# Isolation and characterization of AMP deaminase from mammalian (rabbit) myocardium

Jay K. THAKKAR,* David R. JANERO, Craig YARWOOD, Haamid SHARIF and David HRENIUK
Research Department, Pharmaceuticals Division, CIBA–GEIGY Corporation, Summit, NJ 07901, U.S.A.

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) is a ubiquitous enzyme in eukaryotes, which may play a role in ATP catabolism during myocardial ischaemia. We report isolation of AMP deaminase from rabbit myocardium with a 19% recovery and a 650-fold enrichment, using a newly devised protocol involving sequential cation-exchange, gel-permeation and affinity chromatographies. The cardiac AMP deaminase preparation described was electrophoretically and chromatographically homogeneous and contained one unique N-terminal residue (leucine). The isolated enzyme was sensitive to various cations ($K^+$, $Mg^{2+}$, $Ca^{2+}$). The pH optimum of purified cardiac AMP deaminase was 6.8, its pI was 6.5, and it displayed substrate-specificity toward 5′-AMP. The subunit molecular mass of rabbit heart AMP deaminase on SDS/PAGE (81 kDa) and the holoenzyme molecular mass as estimated by non-denaturing size-exclusion h.p.l.c. (330 kDa) indicated that the native enzyme was a tetramer. Cardiac AMP deaminase displayed a sigmoidal substrate-saturation curve in the presence of 100 mM KCl. Apparent Michaelis constants were a $K_m$ of 5.8 mM AMP and a $V_{max.}$ of 11.1 μmol/min per mg of protein. ATP and ADP were positive allosteric effectors of cardiac AMP deaminase: the apparent $K_m$ was decreased to 1.7 mM by 1.0 mM ATP. The enzyme was inhibited by GTP, coformycin, coformycin 5′-phosphate, palmitoyl-CoA, inorganic phosphate compounds, and the metal chelator *o*-phenanthroline. No inhibition either by product nucleotide (IMP) or by nicotinamide nucleotides was detected when these agents were examined at concentrations up to 2.5 mM. We conclude that this enzyme preparation offers a means by which the kinetic mechanism and regulation of mammalian cardiac AMP deaminase may be directly investigated.

## INTRODUCTION

In all eukaryotic cells studied thus far, AMP deaminase (EC 3.5.4.6) catalyses the essentially irreversible aminohydrolysis of 5′-AMP to equimolar amounts of 5′-IMP and ammonia [1]. Mammalian AMP deaminase from skeletal muscle has been extensively investigated, largely as a result of its very high activity in this tissue [2] and the inherited AMP deaminase deficiency syndrome in humans [3]. Deamination of AMP is the first step in the purine nucleotide cycle, making AMP deaminase a key enzyme for adenylate salvage in working skeletal muscle [4].

In heart muscle (myocardium), AMP deaminase may contribute to the purine nucleotide and nucleoside catabolism associated with myocardial ischaemia [5]. Accelerated AMP flux through this enzyme in the ischaemic heart has been reported to increase myocardial IMP levels and compete with cytosolic 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) for available AMP [6]. Heart muscle-cell IMP production is activated by $\alpha_1$-adrenergic stimulation [7], suggesting that cardiac AMP deaminase is under highly complex regulation *in situ*. Furthermore, cardiac AMP deaminase may be an isoenzyme distinct from the routinely studied skeletal-muscle enzyme [2]. These considerations argue for direct study of AMP deaminase from mammalian heart.

The methods devised for isolation of mammalian AMP deaminase have focused almost exclusively on skeletal muscle [2,8,9]. Direct characterization of mammalian cardiac AMP deaminase is lacking for want of a homogenous enzyme preparation. We describe here our work which has afforded purified AMP deaminase from rabbit myocardium, and we detail the kinetic and biochemical properties of the isolated cardiac enzyme.

## EXPERIMENTAL

### Materials and reagents

Cellulose phosphate cation-exchange resin (P-11) was from Whatman (Clifton, NJ, U.S.A.). Sephacryl S-300 and AMP–Sepharose ($N^6$-aminohexyl-5′-AMP–Sepharose 4B) were from Pharmacia (Piscataway, NJ, U.S.A.). Coformycin was from Calbiochem (San Diego, CA, U.S.A.). Coformycin 5′-monophosphate was kindly supplied by Dr. Vern L. Schramm (Albert Einstein College of Medicine, New York, NY, U.S.A.) [10]. Other reagents, including enzyme substrates, buffer constituents, inorganic phosphate compounds, *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICA ribotide), were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) at the highest available grade. All h.p.l.c. columns were from Waters Chromatography (Bedford, MA, U.S.A.). Electrophoresis and isoelectric-focusing equipment and supplies were from Bio-Rad (Richmond, CA, U.S.A.). Ultrapure water was obtained with a Milli-Q system (Millipore Corp., Bedford, MA, U.S.A.).

### AMP deaminase isolation

The entire isolation protocol, including centrifugations, was carried out at 4 °C. Hearts were removed from male New Zealand White rabbits (~ 25 hearts per enzyme preparation).

Abbreviations used: AMP–Sepharose, $N^6$-aminohexyl-5′-AMP–Sepharose 4B; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; AICA ribotide, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate.

* To whom correspondence should be addressed.

The left-ventricular myocardium was rapidly dissected away and extensively rinsed with buffer A [54 mM $KH_2PO_4$, 35 mM $K_2HPO_4$, 0.1 % (v/v) $\beta$-mercaptoethanol, pH 6.5] containing 0.18 M KCl. The myocardial tissue was blotted, weighed, and added to buffer A containing 0.18 M KCl to form a 10 % (w/v) tissue suspension when homogenized in a precooled Waring blender set at maximum speed; four 1 min homogenizations were conducted. The resulting homogenate was filtered through 4-ply cheesecloth, and the filtrate was centrifuged for 10 min at 121 *g*. The supernatant was recovered and centrifuged for 30 min at 17500 *g*. $(NH_4)_2SO_4$ was slowly added to the resulting supernatant (cytosolic fraction) to give 20 % saturation, and the mixture was centrifuged for 30 min at 17500 *g*. The pellet was discarded, additional $(NH_4)_2SO_4$ was added to the supernatant to give 60 % saturation, and this solution was centrifuged for 30 min at 17500 *g*. The resulting pellet was resupended in 100 ml of buffer A containing 1 M KCl and was extensively dialysed against 2 litres of buffer A containing 0.1 M KCl. The dialysed extract was centrifuged for 15 min at 27500 *g*, and the clear supernatant was added to cellulose phosphate (10 ml of supernatant/g of cellulose phosphate) which had been preactivated by successive washings with: 0.5 M NaOH for 5 min; distilled water for 15 min; 0.5 M HCl for 5 min; distilled water for 15 min; 10-times-concentrated buffer A containing 0.18 M KCl for 5 min; and finally, buffer A containing 0.18 M KCl. The slurry was stirred for 6 h and centrifuged for 5 min at 750 *g*. The pellet was washed three times with buffer A, and the bound AMP deaminase was extracted by three washes (100 ml total volume) with buffer A containing 1.0 M KCl. The extract was dialysed against buffer A containing 0.18 M KCl, and the retentate was concentrated by ultrafiltration with a PM-30 membrane (Amicon, Danvers, MA, U.S.A.). The concentrated extract was applied to a Sephacryl S-300 column (100 cm × 26 mm; $V_0 \sim 90$ ml) which had been pre-equilibrated with buffer A. The column was eluted with 500 ml of buffer A, and 3.5 ml eluate fractions were collected and assayed for AMP deaminase activity. The active fractions were pooled, concentrated to ~ 10 ml, and applied to an AMP–Sepharose column (8.0 cm × 1.5 cm) which had been pre-equilibrated with buffer A. The column was washed with 20 ml of buffer A, and the enzyme was eluted with a linear gradient of 0–1.0 M KCl in buffer A; 2.0 ml fractions were collected and assayed for AMP deaminase activity. The peak active fractions (~ 0.4–0.5 M KCl) were pooled and concentrated by ultrafiltration to ~ 0.2 mg of protein/ml. The concentrate was made 10 % (v/v) in glycerol and stored at 4 °C for no more than 2 weeks. The residual KCl content of the preparation was minimized by dialysis against 10 mM potassium phosphate buffer (pH 6.5) containing 0.1 % (v/v) $\beta$-mercaptoethanol and 10 % (v/v) glycerol at 4 °C for 17 h before use in experiments regarding the influence of cations on enzyme activity.

### Gel electrophoresis and h.p.l.c.

SDS/PAGE was performed as described by Laemmli [11], in 10 % (w/v) polyacrylamide slab gels. The pre-stained low-molecular-mass protein markers were: phosphorylase *b* (106 kDa), BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa). Sample proteins were made visible with Coomassie Brilliant Blue G-250 stain.

Size-exclusion h.p.l.c. was performed on a gradient system with a dual-channel spectrophotometric detector monitoring at 210 and 280 nm (Beckman Instruments, Berkeley, CA, U.S.A.). The system was fitted with a Radial-pak silica pre-column followed by two Protein-pak 300SW analytical gel-filtration columns (7.8 mm × 30 cm each). The non-denaturing mobile phase was an aqueous solution of 100 mM $KH_2PO_4$, pH 7.0. Protein samples were denatured before h.p.l.c. by boiling for 3 min in 2.0 % (w/v) aq. SDS, and the mobile phase for denatured samples consisted of aqueous 100 mM $NaH_2PO_4$, pH 7.0, containing 0.1 % SDS. The protein standards used to calibrate the size-exclusion column were: carbonic anhydrase subunit (29 kDa), catalase subunit (58 kDa), BSA (66.2 kDa), glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) subunit (72 kDa), alcohol dehydrogenase (80 kDa), $\beta$-amylase (202 kDa) and apoferritin (443 kDa).

### Enzyme assays

AMP deaminase was assayed as ammonia formation in 50 mM imidazole/HCl buffer (pH 6.8) containing 100 mM KCl and 5 mM 5′-AMP (final concns.). In some experiments, enzyme, substrate and KCl concentrations were varied, as indicated in the text and Figure legends. All reactions were started by substrate addition. Unless otherwise specified, the enzyme reaction was performed at 30 °C for 10 min in a shaking water bath. Ammonia was quantified with the phenol/hypochlorite reagent [12]. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) was assayed in 54 mM potassium phosphate buffer (pH 7.4) containing 500 $\mu$M adenosine [13] and quantified by microchemical ammonia determination [12]. 5′-Nucleotidase activity was assayed radiometrically [14]. For all enzyme assays, a first-order relationship among enzyme activity, time, and protein concentration was maintained.

### Inhibitor studies

Compounds investigated as potential cardiac AMP deaminase inhibitors were preincubated with the enzyme in 50 mM imidazole/HCl buffer (pH 6.8) containing 100 mM KCl for 20 min at 30 °C before starting the enzyme reaction by adding 5′-AMP to a 5 mM final concentration. The reaction was conducted for 10 min at 30 °C before ammonia assay (above). Contemporaneous 'control' enzyme samples lacking test compound were likewise incubated, and AMP deaminase activity in the samples containing test compound was expressed as the percentage residual activity relative to the controls. Absolute concentrations of coformycin and coformycin 5′-monophosphate were calculated by using a molar absorption coefficient of 8200 at 282 nm.

### pI determination

An aqueous solution containing 200 $\mu$g of purified cardiac AMP deaminase protein, 2.5 % (w/v) pH 3–10 ampholyte mixture and 10 % (v/v) glycerol was subjected to isoelectric focusing in a Bio-Rad Rotofor cell for 4 h at 12 W constant power. Twenty fractions were collected, their pH was measured, and they were subjected to SDS/PAGE (above) for detection of AMP deaminase.

### N-terminal amino acid analysis

Amino acids were analysed as their phenyl isothiocyanate derivatives, by using an automated Pico-Tag Workstation (Waters Chromatography). Standard methods were used for protein hydrolysis and amino acid derivative formation, separation and identification [15–17].

### Protein assay

Protein was quantified with BSA as the standard [18]. Protein in eluates from chromatographic columns used during AMP deaminase isolation was monitored at 280 nm with an on-line spectrophotometric detector.

## RESULTS

### Purification of cardiac AMP deaminase

The activity profile for a typical AMP deaminase isolation from rabbit left-ventricular heart muscle is given in Table 1. All of the cardiac AMP deaminase activity was soluble and associated with the cytosol fraction (i.e. the 17500*g* supernatant derived from the tissue homogenate). The 20–60%-satd.-$(NH_4)_2SO_4$ fraction derived from the cytosol showed enhanced AMP deaminase specific activity as a result of the removal of ~70% of the cytosolic protein, with relatively little decrease in AMP deaminase. Cellulose phosphate batch extraction further decreased the cytosolic protein content, with typical conservation of ~50% of the initial cardiac AMP deaminase activity. Examination of SDS/PAGE of the material eluted from the cellulose phosphate column revealed considerable heterogeneity (Figure 1). Consequently, the cellulose phosphate fraction was subjected to gel-permeation chromatography on Sephacryl S-300 such that the AMP deaminase specific activity increased over 4-fold (Table 1) and the polypeptide heterogeneity of the cellulose phosphate fraction was markedly diminished (Figure 1).

**Table 1 Isolation of rabbit cardiac AMP deaminase**

AMP deaminase was isolated from the left-ventricular myocardium of 25 rabbit hearts as detailed in the text.

| Purification step | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification (fold) | Total yield (%) |
|---|---|---|---|---|---|
| Homogenate | 7078.0 | 71.0 | 0.01 | 1 | 100 |
| Cytosol | 3470.0 | 69.4 | 0.02 | 2 | 98 |
| $(NH_4)_2SO_4$ fractionation (20–60%) | 1075.0 | 54.0 | 0.05 | 5 | 76 |
| Cellulose phosphate chromatography | 50.0 | 35.0 | 0.7 | 70 | 50 |
| Sephacryl S-300 chromatography | 10.0 | 30.0 | 3.0 | 300 | 42 |
| AMP–Sepharose affinity chromatography (gradient elution) | 2.1 | 13.7 | 6.5 | 650 | 19 |

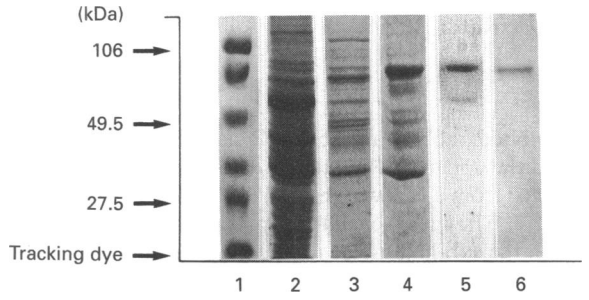


**Figure 1 SDS/PAGE of the cardiac AMP deaminase preparation at different steps of the purification process**

SDS/PAGE of samples of the enzyme preparation at different stages of the purification process was carried out under reducing conditions as described by Laemmli [11], using 10% (w/v) polyacrylamide slab gels. Proteins were stained with Coomassie Brilliant Blue G-250. Lane 1, pre-stained low-molecular-mass markers (phosphorylase *b*, 106 kDa; BSA, 80 kDa; ovalbumin, 49.5 kDa; carbonic anhydrase, 32.5 kDa; soybean trypsin inhibitor, 27.5 kDa; lysozyme, 18.5 kDa); lane 2, cytosol; lane 3, cellulose phosphate fractionation step; lane 4, Sephacryl S-300 fractionation step; lane 5, AMP–Sepharose eluted with 1.0 M KCl; lane 6, AMP–Sepharose fractionation step with KCl gradient elution, showing a band of 81 kDa corresponding to the rabbit heart AMP deaminase subunit.

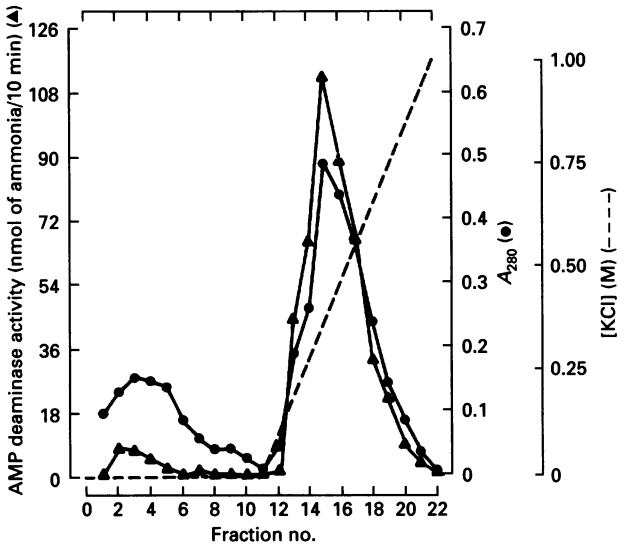


**Figure 2 Elution profile of rabbit heart AMP deaminase from AMP–Sepharose affinity chromatography**

AMP deaminase activity (▲) was quantified by microchemical ammonia assay, and protein (●) was estimated as the $A_{280}$. The enzyme was eluted with a linear gradient of 0–1.0 M KCl in buffer A (----).

The material recovered from the gel-permeation column was subjected to affinity chromatography on AMP–Sepharose. Elution of the AMP–Sepharose column with buffer A containing 1.0 M KCl significantly decreased the heterogeneity of the loaded Sephacryl S-300 fraction, for the AMP–Sepharose eluate contained a single major polypeptide band of apparent molecular mass 81 kDa (Figure 1). However, a second polypeptide with apparent molecular mass 73 kDa was also detected (Figure 1). Re-chromatography of such preparations on AMP–Sepharose did not remove this 73 kDa peptide completely, and inclusion of protease inhibitors (leupeptins, pepstatin A and phenylmethanesulphonyl fluoride) at micromolar levels throughout the isolation procedure described did not prevent its appearance. Densitometric scans of stained SDS/PAGE gels (e.g. Figure 1, lane 5) demonstrated that the 73 kDa peptide was a minor component which constituted < 5% of the total fraction protein.

The lack of electrophoretic homogeneity of the above AMP–Sepharose fraction led us to elute the AMP–Sepharose column first with KCl-free buffer and then with a linear KCl gradient. This procedure yielded two discrete protein peaks from the Sephacryl S-300 fraction, one of which was eluted with 0 M KCl and contained negligible AMP deaminase activity (Figure 2). A linear 0–1.0 M KCl gradient was then established, within which

the bulk AMP deaminase activity emerged as a single discrete peak at ~ 0.4–0.5 M KCl. The peak fractions (14–16) consisted of a single polypeptide band which migrated with apparent molecular mass 81 kDa (Figure 1). The experimentally determined specific activity of AMP deaminase across the peak AMP–Sepharose fractions after gradient elution was $6.5 \pm 0.5$ μmol/min per mg of protein at saturating substrate concentration. This affinity-purified fraction represented some 19% of the total cardiac AMP deaminase activity and ~ 0.16% of total tissue protein (Table 1).

### Molecular properties

In the studies described below, electrophoretically homogeneous AMP deaminase preparations were used which, by SDS/PAGE, consisted of the 81 kDa component under conditions of loading whereby minor species ($\leq 1$% of total) would have been stained and detected. Gel-filtration h.p.l.c. of the cardiac AMP deaminase preparation under non-denaturing conditions resolved a single symmetrical protein peak at an elution volume which corresponded to an apparent molecular mass of 330 kDa. A denatured enzyme sample run on size-exclusion h.p.l.c. evidenced a single peak with apparent molecular mass 80 kDa. The size-exclusion h.p.l.c. profile and the apparent-molecular-mass data from SDS/PAGE indicated that the cardiac AMP deaminase preparation contained a discrete tetrameric protein. Partial amino acid analysis identified a single unique N-terminal residue, leucine, in our AMP deaminase preparation.

### Stability

Isolated cardiac AMP deaminase was routinely stored at a concentration of ~ 0.2 mg of protein/ml in buffer A containing 50 mM KCl and 10% (v/v) glycerol at 4 °C. Under these conditions, there was an ~ 20% loss of activity over a 2-week period. No increase in activity with storage was observed. Dithiothreitol ($\geq 100$ μM final concn.) and/or protease inhibitors did not alter the stability profile. Cardiac AMP deaminase activity was lost with freeze–thawing in the above storage solution.

### pI

The pI of isolated rabbit heart AMP deaminase was 6.5 as determined in a Rotofor isoelectric-focusing cell. Thus the enzyme in aqueous solution at physiological pH bears a net negative charge.

### Reaction time course and substrate specificity

When incubated under the standard conditions detailed in the text, isolated cardiac AMP deaminase showed linear activity with respect to time and enzyme protein. The reaction was specific for 5′-AMP: structurally related molecules (including 3′-AMP, cyclic AMP, ATP, ADP, adenosine and adenine) were not deaminated. No adenosine deaminase or 5′-nucleotidase activity was present in the AMP deaminase preparation.

### pH-dependence

Cardiac AMP deaminase exhibited a well-defined pH profile with a discrete activity optimum at pH 6.8 (50 mM imidazole/HCl buffer). Within the pH range 6.5–7.5, $\geq 80$% of the peak activity remained.

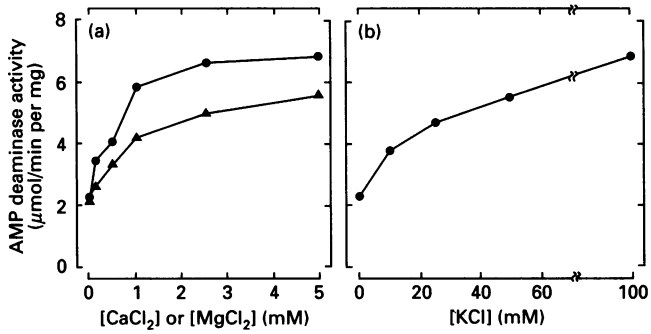


**Figure 3 Activation of rabbit heart AMP deaminase by bivalent and univalent cations**

The enzyme reaction was carried out in 50 mM imidazole/HCl buffer (pH 6.8) with 5 mM AMP substrate and the indicated added concentrations of either $Ca^{2+}$ (●) or $Mg^{2+}$ (▲) (**a**) or $K^+$ (**b**). To minimize the residual KCl content of the purified AMP deaminase preparation, it was dialysed against 10 mM potassium phosphate buffer (pH 6.5) containing 0.1% (v/v) β-mercaptoethanol and 10% (v/v) glycerol at 4 °C for 17 h before use in these studies. Data are means from duplicate assays in three independent experiments.

### Kinetic properties

Rabbit heart AMP deaminase was markedly stimulated by cations (Figure 3). $Mg^{2+}$ and $Ca^{2+}$ activated the enzyme in the low-millimolar range. At higher millimolar concentrations, $K^+$ was the most effective univalent-cation activator. In the presence of 100 mM KCl, full activity was reached, and cardiac AMP deaminase displayed a sigmoidal response to increasing substrate concentration (Figure 4). Transformation of the substrate-saturation data by the Lineweaver–Burk method [19] yielded a linear double-reciprocal plot (Figure 4); the apparent Michaelis constants were $K_m$ 5.8 mM AMP and $V_{max.}$ 11.1 μmol/min per mg of protein.

### Allosteric properties

Cardiac AMP deaminase was allosterically activated by ATP (Figure 4). The apparent $K_m$ as estimated from a Lineweaver–Burk plot was decreased 3.4-fold to 1.7 mM by 1 mM ATP without a change in the apparent $V_{max.}$ at optimal (i.e. 100 mM) KCl concentration. The allosteric effect of 1 mM ATP was abolished by 500 mM KCl. ADP was a somewhat less effective allosteric activator (Figure 4).

### Inhibition

GTP inhibited rabbit cardiac AMP deaminase; 1.0 mM GTP decreased the apparent $V_{max.}$ to 5.5 μmol/min per mg of protein as estimated from a Lineweaver–Burk transformation of the data in Figure 4. The metal chelator *o*-phenanthroline inhibited the enzyme in the sub-millimolar range, whereas millimolar concentrations of inorganic (poly)phosphates were necessary for inhibition (Figure 5). Micromolar concentrations of coformycin, a transition-state inhibitor of adenosine deaminase [20], inhibited cardiac AMP deaminase in the presence of 5 mM AMP (Figure 5). The 5′-monophosphate analogue of coformycin [10] was inhibitory at nanomolar concentrations (Figure 5). Palmitoyl-CoA virtually abolished cardiac AMP deaminase activity at 10 μM, whereas palmitic acid and short-chain CoAs (acetyl, butyryl) were inactive (Figure 5, and results not shown). EHNA, a specific adenosine deaminase inhibitor [21], did not affect cardiac AMP deaminase at concentrations up to 10 μM. The putative AMP deaminase inhibitor AICA ribotide [22] was

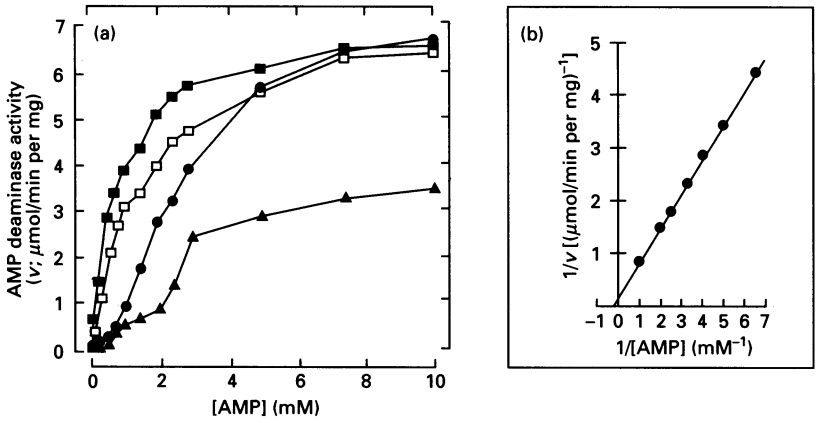


**Figure 4 Michaelis (a) and Lineweaver–Burk (b) plots for rabbit heart AMP deaminase: effects of ATP and GTP**

Each reaction contained (final concns.) 50 mM imidazole/HCl buffer (pH 6.8), 100 mM KCl, enzyme, and AMP as indicated (●). Some reactions also contained 1 mM ADP (□), 1 mM ATP (■) or 1 mM GTP (▲). Initial reaction rates were estimated as ammonia production throughout a 5 min incubation at 30 °C. Data points are means of three experiments, each performed in duplicate.

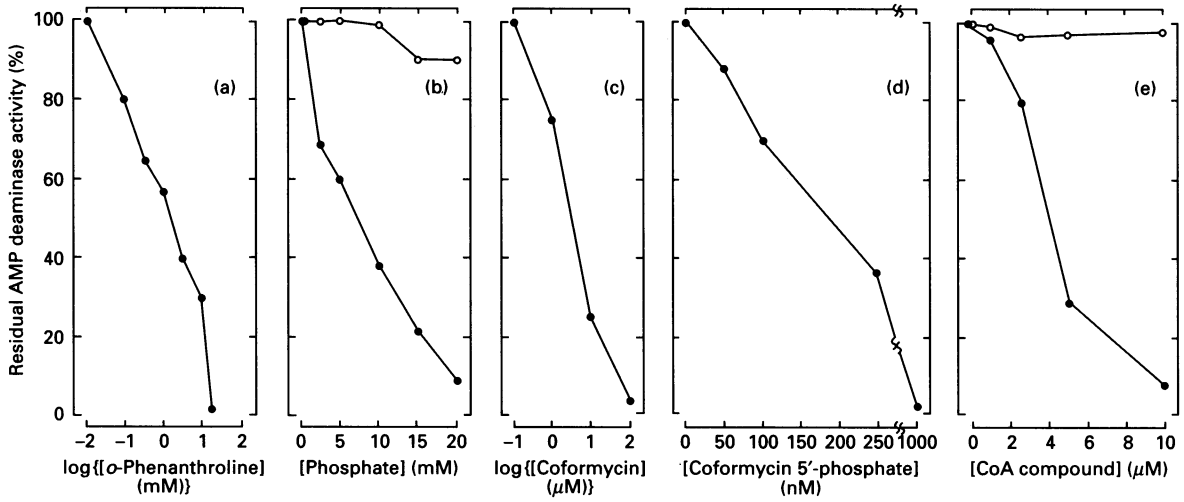


**Figure 5 Inhibitors of rabbit heart AMP deaminase**

Enzyme samples were preincubated for 20 min at 30 °C with a given concentration of *o*-phenanthroline (**a**), pyrophosphate (○) or tripolyphosphate (●) (**b**), coformycin (**c**), coformycin 5′-monophosphate (**d**), butyryl-CoA (○) or palmitoyl-CoA (●) (**e**). AMP was then added (5.0 mM final concn.), and the incubation was continued for another 10 min. AMP deaminase activity in the presence of each agent is expressed as residual activity relative to the enzyme not incubated with test agent. The data are means of two experiments performed in duplicate.

without effect at millimolar concentrations. Inhibition of rabbit cardiac AMP deaminase by product nucleotide (IMP) was not observed up to 2.5 mM IMP concentration. Likewise, no inhibition (or stimulation) by nicotinamide nucleotides was detected at a concentration of 2.5 mM.

## DISCUSSION

The present work constitutes the first study of purified AMP deaminase from mammalian myocardium. The protocol of Smiley et al. [9] for skeletal-muscle AMP deaminase isolation via cellulose phosphate cation-exchange chromatography has been almost universally employed to generate 'enriched' AMP deaminase preparations from avian [23], rat [24], bovine [25], pig [26], human [27] and rabbit [28–30] heart tissue. Molecular characterization of the AMP deaminase activity in such cardiac preparations has not been reported. There is some doubt as to whether the method of Smiley et al. [9] yields a homogeneous AMP deaminase preparation even from skeletal muscle [8]. As our work conclusively demonstrates (Table 1 and Figure 1), cation-exchange chromatography alone is insufficient to generate even a near-homogeneous cardiac AMP deaminase preparation. Two elaborations of the Smiley et al. [9] procedure for heart tissue employed either anion-exchange column chromatography [25] or h.p.l.c. [31] in addition to cellulose phosphate extraction, but these studies also lack electrophoretic and molecular characterization of the cardiac AMP deaminase activity observed. The SDS/PAGE, size-exclusion h.p.l.c. and N-terminal amino acid data presented in the present paper provide compelling evidence for the homogeneity and analytical quality of our cardiac AMP deaminase preparation.

Comparisons may be made between rabbit heart AMP

deaminase as isolated herein and the enzyme from skeletal muscle. Both rabbit cardiac and skeletal-muscle AMP deaminases are tetramers with apparent molecular masses of 316 kDa and 330 kDa respectively [32]. Variable molecular masses from 238 kDa to 326 kDa have been reported for skeletal-muscle AMP deaminase within and across species [33]. The only study other than the present one to specify a molecular mass for cardiac AMP deaminase reported that the bovine heart enzyme was a 160 kDa protein [25]. The literature range of molecular masses probably reflects a proteolytic artifact, for data on human and rat skeletal-muscle AMP deaminase genes allow extrapolation that the holoenzyme molecular mass is at least 320 kDa [34].

The kinetic properties of purified rabbit heart AMP deaminase differ markedly from those of the skeletal-muscle enzyme. Although both require millimolar $K^+$ for full activity, the former is more readily activated by bivalent cations than the latter [9]. The apparent $K_m$ of 5.8 mM AMP for rabbit cardiac AMP deaminase is greater than the $K_m$ of 0.3–0.7 mM generally reported for (rabbit) skeletal-muscle AMP deaminase [29,35], implying that the cardiac isoform has much lower substrate affinity. AMP deaminase activity in enriched cardiac preparations reaches half-maximal reaction velocity at 2–13 mM AMP [23,24,27], suggestive of the low-millimolar $K_m$ that we have observed. An ~ 100-fold difference exists between the $V_{max.}$ of 11.1 μmole/min per mg of protein for isolated rabbit heart AMP deaminase and literature values of > 1000 μmol/min per mg of protein for the (rabbit) skeletal-muscle enzyme [29,33,36]. Enriched cardiac AMP deaminase preparations have reported $V_{max.}$ values of 3–10 μmol/min per mg of protein [23,31], which are somewhat lower than the $V_{max.}$ of purified rabbit heart AMP deaminase.

The activity of isolated rabbit heart AMP deaminase was modulated by several effectors: ADP, ATP, GTP, inorganic (poly)phosphate compounds and fatty acyl-CoAs. Allosteric activation by ADP and ATP and inhibition by GTP, phosphates and fatty acyl-CoA have been observed in enriched cardiac AMP deaminase preparations [23,24,27,28] and with the isolated skeletal-muscle enzyme [37,38]. Quantitatively, however, variable sensitivities toward each effector have been reported. For example, we demonstrate that the $K_m$ of purified rabbit heart AMP deaminase was decreased 3.4-fold by 1.0 mM ATP, whereas a 7-fold decrease was reported for a crude preparation of this enzyme [29]. Non-homogeneous cardiac AMP deaminase exhibited greater sensitivity to millimolar ADP than ATP [28], whereas the isolated rabbit heart enzyme was somewhat more sensitive to ATP (Figure 4). At least some of these discrepancies may reflect contamination of crude AMP deaminase preparations by nucleoside-diphosphate kinase (ATP:nucleoside-diphosphate phosphotransferase, EC 2.7.4.6) and/or adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3.).

Purified rabbit heart AMP deaminase was not affected by 2.5 mM nicotinamide nucleotides or IMP, whereas an enriched preparation from rat heart was inhibited by over 90% by 1.0 mM $NAD^+$ and stimulated slightly by 1.0 mM $NADP^+$ [31]. Conflicting data exist concerning the effect of IMP on skeletal-muscle AMP deaminase: 0.5 mM IMP activated rabbit muscle AMP deaminase [39], yet 1.0 mM IMP inhibited the chick muscle enzyme [40]. Given that 2.5 mM IMP did not inhibit purified rabbit heart AMP deaminase, the cardiac enzyme appears markedly insensitive to product inhibition.

The present work provides the first demonstration of cardiac AMP deaminase inhibition by *o*-phenanthroline, coformycin and coformycin 5′-monophosphate. Inhibition of skeletal-muscle AMP deaminase by *o*-phenanthroline reflects a requirement for bound zinc for catalysis [41]; cardiac AMP deaminase likewise appears to be a zinc metalloprotein. As with skeletal-muscle AMP deaminase [40], the purified cardiac enzyme was inhibited to a much greater degree by the 5′-monophosphate derivative of coformycin than by coformycin itself. These compounds are known transition-state inhibitors of AMP deaminase and, with respect to coformycin, of adenosine deaminase as well [20]. However, the potent adenosine deaminase inhibitor EHNA did not inhibit isolated cardiac AMP deaminase, in line with results reported for the rabbit skeletal-muscle enzyme [42]. We were unable to demonstrate inhibition of rabbit heart AMP deaminase by AICA ribotide at concentrations which purportedly inhibited skeletal-muscle AMP deaminase [22]. As discussed elsewhere [43], interference by AICA ribotide in the u.v.-spectrophotometric assay for AMP deamination may have complicated the results of Baggott et al. [22].

We did not detect multiple molecular forms of AMP deaminase in rabbit heart through gradient elution at either the cation-exchange (i.e. cellulose phosphate) (cf. [25]) or the AMP–Sepharose (Figure 2) step. This finding is reminiscent of data from Ogasawara et al. showing that soluble extracts from rat [1] and rabbit [30] hearts contained a single activity peak on cellulose phosphate. In contrast, one major (~ 85% of total activity) and two minor activity peaks were identified in rat heart [31], and two apparent peaks of bovine heart AMP deaminase activity were eluted from cellulose phosphate [25]. Three distinct AMP deaminase activity peaks have been resolved through cellulose phosphate chromatography of a human heart extract [2]. These data suggest that species-specific cardiac AMP deaminase isoforms may exist. Further experimental work is required to address this point and to define the physiology of AMP deaminase in normal and diseased myocardium.

We thank N. Howie, R. Lappe, and M. Worcel for support of this work, the Netter Scientific Library and Scientific Information Center for literature assistance, and D. Rigel and G. Sandhu for comments.

## REFERENCES

1 Ogasawara, N., Goto, H. and Watanabe, T. (1975) Biochim. Biophys. Acta **403**, 530–537
2 Ogasawara, N., Goto, H., Yamada, Y., Watanabe, T. and Asano, T. (1982) Biochim. Biophys. Acta **298**, 298–306
3 Fishbein, W. N., Armbrustmacher, V. W. and Griffin, J. L. (1978) Science **200**, 545–548
4 Lowenstein, J. M. and Tornheim, K. (1971) Science **171**, 397–400
5 Jennings, R. B. and Steenberger, C. (1985) Annu. Rev. Physiol. **47**, 727–749
6 Van Belle, H., Wynants, J. and Goossens, F. (1988) Basic Res. Cardiol. **80**, 653–660
7 Hu, B., Altschuld, R. A. and Hohl, C. M. (1991) Arch. Biochem. Biophys. **291**, 100–106
8 Coffee, C. J. and Kofke, W. A. (1975) J. Biol. Chem. **250**, 6653–6658
9 Smiley, K. L., Berry, A. M. and Suelter, C. H. (1967) J. Biol. Chem. **242**, 2502–2506
10 Merkler, D. J., Brenowitz, M. and Schramm, V. L. (1990) Biochemistry **29**, 8358–8364
11 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
12 Chaney, A. L. and Marbach, E. P. (1962) Clin. Chem. **8**, 130–132
13 Weisman, M. I., Caiolfa, V. R. and Parola, A. H. (1988) J. Biol. Chem. **263**, 5266–5270
14 Janero, D. R., Yarwood, C. and Thakkar, J. K. (1992) J. Chromatogr. **573**, 207–218
15 Bildingmeyer, B. A., Cohen, S. A. and Tarvin, T. L. (1984) J. Chromatogr. **336**, 93–104
16 Heinrikson, R. L. and Meredith, S. C. (1984) Anal. Biochem. **136**, 65–74
17 Cohen, S. A. and Strydom, D. J. (1988) Anal. Biochem. **174**, 1–16
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
19 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. **56**, 658–666
20 Agarwal, R. P. (1982) Pharmacol. Ther. **17**, 399–429
21 Schaeffer, H. J. and Vogel, D. (1965) J. Med. Chem. **8**, 507–509
22 Baggott, J. E., Vaughn, W. H. and Hudson, B. B. (1986) Biochem. J. **236**, 193–200
23 Kaletha, K., Bogdanowicz, S. and Raffin, J.-P. (1987) Biochimie **69**, 117–123

24 Kaletha, K. and Sklandowski, A. (1979) Biochim. Biophys. Acta **508**, 80–90
25 Sklandowski, A. and Zydowo, M. M. (1988) Acta Biochim. Pol. **35**, 29–37
26 Harmsen, E., Verwoerd, T. C., Achterberg, P. W. and DeJong, J. W. (1983) Comp. Biochem. Physiol. **75B**, 1–3
27 Kaletha, K., Sklandowski, A., Bogdanowicz, S. and Zydowo, M. (1979) Int. J. Biochem. **10**, 925–929
28 Chung, L. and Bridges, W. A. (1976) FEBS Lett. **64**, 338–340
29 Barsacchi, R., Ranieri-Raggi, M., Bergamini, C. and Raggi, A. (1979) Biochem. J. **182**, 361–366
30 Ogasawara, N., Goto, H. and Yamada, Y. (1983) Comp. Biochem. Physiol. **76B**, 471–473
31 Jenkins, R. L., McDaniel, H. G. and Atkins, L. (1991) Biochim. Biophys. Acta **1077**, 379–384
32 Ranieri-Raggi, M. and Raggi, A. (1979) FEBS Lett. **102**, 59–63
33 Ranieri-Raggi, M. and Raggi, A. (1990) Biochem. J. **272**, 755–759
34 Sabina, R. L., Morisaki, T., Clarke, P., Eddy, R., Shows, T. B., Morton, C. C. and Holmes, E. W. (1990) J. Biol. Chem. **265**, 9423–9433
35 Meyer, W. and Follmann, H. (1980) Z. Naturforsch. **35C**, 273–278
36 Zielke, C. W. and Suelter, C. H. (1971) J. Biol. Chem. **246**, 1313–1317
37 Wheeler, T. J. and Lowenstein, J. M. (1979) J. Biol. Chem. **254**, 8994–8999
38 Coffee, C. J. and Solano, C. (1977) J. Biol. Chem. **252**, 1606–1612
39 Ashby, B. and Frieden, C. (1976) J. Biol. Chem. **253**, 8728–8735
40 Bzowska, A. and Shugar, D. (1989) Z. Naturforsch. **44C**, 581–589
41 Raggi, A., Ranieri, M., Taponeco, G., Ronca-Testoni, S., Ronca, G. and Rossi, C. A. (1970) FEBS Lett. **10**, 101–104
42 Agarwal, R. P. and Parks, R. E. (1977) Biochem. Pharmacol. **26**, 663–666
43 Murphy, J., Baker, D. C., Behling, C. and Turner, R. A. (1982) Anal. Biochem. **122**, 328–337

Received 16 July 1992/25 September 1992; accepted 5 October 1992