Modulation of mammalian cardiac AMP deaminase by protein kinase C-mediated phosphorylation

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Using AMP deaminase (AMP aminohydrolase; EC 3.5.4.6) purified from rabbit left-ventricular heart tissue, we report direct investigation of the potential for cardiac AMP deaminase activity to be regulated by kinase-mediated phosphorylation. Rabbit heart AMP deaminase served as a substrate for Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C; PKC) exclusively; no other mammalian protein kinase phosphorylated the enzyme. PKC-dependent AMP deaminase phosphorylation was rapid, linear with respect to time and the concentrations of PKC and AMP deaminase in the reaction, and inhibitable by staurosporine. Upon phosphorylation, the apparent K_m of cardiac AMP deaminase decreased from 5.6 mM to 1.2 mM, without effect on the V_{max} . Whether phosphorylated or not, rabbit heart AMP deaminase was inhibited by 1.0 mM

GTP, which decreased the V_{max} by $\sim 50\%$ in each case. PKC-dependent phosphorylation of cardiac AMP deaminase did not alter the enzyme's allosterism toward millimolar ATP or ADP: both nucleotides at 1.0 mM concentration decreased the apparent K_m to ~ 0.5 mM. Treatment of cardiac phospho-AMP deaminase with either the protein phosphatase calcineurin or alkaline phosphatase generated a dephosphorylated form which displayed molecular and kinetic properties identical with those of the originally isolated enzyme. These data raise the possibility that a phosphorylation–dephosphorylation mechanism may regulate flux through AMP deaminase in the heart under pathological conditions, such as myocardial ischaemia, characterized by PKC activation and adenylate depletion.

INTRODUCTION

In all eukaryotes and vertebrate tissues studied thus far, AMP deaminase (EC 3.5.4.6) catalyses the essentially irreversible hydrolytic deamination of 5'-AMP to yield equimolar amounts of 5'-IMP and ammonia [1]. Of the multiple tissue-specific isoforms identified in mammals, skeletal-muscle AMP deaminase has been the most extensively investigated, a reflection of the very high AMP deaminase activity in this tissue [2] and the inherited AMP deaminase deficiency noted in $\sim 2\%$ of all human skeletal-muscle biopsies [3]. In response to increased work demand, skeletal-muscle AMP deaminase supplies the purine nucleotide cycle with IMP critical to adenylate restoration after exertion [4]. Other important functions proposed for AMP deaminase in skeletal muscle include stabilization of energy charge [5] and indirect modulation of AMP-sensitive metabolic pathways [6].

The very limited activity of the purine nucleotide cycle in well-oxygenated working heart [7] suggests that cardiac AMP deaminase might assume greater significance under injury conditions which elicit myocardial ATP loss. In this regard, cardiac AMP deaminase may modulate the purine nucleotide and nucleoside catabolism characteristic of ischaemic heart disease [8]. Elevated levels of cardiac-tissue IMP found in some experimental models of myocardial ischaemia [9–11] offer indirect evidence for an ischaemia-related enhancement of flux through cardiac AMP deaminase. Furthermore, IMP production in muscle cells isolated from the rat heart and rapidly de-energized by metabolic poisons is potentiated by α_1 -adrenergic stimulation [12] and the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate [13], suggesting that cardiac AMP deaminase activity may be modulated by phosphorylation-dependent reactions. This suggestion gains particular interest through recent reports linking myocardial ischaemia with PKC activation [14,15].

We report here the ability of mammalian cardiac AMP deaminase to undergo kinase-mediated phosphorylation and the effects of this covalent modification on enzyme activity. Our results constitute direct demonstration that phosphorylation of cardiac AMP deaminase specifically by PKC can modulate enzyme activity by markedly increasing the apparent affinity of this AMP deaminase isoform for substrate. The data have implications regarding the regulation of AMP deaminase in the mammalian heart and its potential role in catalysing adenylate degradation during myocardial ischaemia.

EXPERIMENTAL

Materials

Calcineurin, calmodulin, alkaline phosphatase (EC 3.1.3.1), ATPase (EC 3.6.1.3), the catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase, calf thymus histone H1S, okadaic acid and staurosporine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PKC and cyclic GMP (cGMP)-dependent protein kinase were purchased from Calbiochem (La Jolla, CA, U.S.A.) and Promega Biochemicals (Madison, WI, U.S.A.) respectively. Ca^{2+} /calmodulin-dependent protein kinase was kindly provided by Dr. Roger Colbran, Vanderbilt University, Nashville, TN, U.S.A. [γ - ^{32}P]ATP (sp. radioactivity 3000–5000 c.p.m./pmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). All supplies for SDS/PAGE were from Bio-Rad (Richmond, CA, U.S.A.).

AMP deaminase purification

Isolation of AMP deaminase from rabbit left-ventricular myocardium was carried out as detailed previously [16,17]. The enzyme preparation is electrophoretically and chromato-

Abbreviations used: PKC, Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C); cAMP, cyclic AMP; cGMP, cyclic GMP.

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graphically homogeneous and contains a single N-terminal amino acid residue (Figure 1) [16,17].

Phosphorylation of AMP deaminase

Phosphorylation of AMP deaminase by the catalytic subunit of protein kinase A was assayed as modified from Corbin and Reimann [18] in the presence of (final concns.) 50 mM Tris maleate (pH 7.0), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 10 μ M ATP (2–5 μ Ci of [γ - 32 P]ATP) and 5 units/ml catalytic subunit of protein kinase A (sp. activity 41 units/ μ g). After a 15 min incubation at 30 °C, the reaction was terminated either with Laemmli buffer for subsequent SDS/PAGE analysis [19] or with trichloroacetic acid (final concn. 20%, w/v) for quantitative determination of 32 P incorporation into the washed acid-precipitable protein pellet.

Phosphorylation of AMP deaminase by cGMP-dependent protein kinase was assayed as described [20] in the presence of (final concns.) 50 mM HEPES/KOH (pH 7.5), 10 mM $MgCl_2$, 0.2 mM EGTA, 10 μ M ATP (2–5 μ Ci of [γ - 32 P]ATP), 5 units/ml cGMP-dependent protein kinase (sp. activity 2400 units/ μ g) and 0.5 μ M cGMP. After incubation for 15 min at 30 °C, the reaction was terminated with either Laemmli buffer or trichloroacetic acid, for processing as described above.

Phosphorylation of AMP deaminase by Ca^{2+} /calmodulin-dependent protein kinase was assayed in the presence of 50 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM β -mercaptoethanol, 0.4 mM EGTA, 5 units/ml calmodulin kinase (sp. activity 18 units/mg), 1 mM $CaCl_2$, 10 μ M ATP (2–5 μ Ci of [γ - 32 P]ATP) and 30 μ g/ml calmodulin (sp. activity 1000 units/mg) [21]. Reactions were conducted at 30 °C for 15 min and processed as described above.

Phosphorylation of AMP deaminase by PKC was studied in the reaction system of Yasuda et al. [22] in the presence of (final concns.) 50 mM HEPES/KOH (pH 7.0), 10 mM $MgCl_2$, 50 μ M ATP (2–5 μ Ci of [γ - 32 P]ATP), 0.15 mM EGTA, 0.2 mM EDTA, 2 mM $CaCl_2$, 260 μ g/ml phosphatidylserine, 40 μ g/ml 1,2-diolein, 20 nM phorbol 12-myristate 13-acetate and 1 unit/ml PKC (sp. activity 1900 units/mg). After incubation for 15 min at 30 °C, the reaction was terminated and processed for analysis as described above.

Dephosphorylation of phospho-AMP deaminase

Dephosphorylation reactions were performed with minor modifications of methods detailed [23]. Samples were treated with 0.5 unit/ml (final concn.) ATPase (sp. activity 1.1 units/mg) to remove any residual ATP from the PKC reaction. Phospho-AMP deaminase was incubated in 50 mM HEPES/KOH buffer (pH 7.0) in the presence of (final concns.) 2 units/ml calcineurin (sp. activity 4500 units/mg), 5 mM $CaCl_2$ and 1 μ M calmodulin at 30 °C for 2 h. Alternatively, the phosphoenzyme was incubated in 50 mM $Na_2B_4O_7$ /NaOH buffer (pH 9.5) with 2.5 units/ml alkaline phosphatase (final concn.; sp. activity 2500 units/mg) at 30 °C for 2 h. In both cases, reaction samples were analysed for AMP deaminase activity and processed for SDS/PAGE and autoradiography (see below).

Electrophoresis and autoradiography

SDS/PAGE was performed as described by Laemmli [19] in 10%-polyacrylamide slab gels. Samples were prepared for

SDS/PAGE in Laemmli buffer [19]. After electrophoresis, gels were stained with Coomassie Brilliant Blue G-250, dried, and exposed to Kodak XAR-2 film overnight at –80 °C for autoradiography. The film was then developed in an automated processor.

AMP deaminase assay and determination of kinetic constants

AMP deaminase was assayed as ammonia formation in 50 mM imidazole/HCl buffer (pH 6.8) containing (final concns.) 100 mM KCl and 5 mM 5'-AMP. The reaction was initiated upon substrate addition and performed at 30 °C for up to 5 min in a shaking water bath, during which time samples were taken for measurement of ammonia formation [16,17]. Ammonia was quantified by using phenol/hypochlorite [24]. For all AMP deaminase assays, a first-order relationship among reaction time, protein concentration and enzyme activity was maintained. Apparent kinetic constants (K_m , V_{max}) were derived from Lineweaver–Burk transformations [25] of AMP deaminase reaction velocity as a function of substrate concentration.

RESULTS

AMP deaminase purified to electrophoretic and chromatographic homogeneity from left-ventricular rabbit myocardium was used throughout these studies (Figure 1) [16,17]. As analysed by SDS/PAGE followed by autoradiography, cardiac AMP deaminase became rapidly phosphorylated when incubated with PKC and [γ - 32 P]ATP, provided that the requisite cofactors for PKC activity were also present (Figure 1). PKC-mediated phosphorylation of rabbit heart AMP deaminase was linearly dependent on the PKC and AMP deaminase contents of the reaction system over time (Figure 2). A maximal phosphorylation rate of 125 fmol of P_i incorporated/min per μ g of AMP deaminase protein was achieved by 15 min in a phosphorylation

Figure 1 PKC-dependent phosphorylation of cardiac AMP deaminase

AMP deaminase was purified from left-ventricular heart tissue of the rabbit and detected after SDS/PAGE with Coomassie Blue (lane 1). Incubation of cardiac AMP deaminase in a PKC reaction mixture lacking PKC cofactors (i.e. Ca^{2+} and lipids) did not generate phospho-AMP deaminase, as analysed by SDS/PAGE and autoradiography (lane 2). Isolated rabbit heart AMP deaminase became phosphorylated by incubation for 15 min at 30 °C in a complete PKC reaction system consisting of (final concns.) 50 mM HEPES/KOH (pH 7.0), 10 mM $MgCl_2$, 1 unit/ml PKC, 50 μ M ATP (2–5 μ Ci of [γ - 32 P]ATP), 0.15 mM EGTA, 0.2 mM EDTA, 20 nM phorbol 12-myristate 13-acetate, 2 mM $CaCl_2$, 260 μ g/ml phosphatidylserine and 40 μ g/ml 1,2-diolein. After the reaction, the mixture was run on SDS/PAGE with autoradiography of the gel to detect 32 P-labelled protein species, of which the 81 kDa AMP deaminase subunit is evident (lane 3). Pre-stained molecular-mass standards indicated were: phosphorylase *b* (106 kDa), ovalbumin (49.5 kDa) and soybean trypsin inhibitor (27.5 kDa).

Figure 2 Dependence of cardiac AMP deaminase phosphorylation on PKC and AMP deaminase amounts over time

Rabbit heart AMP deaminase (25 μg) was incubated with different amounts of PKC in the complete PKC reaction system described in the text for 15 min at 30 °C [panel a, (●)]. Some reactions contained 10 nM staurosporine [panel a, (○)]. Alternatively, different amounts of cardiac AMP deaminase protein up to 25 μg were incubated with 40 m-units of PKC in the complete PKC reaction system for either 15 min [panel b] or for different times up to 60 min [panel c]. Reactions were stopped by adding trichloroacetic acid to a final concentration of 20% (w/v) and analysed for net incorporation of ^{32}P label into acid-precipitable protein [i.e. cardiac AMP deaminase (Figure 1, lane 3)]. Each data point is the mean of three independent determinations; the range about each mean is < 10%.

Figure 3 Lineweaver-Burk plots for rabbit heart AMP deaminase before and after PKC-dependent phosphorylation

The substrate-concentration-dependence of the reaction rate of cardiac AMP deaminase, either as isolated from rabbit myocardium (●) or as isolated and then phosphorylated *in vitro* by PKC (■), is given here in a Lineweaver-Burk transformation. Data for phospho-AMP deaminase, after dephosphorylation by calcineurin, are also given (○). Each data point is the mean of at least three independent determinations; the range about each mean is < 10%.

reaction containing 25 μg of AMP deaminase and 80 m-units of PKC. These data allow estimation that approx. 1 phosphate molecule is incorporated per holoenzyme. Inclusion of staurosporine, a potent PKC inhibitor [26], at a final concentration of 10 nM blocked the PKC-mediated phosphorylation of rabbit heart AMP deaminase (Figure 2). Cardiac AMP deaminase was not a substrate for the catalytic subunit of protein kinase A,

cGMP-dependent protein kinase or Ca^{2+} /calmodulin-dependent protein kinase under reaction conditions whereby known exogenous substrates (e.g. histone) were readily phosphorylated (results not shown).

PKC-dependent phosphorylation altered the kinetic properties of cardiac AMP deaminase. Michaelis plots indicated that phospho-AMP deaminase reached half-maximal experimental reaction velocity at a lower substrate concentration than did the non-phosphorylated form, whereas the maximal measured velocity attained in both cases was $\sim 6.4 \mu\text{mol}/\text{min per mg}$. As calculated from Lineweaver-Burk transformations (Figure 3), AMP deaminase isolated from rabbit myocardium showed an apparent K_m of 5.6 mM AMP and an apparent V_{max} of $10.6 \mu\text{mol}/\text{min per mg}$ of protein. After phosphorylation by PKC, the apparent K_m decreased to 1.2 mM AMP without significant change in the V_{max} . Treatment of cardiac phospho-AMP deaminase with either the protein phosphatase calcineurin or alkaline phosphatase generated a non-phosphorylated form having the same electrophoretic, chromatographic and kinetic properties displayed by AMP deaminase when purified from normal rabbit myocardium (Figure 3 and results not shown). Calcineurin treatment itself did not affect the molecular or

Table 1 Nucleotide effectors and phosphorylation state of rabbit heart AMP deaminase

AMP deaminase phosphorylation state	Effector			
	None	1.0 mM ATP	1.0 mM ADP	1.0 mM GTP
Non-phosphorylated				
K_m (mM)	5.6	1.6	1.9	5.4
V_{max} ($\mu\text{mol}/\text{min per mg}$ of protein)	10.6	11.0	10.9	5.1
PKC-phosphorylated				
K_m (mM)	1.2	0.4	0.5	1.3
V_{max} ($\mu\text{mol}/\text{min per mg}$ of protein)	10.1	10.5	10.6	5.4

kinetic properties of isolated cardiac AMP deaminase (results not shown). The presence of okadaic acid during AMP deaminase isolation at a concentration ($2.5 \mu\text{M}$) sufficient to block serine/threonine phosphoprotein phosphatases [27] likewise did not affect the enzyme's K_m or V_{max} (results not shown).

PKC-dependent phosphorylation did not alter the allosteric nature of cardiac AMP deaminase (Table 1). In the presence of 100 mM KCl, 1.0 mM ATP decreased the respective K_m values of the non-phosphorylated and phosphorylated forms of rabbit heart AMP deaminase by some 3-fold, with no significant effect on the respective V_{max} values. ADP exerted a similar allosteric activation of cardiac AMP deaminase, whether phosphorylated by PKC or not. GTP inhibition of cardiac AMP deaminase was also independent of the phosphorylation state of the enzyme: 1.0 mM GTP decreased the respective V_{max} values by $\sim 50\%$ (Table 1).

DISCUSSION

Ischaemia may elicit cardiac IMP formation [9,10], and the standard PKC activator phorbol 12-myristate 13-acetate potentiates IMP production in cardiomyocytes de-energized with metabolic poisons [13]. These results have led to speculation that mammalian cardiac AMP deaminase activity may be modulated by post-translational phosphorylation [13]. Such speculation, however, was advanced despite the variable association between myocardial ischaemia and ATP degradation to IMP [11]. Furthermore, ATP depletion in poisoned cardiomyocytes is much faster than during ischaemia/anoxia, and involves bioenergetic derangements (e.g. mitochondrial uncoupling) not necessarily found in acute myocardial ischaemia [8]. There is also a conspicuous lack of any evidence that the cardiac isoform of mammalian AMP deaminase serves as a substrate for protein kinases.

Our studies provide compelling demonstration that rabbit heart AMP deaminase can be phosphorylated rapidly and specifically by PKC, thereby activating the enzyme through a marked (~ 5 -fold) increase in the apparent affinity of the cardiac isoform for AMP substrate. Phosphorylation of AMP deaminase by PKC was observed only in the presence of PKC cofactors. Neither the allosterism of cardiac AMP deaminase toward ATP and ADP nor its inhibition by GTP was altered by PKC-dependent phosphorylation. As a whole, these data imply that activation of cardiac AMP deaminase by PKC does not occur through phosphorylation at an allosteric nucleotide-binding site and support a molecular mechanism involving enhancement of substrate affinity through phosphorylation of active-site amino acid residue(s), as has been observed for some other enzymes of intermediary metabolism [28].

Rat skeletal-muscle AMP deaminase has been reported to serve as a PKC substrate, its apparent K_m decreasing from 0.6 mM AMP to 0.2 mM AMP upon phosphorylation [29]. Thus the K_m of 1.2 mM for cardiac phospho-AMP deaminase indicates that PKC-dependent phosphorylation brings its substrate affinity near the 0.3–0.7 mM K_m range reported for various skeletal-muscle AMP deaminase isoforms [30,31]. With allosteric activation by 1.0 mM ATP or ADP, cardiac phospho-AMP deaminase displays a K_m equivalent to that of the skeletal-muscle isoform. However, the V_{max} of rabbit heart AMP deaminase, whether phosphorylated or not, remains ~ 100 -fold lower than reported V_{max} values $> 1000 \mu\text{mol/min per mg}$ for the rabbit skeletal-muscle enzyme [1,32]. The cardiac isoform of AMP deaminase clearly retains a distinctive kinetic profile with respect to skeletal-muscle AMP deaminase, regardless of its phosphorylation state.

Dephosphorylation of rabbit heart phospho-AMP deaminase generated an enzyme with molecular and kinetic properties identical with those of the AMP deaminase isolated from the non-ischaemic source tissue, and neither phosphatase treatment nor the presence of the potent phosphatase inhibitor okadaic acid during the isolation affected the properties of AMP deaminase purified from rabbit heart. These findings imply that healthy myocardium contains predominantly non-phosphorylated AMP deaminase of low substrate affinity. They also suggest a mechanism whereby phosphorylation–dephosphorylation of cardiac AMP deaminase *in situ* could regulate its contribution to tissue adenylate depletion. The marked decrease in the K_m of cardiac AMP deaminase upon PKC-dependent phosphorylation and the further allosteric decrease in the K_m of phospho-AMP deaminase by ADP would imply that such a regulatory mechanism might act as a patho-physiologically important enhancer of flux through AMP deaminase during disease states, such as myocardial ischaemia, involving ATP degradation [8]. The concept of an ischaemia-related activation of cardiac AMP deaminase through direct PKC-dependent phosphorylation gains attractiveness from the PKC activation observed during acute myocardial ischaemia [15,33]. Although the mechanisms by which PKC is activated during myocardial ischaemia are not well understood [15], the present study invites evaluation of the potential role of PKC as a regulator of adenylate catabolism through direct modulation of the catalytic activity of AMP deaminase in the ischaemic heart.

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

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Received 16 October 1992/19 November 1992; accepted 2 December 1992