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AMP DEAMINASE FROM RABBIT SKELETAL MUSCLE:

THE EFFECT OF MONOVALENT CATIONS ON CATALYTIC ACTIVITY AND MOLECULAR WEIGHT

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SUMMARY

1. The effects of monovalent cations on the kinetic properties and molecular weight of rabbit muscle AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) have been determined and the subunit structure of the enzyme has been examined.

2. The enzyme is activated by monovalent cations at non-saturating AMP concentrations. K^+ is the most effective, followed by Na^+, Li^+, NH_4^+ and Rb^+ in that order. No stimulation was observed with Cs^+ up to 0.5 M. The response to these cations is non-hyperbolic, and both the half-saturating concentrations and the maximal stimulation are different for different cations.

3. On the removal of activating cations, and at protein concentrations of less than 1 mg/ml the enzyme reversibly dissociates, giving a peak of activity in sucrose density gradient centrifugation corresponding to a molecular weight of approximately 80 000.

4. Sedimentation to equilibrium in an analytical ultracentrifuge in the absence of activating ions showed a marked increase in the apparent molecular weight with increasing protein concentration. The apparent molecular weight varied from $<100\,000$ to $>250\,000$ in the range 1.6–10 mg protein/ml.

5. After reduction and alkylation, AMP deaminase migrated in sodium dodecyl sulphate–polyacrylamide gels as a single component with molecular weight of $79\,000 \pm 2000$.

INTRODUCTION

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) from rabbit skeletal muscle

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is activated by Li^+, Na^+, K^+, NH_4^+ and Rb^+ (SMILEY *et al.*[1]) at non-saturating concentrations of AMP. K^+ decreases the AMP concentration for half maximal activity and changes the Hill-plot slope from 2.2 to 1.1, but does not change the maximal velocity (SMILEY AND SUELTER[2]).

During a study, in this laboratory, of nucleotide analogues as substrates and activators of AMP deaminase, it was observed that removal of K^+ resulted in a reduction in the apparent molecular weight of the enzyme as determined by gel filtration (M. R. ATKINSON, unpublished results). This suggested that activation by monovalent cations might be associated with a change in the state of aggregation of the enzyme.

This paper reports more detailed kinetic studies of the activation by monovalent cations, and centrifugation and electrophoresis studies indicating the existence of a monomer–dimer–tetramer system, with increasing subunit association at higher concentrations of K^+ and Na^+.

METHODS

AMP deaminase

The enzyme was prepared from rabbit skeletal muscle as described by SMILEY *et al.*[1] up to the elution from cellulose phosphate. At this stage the enzyme had a specific activity of 80–90 μmoles AMP deaminated/min per mg of protein when assayed at 30° with 50 μM AMP in 0.1 M succinate (K^+, pH 6.5) as described by SMILEY *et al.*[1]. The specific activity in this system with saturating levels of AMP was about 1.5 mmoles AMP deaminated/min per mg protein. No contaminants were detected on electrophoresis in sodium dodecyl sulphate–polyacrylamide gels as described by WEBER AND OSBORN[3], after reduction and alkylation. The native enzyme did not readily enter polyacrylamide gels under a variety of conditions.

AMP deaminase was freed of K^+ by passing 1-ml portions (concentrated by ultrafiltration to about 5 mg/ml) through a column (12 cm × 2 cm) of Sephadex G-50 in 5 mM 2-mercaptoethanol–0.55 M Tris–0.05 M succinate (Cl^-, pH 6.5); the K^+ concentration in the eluted enzyme, measured by flame photometry, was about 10^{-5} M.

Kinetic studies

Initial rates of determination of AMP at 30° were measured from the decrease in extinction at 265 nm ($\Delta\varepsilon^{1\,cm}_{1\,mM} = 8.86$; SMILEY *et al.*[1]) in a Zeiss PMQ II spectrophotometer in a system containing 50 μM AMP (Tris salt)–5 mM 2-mercaptoethanol–0.1 M succinate (Tris, pH 6.5). Except where indicated, reactions were started by adding the enzyme (1–10 μg/ml).

Sucrose density gradient centrifugation

The molecular weight of the native enzyme under various conditions was determined by centrifugation in 5–20% linear sucrose gradients (5 ml) as described by MARTIN AND AMES[4]. Gradients contained 0.55 M Tris–0.05 M succinate (Cl^-, pH 6.5) and 0.5 M KCl (or NaCl)–0.05 M succinate (Tris, pH 6.5) combined to give the required alkali metal ion concentration, and 5 mM 2-mercaptoethanol. AMP deaminase and marker enzymes were equilibrated with the appropriate buffer solutions by gel filtra-

tion (as above) or by extensive dialysis under N_2 at 4°. 0.22-ml fractions were collected into 0.5 ml of 0.5 M KCl–0.05 M succinate (Tris, pH 6.5). AMP deaminase and marker enzymes were detected by their catalytic activity. Marker enzymes used were pancreatic deoxyribonuclease (deoxyribonucleate oligonucleotidohydrolase, EC 3.1.4.5) (mol. wt. 31 000, ref. 5), yeast alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) (mol. wt. 151 000, ref. 6), pig heart lipoamide dehydrogenase (reduced-NAD:lipoamide oxidoreductase, EC 1.6.4.3) (mol. wt. 110 000, ref. 7) and rabbit muscle pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) (mol. wt. 237 000, ref. 8).

Measurement of subunit molecular weight

AMP deaminase was reduced in 10 mM 2-mercaptoethanol–6 M guanidinium chloride (or 8 M urea)–0.1 M phosphate (Na^+, pH 7.0) containing 1% sodium dodecyl sulphate at 60° for 5 h and was then alkylated by addition of sodium iodoacetate to 20 mM at pH 8–9 for 1 h at 25° in the dark. After dialysis against 0.1% sodium dodecyl sulphate–10 mM phosphate (Na^+, pH 7.0) the reduced and alkylated enzyme was subjected to electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulphate, as described by WEBER AND OSBORN[3]. Mobilities were compared with those of reference compounds prepared by reduction and alkylation of bovine pancreas carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.2.1), ovalbumin, rabbit muscle pyruvate kinase, bovine serum albumin and rabbit muscle phosphorylase *b*. Subunit molecular weights for these proteins were from WEBER AND OSBORN[3].

Enzymes

Phosphorylase *b* (FISCHER AND KREBS[9]), lipoamide dehydrogenase (MASSEY *et al.*[10]) and ovalbumin [11]were prepared in this laboratory. Bovine pancreatic deoxyribonuclease was obtained from Worthington. Other proteins were obtained from Sigma.

RESULTS

Kinetic activation of AMP deaminase by monovalent cations

The response of AMP deaminase activity, at 50 μM AMP, to varying concentrations of alkali metal and ammonium ions is shown in Fig. 1. K^+ is the most effective activator, followed by Na^+, Li^+, NH_4^+ and Rb^+ in decreasing order of effectiveness. Cs^+ gave no stimulation at 0.1 or 0.5 M. The maximum velocities obtained in the presence of K^+, Na^+, Li^+, NH_4^+ and Rb^+ were respectively 80, 78, 68, 65 and 55 μmoles IMP formed per min per mg. That the response to all of these ions is sigmoidal was particularly evident from the characteristic upward curvature at low activator concentrations observed in double reciprocal plots of the data in Fig. 1. Hill coefficients (n) were in the range 1.4–1.9 for all the activating ions. Sigmoidal response curves were obtained regardless of whether reactions were started with enzyme, or with AMP 30 sec after the addition of enzyme, and so are unlikely to be due to slow activation (*cf.* SUELTER *et al.*[12]) at low cation concentrations. Values for the concentration of activator giving half-maximal stimulation ($K_{0.5}$), determined graphically by extrapolation of plots of 1/velocity *vs.* 1/[activator]^n, were 6.8, 60, 52, 55 and 59 mM

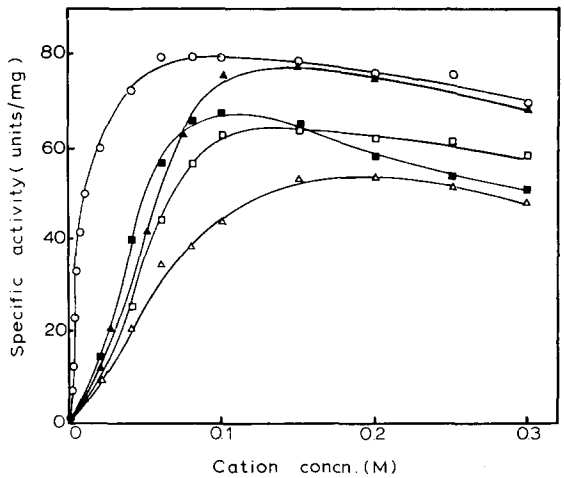


Fig. 1. Activation of AMP deaminase by monovalent cations. Assays contained 0.1 M succinate (Tris, pH 6.5), 5 mM 2-mercaptoethanol, 50 μM AMP (Tris salt) and the indicated concentration of cation as the chloride. The reaction was started by the addition of enzyme, freed from activating cations by gel filtration, and followed spectrophotometrically at 265 nm. ○—○, K^+; ▲—▲, Na^+; ■—■, Li^+; □—□, NH_4^+; △—△, Rb^+.

for K^+, Na^+, Li^+, NH_4^+ and Rb^+ respectively. Data obtained at cation concentrations above 0.1 M, where some inhibition occurs, were omitted from the calculations.

The value of $K_{0.5}$ may depend on the ionic strength in the assay; when assays were done at higher (constant) ionic strength in a system containing 50 μM AMP

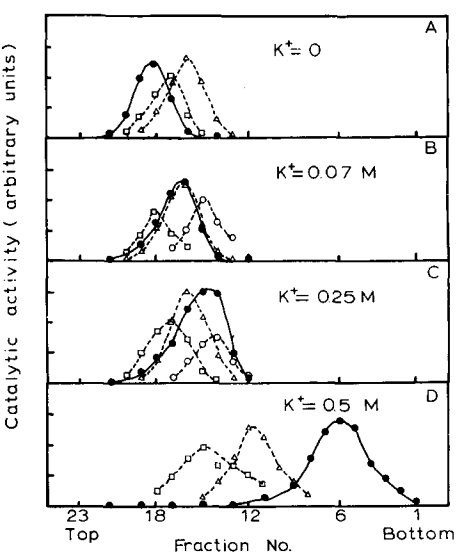


Fig. 2. Sedimentation of AMP deaminase in sucrose density gradients. The figure shows activity profiles of AMP deaminase (●—●) and marker enzymes: lipoamide dehydrogenase (□---□); alcohol dehydrogenase (△---△); and pyruvate kinase (○---○), in sucrose density gradients in the presence of the indicated concentrations of K^+. Gradients and samples were prepared as described in METHODS and centrifuged at 35 000 rev./min (100 000 × *g*) in a 3 × 5-ml swing-out rotor in a MSE-50 centrifuge at 5°, A, B and C for 18 h, D for 21 h.

(Tris)–5 mM 2-mercaptoethanol and 0.44 M Tris–0.04 M succinate (Cl^-, pH 6.5) and 0.4 M KCl–0.04 M succinate (Tris, pH 6.5) combined in appropriate proportions to give the required concentration of K^+, a value of 2.7 mM was obtained for the $K_{0.5}$ for this ion. Furthermore, the inhibition at high concentrations of ions, shown in Fig. 1, is not observed, at least for KCl up to 0.4 M, when assays are done at constant ionic strength, suggesting that inhibition is a non-specific effect of high ionic strength.

Effects of cations on sedimentation in sucrose density gradients

AMP deaminase, freed from activating cations as described in METHODS, sedimented in alkali-metal free sucrose density gradients at a position intermediate between deoxyribonuclease (mol. wt. 31 000) and lipoamide dehydrogenase (mol. wt. 110 000). The molecular weight was calculated to be 81 000 ± 8000 (7 measurements). In contrast, in 0.5 M K^+, the enzyme sedimented at a rate corresponding to a molecular weight of approx. 280 000. The dissociation–association process was fully reversible; enzyme which had been freed from K^+ and then had K^+ added again to 0.5 M behaved identically with enzyme loaded on to the gradient without desalting.

Some representative gradient activity profiles are shown in Fig. 2. At K^+ concentrations between 0.03 and 0.1 M the enzyme sedimented as a symmetrical peak level with alcohol dehydrogenase (mol. wt. 150 000). Single but asymmetric peaks were obtained at K^+ concentrations between 0.005 and 0.03 M and between 0.1 and 0.3 M (*cf.* Fig. 2c), indicating the presence of more than one species in equilibrium.

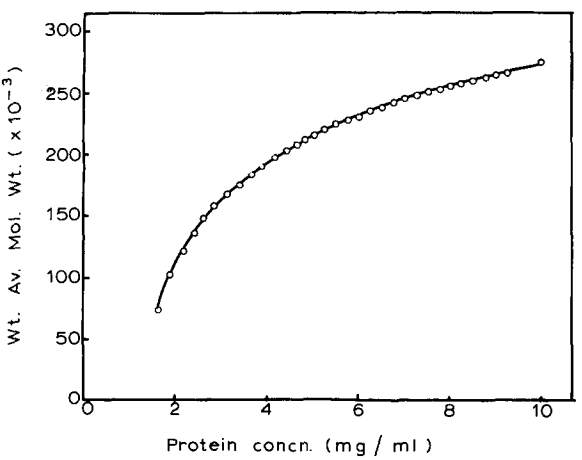


Fig. 3. Equilibrium centrifugation. The enzyme was freed from activating cations by extensive dialysis, at 4° under N_2, against 0.25 M Tris–0.05 M succinate (Cl^-, pH 6.5) containing 5 mM 2-mercaptoethanol. The final K^+ concentration, determined by flame photometry, was $2 \cdot 10^{-5}$ M. The enzyme was loaded at a concentration of 3.74 mg/ml, specific activity 76, and centrifuged to equilibrium at 6166 rev./min at 20° in a Spinco Model E analytical ultracentrifuge using interference optics. The graph relates protein concentration and weight average molecular weight at different positions in the cell. The weight average molecular weight, M_x at each point, x, in the cell was determined using the equation (ref. 14):

$$M_x = \frac{RT\,(dc/dx)}{(1 - \bar{v}\varrho)\,\omega^2 x c}$$

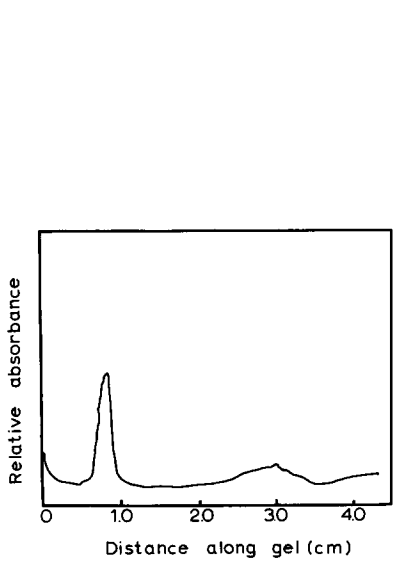
where c is the total concentration of enzyme in refractive index units, ϱ is the solvent density and ω is the angular velocity. The value for the partial specific volume, $\bar{v}$, used in the calculation was from WOLFENDEN *et al.*[15].

The biphasic nature of the increase of mean molecular weight with increasing K^+ concentration, with a plateau region between 0.03 and 0.1 M, implies that the association process involves two distinct steps. Similar results were obtained in experiments where NaCl replaced KCl in the gradients, except that higher concentrations were required to cause aggregation (approx. 0.2 M for formation of the 150 000-mol. wt. form, and more than 0.5 M for formation of the approx. 280 000-mol. wt. form).

The AMP deaminase concentration in peak fractions was approx. 0.25 mg/ml, and recovery of activity from the gradients was essentially complete. The slow loss of activity in the presence of mercaptoethanol recently reported by ZIELKE AND SUELTER[13] was not observed under our conditions.

Concentration dependent association in the absence of activating cations

The enzyme also undergoes a concentration-dependent association at a concentration of activating cations less than 10^{-3} of that required to cause aggregation in the sucrose density gradient studies. Fig. 3 shows the variation of the weight average molecular weight with protein concentration down the cell after sedimentation to equilibrium in an analytical ultracentrifuge fitted with interference optics*. The apparent molecular weight varied from $<100\,000$ to $>250\,000$ as the enzyme concentration increased from 1.6 to 10 mg/ml down the cell.



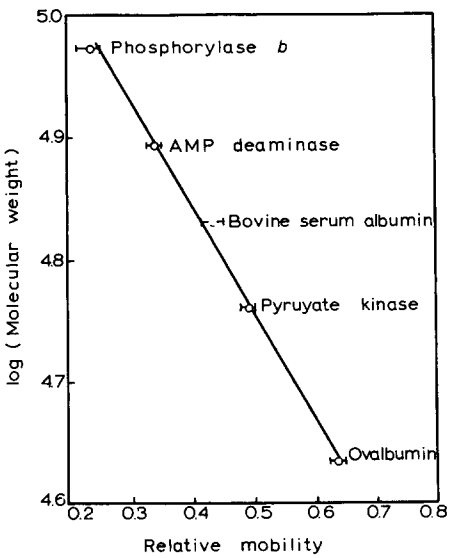


Fig. 4. Electrophoresis of AMP deaminase in sodium dodecyl sulphate–polyacrylamide gels. The figure shows a densitometer trace of AMP deaminase prepared and run as described in METHODS, and stained with Coomassie Blue as described by WEBER AND OSBORN[3].

Fig. 5. Molecular weight estimation of AMP deaminase in sodium dodecyl sulphate–polyacrylamide gels. Proteins were prepared and run as described in METHODS. Mobilities shown are relative to carboxypeptidase A. The standard deviation, shown for each protein, is the result of 12 separate estimations.

* We wish to gratefully acknowledge the assistance of Mr. P. A. Baghurst of the Waite Agricultural Research Institute, Adelaide, South Australia in performing this experiment and analysing the data.

Subunit molecular weight

AMP deaminase denatured, reduced and alkylated as described in METHODS migrated as a single band on sodium dodecyl sulphate–polyacrylamide gels (Fig. 4). Fig. 5 shows relative mobilities *vs.* the log of the molecular weight for a number of marker proteins. From this curve the subunit molecular weight of AMP deaminase was determined to be 79 000 ± 2000 (12 measurements).

DISCUSSION

Previously determined values of the molecular weight of AMP deaminase are 320 000 (LEE[16]) and 270 000 (WOLFENDEN *et al.*[15]). Our results imply that these values correspond to a tetramer. Consistent with this is the report that the purified enzyme contains approx. 3 g atoms of Zn^{2+}/mole (278 000 mol. wt.), and enzyme freed from Zn^{2+}, then reconstituted, contains 4 g atoms of Zn^{2+}/mole (ZIELKE AND SUELTER[13]).

The tetramer reversibly dissociates to monomers in the absence of activating cations and at concentrations of less than 1 mg/ml. The good agreement of the values for the monomer molecular weight obtained in sucrose density gradient and sodium dodecyl sulphate-electrophoresis experiments under strongly denaturing conditions implies that the monomer obtained on removal of activating monovalent cations is the fundamental subunit of the enzyme. The results presented indicate that the subunits are homogeneous with respect to molecular weight within the limits of resolution of the method (5–10%), but further work is required to determine whether they are identical.

The sedimentation to equilibrium experiment provides clear evidence for a concentration dependent subunit association in the absence of activating cations, although a single experiment does not enable quantitative determination of the minimum and maximum molecular weights, or the precise nature of the association process, and may be subject to systematic error if the partial specific volume changes on polymerisation (HOWLETT *et al.*[17]). The values obtained for apparent molecular weight agree reasonably with those for monomer: tetramer, determined by other methods.

As shown in the sucrose density gradient centrifugation experiments, association in response to increasing K^+ concentration is biphasic and appears to involve a dimer as a distinct intermediate. Either an isologous association of identical subunits (MONOD *et al.*[18]) or the involvement of 2 pairs of non-identical subunits, as in haemoglobin, could explain this behaviour (KLOTZ *et al.*[19]), and would be consistent with the finding of 2 binding sites for GTP and 4 binding sites for ATP per approx. 270 000 molecular weight by TAMOZAWA AND WOLFENDEN[20]. The fact that Na^+ also causes aggregation implies that this may be a general effect of activating monovalent cations. Tris, predominantly in the cationic form at this pH, does not activate or cause subunit association over a comparable concentration range.

Since the enzyme appears to exist, under these conditions, as an equilibrium mixture of the various states of aggregation, and since the distribution is strikingly affected by the catalytic activators, Na^+ and K^+, it seemed possible that polymerization could be an intrinsic part of the regulatory mechanism, as in the scheme of NICHOL *et al.*[21]. A model of this kind is also consistent with the change in the kinetic order of the reaction by K^+ (SMILEY AND SUELTER[2]). Comparison of the effects of

Na^+ and K^+ on the sedimentation of the enzyme in sucrose density gradients and on the velocity of the enzymic reaction, shows some correlation between activation and apparent dimerization. However, full activation occurs at K^+ and Na^+ concentrations only about half of those required for near complete conversion to the dimer. It must be pointed out that the results from sucrose density gradient and kinetic experiments are not completely comparable: the two kinds of experiments differ in temperature, enzyme concentration, and the presence of AMP, all of which may significantly affect the equilibrium.

In preliminary experiments we have found that other modifiers of AMP deaminase activity, ATP, an activator in these conditions, and the inhibitors GTP and 2,3-diphosphoglycerate (Tris salts) affect the concentration of AMP giving half maximal rate ($S_{0.5}$) without significantly affecting v_{max}. However, we have found no simple correlation between the apparent state of aggregation of the enzyme and its $S_{0.5}$ value or the kinetic order of the reaction, in the presence of these compounds. For this reason, the significance of the subunit association in relation to the regulatory mechanism is still not completely resolved.

It has recently been reported[22] that the tetrameric enzyme formyltetrahydrofolate synthetase, which requires NH_4^+ or K^+ for maximal activity, also dissociates to monomers in the absence of these cations and reassociates on their re-addition. In this case, as with AMP deaminase, the kinetic effect of the cation is to increase the apparent affinity of the enzyme for a substrate (formate) without affecting v_{max}.

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