a four-subunit molecule [15,16]. Boosman et al. [15] have shown by gel filtration chromatography in guanidine hydrochloride and electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels that AMP deaminases of rabbit and chicken muscle are composed of four nearly identical subunits. The existence of five isozymes in rat brain might be explained on the basis of a hybridization between two parent enzymes (heart and kidney types), each with four subunits. It seems clear that the heart, kidney and muscle enzymes are different basic types from one another and they may represent parent enzymes. In parallel with the alldolase [17] and phosphofructokinase [18] nomenclature systems, the muscle enzyme is designated as AMP deaminase A; the kidney enzyme, which is also the species found exclusively in liver, AMP deaminase B. The other species present in the heart is designated as AMP deaminase C. If we assume that there exist three types of monomer and assuming that all forms are tetramer, it is possible to have 15 isozymes of AMP deaminase; 3 homopolymers, 9 isozymes containing two types of monomer, and 3 isozymes containing three different kinds of monomer. However, the immunological and chromatographic studies indicate that six isozymes are present in rat tissues; three parent isozymes (A, B, C) and three B-C hybrids. The apparent absence of the muscle type in brain, lung and spleen, where the five isozymes occur, would suggest that AMP deaminase A monomer is synthesized only in the muscle tissue and not taking part in these hybridizations.

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