Cardiac adenylate deaminase: molecular, kinetic and regulatory properties under phosphate-free conditions

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Adenylate deaminase (EC 3.5.4.6) may help to regulate the adenine nucleotide catabolism characteristic of such disease states as myocardial ischaemia. We report analysis of the molecular, kinetic and allosteric properties of rabbit heart adenylate deaminase when extracted and purified under phosphate-free conditions (i.e., with HEPES/KOH). The enzyme's subunit molecular mass (~ 81 kDa), pI (6.5), substrate specificity for 5'-AMP, and activation by K⁺ were identical in the absence or presence of phosphate. At each chromatographic step during isolation without phosphate, cardiac adenylate deaminase showed a lower apparent activity as compared with the enzyme prepared with phosphate present. Kinetic constants for the phosphate-free rabbit heart adenylate deaminase preparation (K_m 0.54 mM AMP; V_{max} 1.4 μ mol/min per mg of protein) were ~10-fold lower than those of the enzyme isolated with phosphate. The same irreversible decrease in kinetic constants could be achieved by dialysing phosphate from the phosphate-containing enzyme preparation. The relationship between

enzyme activity and substrate concentration was sigmoidal in the presence of phosphate, but hyperbolic in its absence. Cardiac adenylate deaminase under phosphate-free conditions was no longer allosterically activated by ATP and ADP, yet remained inhibitable by GTP. Enzyme inhibition by the transition-state mimic coformycin was not influenced by phosphate status. The phosphate-free preparation of rabbit heart adenylate deaminase was markedly labile and extremely susceptible to proteolysis by trypsin or chymotrypsin. The inactivation kinetics and fragmentation pattern in response to controlled proteolysis depended on whether the enzyme had been isolated with or without phosphate present, suggesting a conformational difference between the two enzyme preparations. These data constitute direct evidence that the absence of phosphate irreversibly converts cardiac adenylate deaminase into a pseudo-isoenzyme with distinct kinetic, regulatory and stability properties.

INTRODUCTION

Adenylate deaminase (AMP deaminase, AMP aminohydrolase; EC 3.5.4.6) is a ubiquitous enzyme catalysing the essentially irreversible deamination of 5'-AMP to equimolar amounts of IMP and ammonia [1]. Although particularly critical to adenylate salvage in working skeletal muscle as a component of the purine nucleotide cycle [2], adenylate deaminase in heart muscle appears to support the adenine nucleotide catabolism associated with disease states such as myocardial ischaemia [3,4]. Furthermore, by competing with 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) for available AMP, cardiac adenylate deaminase may modulate the local production of adenosine, a cardioprotective mediator of vascular and cardiomyocyte function [4,5].

Adenylate deaminase has been isolated to apparent electrophoretic homogeneity from the skeletal muscle of several species, including rat [1], rabbit [6] and man [7]. The cardiac isoform has lacked direct analysis for want of an analytical-quality preparation. Instead, indirect investigation of purported adenylate deaminase activity in either myocardial tissue [5,8] or crude heart-muscle fractions [8,9] has been made. As part of a targeted analysis of cardiac ischaemic injury and its prevention [4,10,11], we have recently reported the first successful isolation of adenylate deaminase from mammalian (rabbit) heart muscle to electrophoretic and molecular homogeneity [12]. This preparation has proved useful in detailing the enzyme's regulation through allosterism [12] and protein kinase C-mediated phosphorylation [13].

Virtually all procedures for skeletal-muscle adenylate deaminase purification employ phosphate-containing isolation media [1,6,7], as does our novel protocol for isolating the rabbit heart enzyme [12]. Recent data on a crude adenylate deaminase extract from rat heart suggest that the enzyme's kinetics may vary with the phosphate status of the extraction medium [8]. We detail here a systematic evaluation of the properties of rabbit heart adenylate deaminase isolated under phosphate-free conditions (i.e., in HEPES/KOH) as compared with the enzyme isolated in the presence of phosphate [12]. Our data offer direct demonstration that phosphate enhances enzyme activity and stability and confers resistance to proteolysis. In the absence of phosphate, cardiac adenylate deaminase is irreversibly converted into a pseudo-isoenzyme with distinct kinetic and regulatory properties.

EXPERIMENTAL

Materials and reagents

Trypsin [treated with tosylphenylalanylchloromethane ('TPCK'); sp. activity 12000 units/mg] and chymotrypsin [treated with tosyl-lysylchloromethane ('TLCK'); sp. activity 2000 units/mg] were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ¹²⁵I-Protein G (sp. radioactivity 20 μ Ci/ μ g) was from New England Nuclear (Boston, MA, U.S.A.). Polyclonal antibodies against purified rabbit heart adenylate deaminase were raised in sheep by standard procedures [14]. All other reagents were from sources previously specified [4,10–13].

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Isolation of adenylate deaminase from rabbit heart

The isolation protocol employing 50 mM potassium phosphate buffer (pH 6.8) containing 0.1 M KCl and 0.1 % (v/v) β -mercaptoethanol has been detailed [12]. Two approaches were used to obtain phosphate-free enzyme. In the first, 50 mM Hepes/KOH (pH 6.8) containing 0.1 M KCl and 0.1 % (v/v) β -mercaptoethanol was substituted for potassium phosphate (see above) in the detailed purification protocol [12]. Alternatively, cardiac adenylate deaminase isolated in the presence of phosphate [12] was exhaustively dialysed against several changes of 50 mM Hepes/KOH (pH 6.8) containing 0.1 M KCl and 0.1 % β -mercaptoethanol at 4 °C.

Protein electrophoresis

SDS/PAGE was performed on 10 % (w/v) polyacrylamide slab gels [15]. The prestained low-molecular-mass protein markers were as identified in the legend to Figure 4. Sample proteins were made visible with Coomassie Brilliant Blue G-250 stain.

Immunoblotting

Proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose membranes. After transfer, the membranes were blocked with 3 % (w/v) BSA for 2 h, and the blots were treated for 1 h with sheep antiserum containing polyclonal antibodies raised against rabbit heart adenylate deaminase at a 1:1000 (v/v) dilution in PBS. The blots were then washed with PBS and incubated with 125 I-Protein G for 1 h. The blots were re-washed and dried before being processed for autoradiography [13].

pI determination

Purified cardiac adenylate deaminase [200 μ g of protein in either 50 mM potassium phosphate or Hepes/KOH buffer containing 2.5 % (w/v) pH 3–10 ampholyte mixture and 10 % (v/v) glycerol] was subjected to isoelectric focusing in a Rotofor cell (Bio-Rad, Richmond, CA, U.S.A.) for 4 h at 12 W constant power. Upon voltage stabilization at 1200 V, 20 fractions were collected across the ampholyte gradient. The pH of each fraction was determined, and its adenylate deaminase content was analysed by SDS/PAGE (see above).

Enzyme assay

Adenylate deaminase activity was assayed at 30 °C as ammonia formation in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl and 5 mM AMP (final concns.) [12]. Ammonia was quantified colorimetrically with phenol/hypochlorite reagent [12]. Product formation was linear with time in this assay.

Inhibitor studies

Coformycin was preincubated with cardiac adenylate deaminase in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl for 5 min at 30 °C before the enzyme reaction was started by adding AMP to 5 mM final concentration. Reactions were conducted for 10 min at 30 °C before ammonia assay. Contemporaneous 'control' enzyme samples lacking coformycin were likewise incubated. Adenylate deaminase activity in the samples containing coformycin was expressed as percentage residual activity relative to the control.

Proteolysis studies

Adenylate deaminase was incubated at 30 °C with a known amount (2 or 20 μ g) of either trypsin or chymotrypsin in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl and 0.1 % β -mercaptoethanol. Preliminary experiments with a standard spectrophotometric assay [16] demonstrated that the levels of (chymo)trypsin used in these incubations were not limiting to the proteolysis observed (results not shown). At appropriate times, samples were withdrawn and diluted into assay mixture (above) to determine residual adenylate deaminase activity. Enzyme from parallel protease-free incubations was used to quantify the basal activity at each sampling time.

Protein assay

Protein was quantified colorimetrically with IgG as the standard [17].

RESULTS

Three chromatography steps are required to purify adenylate deaminase from the cytosol of rabbit cardiac muscle [12]. The experimentally determined catalytic activity of rabbit heart adenylate deaminase in each of the resulting chromatographic fractions was considerably (> 7-fold) lower when phosphate was absent during the purification (Table 1). Adenylate deaminase activity in the concentrated cytosol used as the tissue source for subsequent chromatographic enzyme isolation was the same whether the tissue had been homogenized in phosphate or in phosphate-free buffer (Table 1), likely reflecting the endogenous tissue phosphate present compounded by cytosol concentration before application to the cellulose phosphate column [12]. At saturating substrate concentration, the apparent specific activity of phosphate-free cardiac adenylate deaminase across the peak affinity-purified fractions was 0.8 μ mol/min per mg of protein, compared with 6.5 μ mol/min per mg of protein when isolated with phosphate. Regardless of the presence or absence of phosphate, the affinity-purified fraction consisted virtually exclusively ($\geq 95\%$) of a single ~ 81 kDa polypeptide by SDS/PAGE. Cardiac adenylate deaminase showed absolute substrate specificity for 5'-AMP with or without phosphate, and the pI was 6.5 in either case.

Table 1 Apparent activities of cardiac adenylate deaminase in selected fractions during enzyme isolation in the presence or absence of phosphate

Adenylate deaminase was isolated from the left-ventricular myocardium of 25 rabbit hearts as detailed in [12] by using either 50 mM potassium phosphate or 50 mM Hepes/KOH buffer, both at pH 6.8 and containing 0.1 M KCl and 0.1 % β -mercaptoethanol. Data are means from triplicate assays in three independent experiments; the range about each mean is < 10%. Abbreviation: AMP–Sephacryl, N^6 -aminohexyl-5'-AMP–Sephacryl.

Purification step	Enzyme activity (units/mg)	
	Potassium phosphate	Hepes/KOH
Concentrated cytosol	0.01	0.01
Cellulose phosphate chromatography	0.7	0.02
Sephacryl S-300 chromatography	3.0	0.4
AMP–Sephacryl affinity chromatography	6.5	0.8

Figure 1 Michaelis (a) and Lineweaver-Burk (b) plots for rabbit heart adenylate deaminase isolated under phosphate-free conditions

Each reaction contained (final concns.) 50 mM imidazole/HCl buffer (pH 6.8), 0.1 M KCl, enzyme, and AMP as indicated. Initial reaction rates were estimated as ammonia production throughout a 10 min incubation at 30 °C. Apparent kinetic constants were derived from computer-assisted regression analysis [ENZFITTER program (Elsevier, Amsterdam, The Netherlands)] of the data in (b), for which the correlation coefficient was 0.98. Each data point is the mean of triplicate assays in at least three independent enzyme preparations; the range about each mean is < 10%.

With 0.1 M KCl present, phosphate-free cardiac adenylate deaminase displayed a hyperbolic activity response to increasing substrate concentration (Figure 1), unlike the sigmoidal response of the enzyme with phosphate present [12]. Transformation of the concentration-dependent activity data for 0.1–5.0 mM AMP by the Lineweaver-Burk method [18] yielded a linear double-reciprocal plot (Figure 1). The apparent kinetic constants of the phosphate-free enzyme were: K_m , 0.54 mM AMP; V_{max} , 1.4 $\mu\text{mol/min per mg}$ of protein. These values are at least 8-fold lower than the kinetic constants of rabbit heart adenylate deaminase prepared with phosphate [12]. After extensive dialysis against non-phosphate (i.e. Hepes/KOH) buffer, cardiac adenylate deaminase as isolated in the presence of phosphate assumed the activity profile, hyperbolic relationship to AMP concentration, and kinetic constants of the phosphate-free enzyme. However, dialysis of phosphate-free adenylate deaminase against phosphate buffer did not affect the enzyme's apparent catalytic activity or kinetic properties.

Over a wide substrate concentration range with ≥ 0.1 M KCl, the activity of rabbit heart adenylate deaminase in the absence of phosphate was not influenced by ATP or ADP at nucleotide concentrations sufficient for pronounced allosteric activation of the enzyme prepared with phosphate (Figure 2). At low (i.e. ≤ 20 mM) KCl concentrations, maximal activity of the phosphate-free adenylate deaminase preparation required 1.0 mM ATP, but no synergism was evident (Figure 2). GTP (1.0 mM) inhibited adenylate deaminase activity whether phosphate was present or not, in the latter case decreasing the

Figure 2 Influence of nucleotides and K^+ on the catalytic activity of rabbit heart adenylate deaminase isolated under phosphate-free conditions

(a) The enzyme reaction was carried out in 50 mM imidazole/HCl buffer (pH 6.8) containing (final concn.) 0.1 M KCl and AMP as indicated (○). Some reactions also contained 1 mM GTP (▼), 1 mM ADP (●) or 1 mM ATP (▽). (b) To eliminate the residual KCl content of the purified adenylate deaminase preparation, it was rapidly dialysed against 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1% β -mercaptoethanol and 10% (v/v) glycerol at 4 °C before use [12]. The enzyme was then made to react with AMP as indicated in 50 mM imidazole/HCl buffer (pH 6.8) containing 20 mM KCl (◆), 0.2 M KCl (□), 20 mM KCl + 1 mM ATP (▲), or 0.2 M KCl + 1 mM ATP (△). Data are means from duplicate assays in at least two independent experiments; the range about each mean is < 10%.

measured reaction velocity by $\sim 60\%$ at saturating substrate concentration (Figure 2). Regardless of the phosphate status of the enzyme, K^+ was an effective univalent-cation activator (Figure 2). Comparable $MgCl_2$ concentrations did not influence enzyme activity, thereby excluding the possibility of an ionic-strength/non-specific ion effect in these KCl studies. Concentration-dependent inhibition of the enzyme by the transition-state mimic cofomycin, however, was comparable in the absence (results not shown) or presence [12] of phosphate.

As isolated under phosphate-free conditions, rabbit heart adenylate deaminase was extremely susceptible to proteolysis by trypsin (Figure 3) or chymotrypsin (results not shown). For example, $\sim 70\%$ loss of basal adenylate deaminase activity was observed within 20 min after incubation of the phosphate-free enzyme with 2 μg of trypsin, whereas the enzyme prepared with phosphate was only minimally affected ($\sim 10\%$ activity loss). The marked proteolytic sensitivity of cardiac adenylate deaminase prepared without phosphate remained when the amount of trypsin was increased 10-fold (Figure 3). Basal adenylate deaminase activity in parallel 'control' (i.e. trypsin-free) incubations at 30 °C declined when phosphate had been absent during enzyme isolation. By 30 min, basal cardiac adenylate deaminase activity of the phosphate-free enzyme was decreased by $\sim 50\%$, whereas the enzyme prepared with phosphate showed full activity (results not shown). In the presence of trypsin, however, no activity was detectable at the

Figure 3 Effect of trypsin on the activity of rabbit heart adenylate deaminase as isolated in either the absence (a) or presence (b) of phosphate

Enzyme samples were incubated with ($\blacktriangle$, 2 μ g; $\blacksquare$, 20 μ g) or without ($\bullet$) trypsin in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl and 0.1% β -mercaptoethanol. At the times indicated, samples were withdrawn and assayed for adenylate deaminase activity. The activity of trypsin-treated adenylate deaminase is expressed relative to non-proteolysed enzyme. Data are means from duplicate assays in three independent experiments, the range about each mean is < 10%.

Figure 4 Immunoreactive proteolytic products derived from trypsin treatment of phosphate-containing and phosphate-free cardiac adenylate deaminase preparations

Rabbit heart adenylate deaminase as isolated in the presence (lanes 2–5) or absence (lanes 6–9) of phosphate was incubated in 50 mM imidazole/HCl buffer (pH 6.8) with or without trypsin for the time periods indicated. At each sampling time, samples were taken from these incubations and subjected to SDS/PAGE under reducing conditions [15]. The resolved peptides were electrotransferred to nitrocellulose membranes, which were processed for Western blotting by using 125 I-Protein G and antibody raised against rabbit heart adenylate deaminase. The washed and dried membranes were then analysed by autoradiography. Prestained molecular-mass markers (lane 1) were: phosphorylase *b* (106 kDa), BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa) and soybean trypsin inhibitor (27.5 kDa).

30 min sampling time for enzyme isolated in the absence of phosphate (Figure 3). Even at 4 °C, cardiac adenylate deaminase isolated without phosphate was labile under conditions whereby the enzyme prepared with phosphate remained stable for ~ 2 weeks [12].

Analysis of the controlled proteolysis reactions by SDS/PAGE and immunoblotting revealed a complete loss of immunoreactive ~ 81 kDa adenylate deaminase subunit within 30 min from the

phosphate-free preparation incubated with 20 μ g of trypsin (Figure 4). By contrast, some of the original adenylate deaminase subunit (Figure 4) and ~ 40 % of the basal activity (Figure 3) remained 30 min after the identical trypsin treatment of the phosphate-containing enzyme preparation. Proteolysis of cardiac adenylate deaminase which had been isolated with phosphate by 20 μ g of trypsin generated within 30 min a heterogeneous group of immunoreactive peptides in the ~ 40–65 kDa mass range, whereas the identical trypsinolysis of the phosphate-free enzyme converted virtually all of the ~ 81 kDa subunit into a discrete ~ 60 kDa fragment (Figure 4).

DISCUSSION

Millimolar inorganic phosphate (i.e. ortho- and pyro-phosphate) and select phosphorylated metabolites (e.g. ATP, ADP, GTP, phosphocreatine) can alter the apparent activity of purified yeast [19] and mammalian (i.e., skeletal-muscle) [6,20,21] adenylate deaminase. As established by the initial attempts to isolate the skeletal-muscle isoform [6], millimolar-strength phosphate is an integral component of the solutions used for adenylate deaminase purification. A potential complication thus arises that such adenylate deaminase preparations may artifactually reflect an enzyme whose activity had been compromised through the presence of phosphate. Another issue has been raised that lack of phosphate masks the enzyme's response to nucleotide effectors [8]. These considerations led us to conduct the first direct analysis of the molecular, kinetic and regulatory properties of mammalian adenylate deaminase isolated under phosphate-free conditions. Rabbit heart adenylate deaminase was selected for study, since our recent purification of this enzyme in the presence of phosphate [12,13] affords a sound comparative basis.

Certain properties of rabbit cardiac adenylate deaminase (subunit molecular mass, pI, substrate specificity, activation by K^+ , inhibition by GTP and coformycin) remained unaffected by isolation in the absence of phosphate. The enzyme's kinetic properties, however, were radically altered. Without phosphate, the relationship between enzyme activity and substrate concentration changed from sigmoidal [12] to hyperbolic. As compared with cardiac adenylate deaminase isolated in the presence of phosphate without effectors [12], the phosphate-free enzyme showed an ~ 10-fold greater substrate affinity (as K_m) and an ~ 8-fold lower V_{max} . and measured catalytic activity, the latter evident in all chromatographic fractions. The lower K_m of the phosphate-free cardiac enzyme approximates to the K_m of 0.3–0.7 mM AMP generally reported for (rabbit) skeletal-muscle adenylate deaminase [6,7,20–22]. Non-cardiac and cardiac adenylate deaminases isolated in the presence of phosphate and subsequently incubated with millimolar inorganic phosphate display a several-fold decrease in V_{max} /measured activity [7,21,23–25]. Under this condition, the activity decrease is likely to reflect phosphate binding at the catalytic site as a competitive inhibitor with respect to AMP [19]. The absence of phosphate in our Hepes/KOH preparation clearly precludes such a mechanism as the basis of the activity loss observed.

At inhibitory concentrations, inorganic phosphate generally increases [21,23,24] or leaves unaltered [7,25] the K_m /substrate half-saturation value ($S_{0.5}$) of (non-cardiac) adenylate deaminases prepared with phosphate, in contrast with the ~ 10-fold K_m decrease brought about by isolation of rabbit heart adenylate deaminase under phosphate-free conditions. Since the absence of phosphate also decreased the V_{max} , and the measured activity of cardiac adenylate deaminase, active-site AMP binding under phosphate-free conditions appears tightened so as to restrict

substrate turnover/product release, perhaps because the molecular conformation differs in the absence and presence of phosphate. This reasoning is supported by data suggesting that (poly)phosphate binding to yeast adenylate deaminase modulates breakdown of the enzyme-substrate complex [24]. A conformational difference would be consistent with the greater susceptibility of phosphate-free cardiac adenylate deaminase to controlled proteolysis, its distinct proteolytic pattern, and its altered stability.

A crude rat heart adenylate deaminase extract prepared with imidazole/HCl buffer was reported to have an ~2-fold greater apparent activity and an ~25-fold lower K_m than a parallel extract in phosphate buffer [8]. This activity increase clearly differs from our result with purified rabbit heart adenylate deaminase, whose activity declined when prepared in the absence of phosphate. The lack of homogeneity of the rat heart enzyme and its abnormally low reported activity even in the presence of phosphate [8] complicate, if not confound, explanation of this seeming discrepancy. With 0.1 M KCl present, IMP production by the crude rat heart extract in imidazole/HCl appeared unaffected by millimolar ATP [8]. The resulting implication that cardiac adenylate deaminase prepared under phosphate-free conditions would no longer be modulated by positive allosteric effectors has been directly demonstrated herein with respect to both ATP and ADP. At low (≤ 20 mM) KCl concentrations, however, millimolar ATP did increase the measured activity of phosphate-free rabbit-heart adenylate deaminase. Changes in K^+ concentration have been reported to modify the response of yeast adenylate deaminase toward ATP, but not effector binding itself [19].

Rabbit heart adenylate deaminase isolated with phosphate assumed the kinetic and regulatory properties of the phosphate-free enzyme upon dialysis against phosphate-free buffer, whereas the reverse did not occur. Consequently, the molecular effects of phosphate absence/removal do not permit restoration of catalytic activity, a sigmoidal relationship to substrate concentration, or allosteric regulation. Multiple phosphate-binding sites exclusive of the active site have been identified in yeast adenylate deaminase [19]. Lack of occupancy of such putative sites on the cardiac enzyme during isolation or after phosphate removal via dialysis may have irreversibly altered its kinetic, regulatory and stability properties. These data raise the possibility that purported cardiac adenylate deaminase isoforms described by some [26–28], but not all [1,12,29], investigators may represent minor pseudo-isoenzyme species generated as a consequence of the irreversible molecular alteration of one basic tissue-specific isoform. Physico-chemical analysis of rabbit heart adenylate deaminase with and

without phosphate would be warranted to help to characterize potential molecular changes responsible for the loss of catalytic competency and allostereism in the absence of phosphate.

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