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AMP deaminase from anterior lobe of bovine pituitary: purification and properties

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AMP deaminase (EC 3.5.4.6) from anterior lobe of bovine pituitary has been purified for the first time. Six molecular forms of the enzyme were eluted from phosphocellulose P11 with a KCl concentration gradient. By two stage gel chromatography individual molecular forms were purified to electrophoretic homogeneity. Comparison of some physico-chemical and kinetic properties of the preparations obtained showed high similarity of their properties to those of AMP deaminase from other animal tissues already described. All the isoforms were found to be Zn^{2+}-dependent.

AMP deaminases have been shown to exist in many human and animal tissues. They deaminate AMP, producing IMP and ammonia. Some of them, isolated from brain, liver, spleen and lungs, occur in several molecular forms [1, 2]. The results of Ogasawara *et al.* [3, 4], as well as ours [5], demonstrated the existence of 5 isozymes in rat brain. However, some other authors isolated the enzyme from bovine brain as single isoform [6, 7]. In our previous paper [8] isolation of six isoforms of AMP deaminase from bovine pituitary and from brain gray matter has been described. In the present paper some properties of AMP deaminase isoforms from the bovine pituitary anterior lobe (PAL[1]) are presented. The enzyme was inactivated by EDTA treatment and its activity was restored in the presence of several divalent metal ions. Zn^{2+} being most effective for all isozymes investigated.

MATERIALS AND METHODS

Reagents and apparatus. Phosphocellulose P11 was obtained from Whatman (England), 2-mercaptoethanol, bovine serum albumin, ADP and ATP were from Sigma (U.S.A.), AMP and GTP — from Serva (Germany); reagents for electrophoresis — from Reanal (Hungary), Sephadex G-200 and Sepharose 6B — from Pharmacia (Sweden). Catalase from bovine heart, adrenodoxine from adrenal cortex, and AMP deaminase from rat skeletal muscle were obtained in homogeneous form in our laboratory. All other reagents were of the highest purity available. Spectrophotometric and fluorescence measurements were performed on Specord M-40 (Germany) spectrophotometer and on Perkin-Elmer spectrofluorimeter MPF-44A

[1]Abbreviations: PAL, pituitary anterior lobe; P11, phosphocellulose P11.

(U.S.A.), respectively, in 10 mm rectangular cells, at ambient temperature.

Enzyme isolation and assay. AMP deaminase from PAL was isolated by two step affinity chromatography on phosphocellulose P11 as described previously [8]. The procedure consisted of homogenization in 20 mM phosphate buffer, pH 7.0, containing 50 mM KCl and 1 mM dithiothreitol, centrifugation, adsorption on phosphocellulose P11 and elution with a KCl 0.3–2.0 M concentration gradient. Then rechromatography on P11 was performed. In the experiments presented here, the enzyme was further purified by gel filtration on Sephadex G-200 and Sepharose 6B.

AMP deaminase activity assay mixture in the final volume of 0.8 ml contained: 40 mM imidazole buffer, pH 7.0, 100 µl of enzyme solution (usually 50 µg of protein) and, where indicated, either 2 mM ADP or 2 mM ATP. After preincubation for 5 min at 37°C the reaction was started by the addition of AMP to the final concentration 4 mM (or as indicated) and the mixture was incubated for 10 min at 37°C. The reaction was terminated by the addition of 0.5 ml ethanol. The ammonia liberated was determined by the method of Sugawara & Oyama [9] with some modifications described previously [8].

Preparation of apoenzyme. This procedure was performed in two ways: either by incubating the enzyme in 10 mM phosphate buffer, pH 7.0, containing 0.6 M KCl and 1 mM EDTA, or by dialysing the enzyme solution against 100 volumes of the same mixture containing EDTA. Both the incubation and the dialysis were prolonged for 23 h, then EDTA was removed by dialysing the samples against 100 volumes of the same mixture but containing no EDTA. As control, the enzyme preparations subjected to the identical treatment, but in the buffer solution without EDTA, were used.

Analytical procedures. Protein was estimated by the method of Bradford [10] with bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide was performed as described by Weber & Osborn [11]. Prior to electrophoresis protein samples were dialysed against 10 mM sodium phosphate buffer, pH 7.0, and heated for 5 min in a boiling water bath in the presence of 5.0 M urea, 1% SDS and 1% 2-mercaptoethanol. To investigate the ability of metal ions to restore the activity of apoenzyme the sulphate salts of zinc, copper, magnesium, manganese and iron were used. The assay mixtures containing respective metal ions were preincubated for 10 min at 37°C, then the reaction was initiated by the addition of substrate.

RESULTS AND DISCUSSION

The application of the KCl concentration gradient from 0.3 to 2.0 M in order to elute proteins of bovine pituitary adsorbed on P11, resulted in six maxima of AMP deaminase activity coinciding with protein peaks (Fig. 1). The six molecular forms obtained differed in their specific activities, and their half-saturation ($S_{0.5}$) constants ranged from 3.0 mM to 12.0 mM [8]. The sequential gel filtration of isoforms through Sepharose 6B and Sephadex G-200 caused the removal of, respectively, high and low molecular mass contaminations. Identical elution profiles were obtained for all isoforms of the enzyme. AMP deaminase activity was eluted as a single peak in either gel filtration. Comparison of the elution volumes of the active fractions and of standard proteins such as catalase (M_r 22000), bovine serum albumin (M_r 67000), adrenodoxin (M_r 300000) and rat skeletal muscle AMP deaminase (M_r 300000), demonstrated that all the molecular forms of AMP deaminase from PAL have the same relative

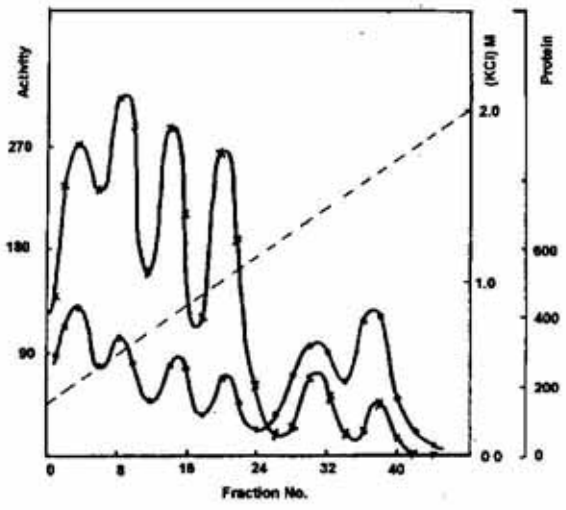

Fig. 1. Phosphocellulose column chromatography of bovine PAL AMP deaminase: ×, AMP deaminase activity (nmol/min × mg^{-1}); ●, protein (µg per ml of fraction); —, KCl gradient.

molecular mass of about 300000. The active fractions obtained after two stages of gel filtration were concentrated by adsorption on a small phosphocellulose column, followed by elution with 1.7 M KCl in 10 mM phosphate buffer, pH 7.0. Storage for 10 days at 4°C of the enzyme preparations obtained in this way caused a negligible loss of activity.

When all the six purified AMP deaminase fractions were mixed, adsorbed again on a phosphocellulose P11 column and eluted with the above mentioned KCl gradient, the same six peaks of AMP deaminase activity appeared, indicating that these fractions are in fact distinct molecular forms of the enzyme.

The SDS-PAGE with the above listed standard proteins showed that each of the six molecular forms obtained from the second P11 column, exhibited the main protein band corresponding to relative molecular mass of 75000. Comparing this with the gel filtration results we may suppose that every native isoform of AMP deaminase from bovine PAL has a tetrameric structure with relative molecular mass of monomers equal to 75000.

Several catalytic properties of the isozymes obtained were studied. In Fig. 2 the substrate saturation plots for the isoform 2 are presented. In the absence of any effector added the curve had sigmoidal shape (curve 1). In the presence of ADP or ATP the plot became more hyperbolic (curves 2 and 3, respectively). A simultaneous decrease of the $S_{0.5}$ value was observed. Similar results were obtained for all the isoforms. This means that both ATP and ADP are allosteric activators of all the AMP deaminase isoforms, ATP being more effective than ADP.

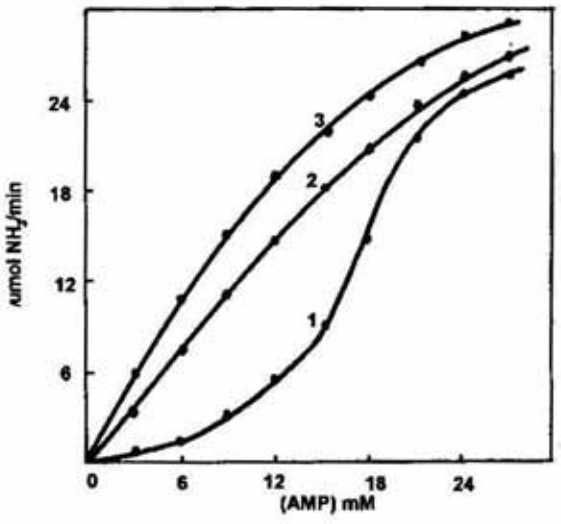

Fig. 2. Dependence of the enzymatic reaction rate of isoform 2 on AMP concentration.
The reactions were carried out in the absence (curve 1) and the presence of either ADP (curve 2) or ATP (curve 3) at concentrations of 2 mM.

The ATP apparent affinity constant determined by the double reciprocal plot (1/V *versus* 1/[ATP]) was equal to 0.125 mM at 4 mM AMP concentration (not shown). GTP was an effective inhibitor of the enzymatic reaction; 0.5 mM GTP inhibited the reaction catalyzed by the isoform 2 down to 50% of the initial velocity. The inhibitory constant determined for enzyme activated by ATP and calculated from Dixon plot [12] was equal to 0.03 mM (not shown). The obtained kinetic constants are in accordance with the values usually observed for AMP deaminase from bovine brain [6, 13].

It is known that monovalent cations are effective activators of AMP deaminases [14,15]. In our experiments the plots of the reaction rate *versus* KCl concentration were for all enzyme isoforms, bell-shaped with optimum concentration in the range of 0.1–0.2 KCl (not shown) in agreement with the data for other known AMP deaminases.

The ability of divalent metal ions to restore the activity of apoenzyme from which the metal ions had been removed by treatment with EDTA, was also studied. In Fig. 3 the effect of metal ions concentration on the activity of the apoenzyme isoform 2 is presented. The apoenzyme retained 30% of the control activity, i.e. of the activity taken as 100 of a sample which underwent all the treatments in the absence of EDTA and which was kept in the presence of 0.6 M KCl to prevent enzyme denaturation [16]. Although three of the ions used were able to restore the activity of the apoenzyme, only Zn^{2+} ions restored it up to 100% of the control value. Fe^{2+} and Mn^{2+} restored the activity up to 70% and 80%, respectively. In contrast, Mg^{2+} and Cu^{2+} did not restore the activity of apoenzyme at all. Similar results were obtained for all the isoforms of the bovine PAL AMP deaminase. We conclude, therefore, that AMP deaminase isoforms of bovine pituitary anterior lobe, like the enzymes from other human and animal tissues [16–20] are Zn^{2+}-dependent.

Since AMP deaminase from bovine pituitary has been isolated and investigated for the first

time, it is worth to compare its properties with those of the enzymes isolated from other tissues. The results obtained in our experiments show their high similarity in main physicochemical properties such as the absorption spectra, relative molecular mass, subunit composition of holoenzyme, complete restoration of the activity of apo-AMP deaminase by Zn^{2+} and partial by Mn^{2+} and Fe^{2+} ions. Kinetic and regulatory properties were also similar: ATP and ADP are effective activators but GTP is an inhibitor of enzymatic deamination of AMP; the plot of the rate of AMP deamination *versus* substrate concentration is sigmoidal and becomes hyperbolic in the presence of either ADP or ATP; a simultaneous decrease of $S_{0.5}$ and Hill's coefficient was observed (cf. [8]). Unlike in the case of AMP deaminases from some tissues, and like in those from brain [15], the maximum activating concentrations of KCl did not transform the sigmoidal substrate saturation plot of the pituitary enzyme to a hyperbolic one.

In the earlier papers of Rhoads [7], Ito *et al.* [21], and Setlow & Lowenstein [6] a single peak of AMP deaminase activity was reported to exist in calf and sheep brain. On the other hand, five molecular forms of AMP deaminase from rat brain were obtained by Ogasawara *et al.* [3, 4] and by Haroutunian & Lowenstein [5]. These isoforms were eluted from P11 in the KCl concentration range of 0.3–0.8 M. They differed in the relative amounts, $S_{0.5}$ values, kinetic and immunological properties. According to Ogasawara's classification based on chromatographic, immunological and kinetic properties, three parental forms of AMP deaminase (A, B, and C) occur in mammalian tissues. Ogasawara *et al.* [4] conclude that rat brain contains two parent enzymes, B and C, and their three hybrids.

In our experiments the application of a wider range of the KCl gradient and of a sensitive fluorimetric method for determination of the ammonia liberated, resulted in finding six forms of AMP deaminase in bovine PAL, eluted in the KCl concentration range of 0.5–1.7 M. The obtained isoforms differed in their relative amounts and the $S_{0.5}$ values. However, to find to what types of Ogasawara's classification they correspond, requires further studies.

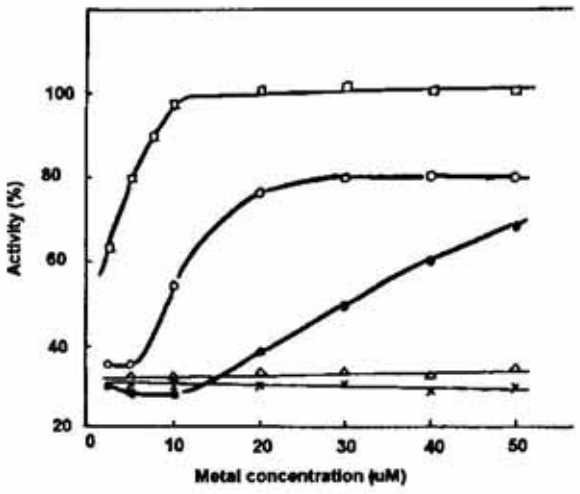

Fig. 3. Dependence of the reactivation of apo-AMP deaminase on the concentration of metal ions: Zn^{2+} (□), Mn^{2+} (○), Fe^{2+} (●), Mg^{2+} (×) and Cu^{2+} (Δ). The enzyme was inactivated by EDTA down to nearly 30% of control values. Concentration of the enzyme in assay mixture was 0.7 μM

REFERENCES

1. Ogasawara, N., Goto, H., Yamada, Y., Watanabe, T. & Asano, T. (1982) AMP deaminase isozymes in human tissues. *Biochim. Biophys. Acta* **714**, 298–306.
2. Ogasawara, N., Goto, H., Watanabe, T., Kawamura, Y. & Yoshino, M. (1974) Multiple forms of AMP deaminase in various rat tissues. *FEBS Lett.* **44**, 63–66.
3. Ogasawara, N., Goto, H. & Watanabe, T. (1975) Isozymes of rat brain AMP deaminase: developmental changes and characterizations of five forms. *FEBS Lett.* **58**, 245–248.
4. Ogasawara, N., Goto, H. & Watanabe, T. (1975) Isozymes of rat AMP deaminase. *Biochim. Biophys. Acta* **403**, 530–537.
5. Haroutunian, A. & Lowenstein, J. (1977) Multiple forms of rat brain AMP deaminase. *Doklady Akademii Nauk S.S.S.R.* **234**, 951–954.
6. Setlow, B. & Lowenstein, J. (1967) Adenylate deaminase. II. Purification and some regulatory properties of the enzyme from calf brain. *J. Biol. Chem.* **242**, 607–615.
7. Rhoads, D. (1969) *Studies on adenylate deaminase from calf brain.* Ph.D. Thesis, Brandeis University.
8. Sharoyan, S., Mardanian, S. & Haroutunian, A. (1992) Isolation of AMP deaminase molecular forms from the cattle nerve tissue. *Neurokhimiya* **11**, 158–165.

9. Sugawara, K. & Oyama, F. (1981) Fluorogenic reaction and specific microdetermination of ammonia. *J. Biochem. (Tokyo)* **89**, 771–774.
10. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
11. Weber, K. & Osborn, M. (1969) The reliability of molecular weigh determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406–4412.
12. Dixon, M. (1953) The determination of enzyme inhibitor constants. *Biochem. J.* **55**, 170–171.
13. Setlow, B. & Lowenstein, J. (1968) Adenylate deaminase. IV. Nucleotide specificity of the enzyme from calf brain with special reference to guanosine triphosphate. *J. Biol. Chem.* **243**, 3409–3415.
14. Setlow, B. & Lowenstein, J. (1968) Adenylate deaminase. V. Effect of alkali metal and magnesium ions on activity. *J. Biol. Chem.* **243**, 6216–6221.
15. Haroutunian, A. (1974) AMP-aminohydrolase of brain and other organs and the regulation of its activity. *Problems of Brain Biochemistry* (in Russian) **9**, 251–272.
16. Raggi, A., Ranieri, M., Taponeco, G., Ronca-Testoni, S., Ronca, G. & Rossi, C. (1970) Interaction of rat muscle AMP aminohydrolase with chelating agents and metal ions. *FEBS Lett.* **10**, 101–104.
17. Zielke, C. & Suelter, C. (1971) Rabbit muscle adenosine 5′-monophosphate aminohydrolase. Characterization as a zinc metalloenzyme. *J. Biol. Chem.* **246**, 2179–2186.
18. Makarewicz, W. (1974) Purification and properties of AMP-aminohydrolase from human placenta. *Acta Biochim. Polon.* **21**, 125–135.
19. Stankiewicz, A. & Makarewicz, W. (1975) AMP deaminase of human skeletal muscle. Divalent metal cations and catalytic activity. *Biochem. Medicine* **13**, 197–212.
20. Stankiewicz, A., Spychała, J., Składanowski, A. & Żydowo, M. (1979) Comparative studies on muscle AMP deaminase. I. Purification, molecular weight, subunit structure and metal content of the enzymes from rat, rabbit, hen, frog and pikeperch. *Comp. Biochem. Physiol.* **62B**, 363–369.
21. Ito, K., Yamamoto, H. & Mizugaki, M. (1988) Purification and general properties of AMP deaminase from sheep brain. *J. Biochem. (Tokyo)* **103**, 259–262.