Oxidative modulation and inactivation of rabbit cardiac adenylate deaminase

David R. JANERO* and Craig YARWOOD†

Research Department, Ciba Pharmaceuticals, Summit, NJ 07901, U.S.A.

Oxidative stress and adenine nucleotide catabolism occur concomitantly in several disease states, such as cardiac ischaemia–reperfusion, and may act as synergistic determinants of tissue injury. However, the mechanisms underlying this potential interaction remain ill-defined. We examined the influence of oxidative stress on the molecular, kinetic and regulatory properties of a ubiquitous AMP-catabolizing enzyme, adenylate deaminase (AMPD) (EC 3.5.4.6). To this intent, rabbit heart AMPD and an H_2O_2 /ascorbate/iron oxidation system were employed. Enzyme exposure to the complete oxidation system acutely impaired its catalytic activity, lowered the V_{max} by 7-fold within 5 min, and rendered the enzyme unresponsive to nucleotide effectors. Irreversible AMPD inactivation resulted within about 15 min of oxidative insult and was not prevented by free-radical scavengers. Oxidative stress did not affect the molecular

mass, tetrameric nature, K_m , immunoreactivity or trypsinolytic pattern of the enzyme; nor did it induce carbonyl formation, Zn^{2+} release from the holoenzyme or net AMPD S-thiolation. This injury pattern is inconsistent with a radical-fragmentation mechanism as the basis for the oxidative AMPD inactivation observed. Rather, the sensitivity of the enzyme to both S-thiolation and thiol alkylation and the significant (3 of 9/mol of denatured enzyme) net loss of DTNB-reactive thiols on exposure to oxidant strongly implicate the conversion of essential thiol moieties into stable higher-oxidation states in the oxidative inactivation of cardiac AMPD. The altered thiol status of the enzyme on oxidative insult may prohibit a catalytically permissible conformation and, in so doing, increase AMP availability to 5'-nucleotidase *in vivo*.

INTRODUCTION

A major component of cardiac ischaemic injury is net heart muscle ATP catabolism to nucleosides, which themselves may be degraded to purines [1]. AMP is a key catabolite as substrate of both 5'-nucleotidase (EC 3.1.3.5) and adenylate deaminase (AMPD) (EC 3.5.4.6). The nucleotidase generates adenosine, a cardioprotectant and precursor for 'salvage' adenylate biosynthesis, whereas AMPD irreversibly converts AMP into IMP [2]. Thus AMPD may influence myocardial adenine nucleotide metabolism during ischaemia through both substrate availability to 5'-nucleotidase and net utilization of tissue adenine nucleotides [3].

Although timely restoration of nutritive blood flow is essential for the salvage of ischaemic myocardium, reperfusion-induced oxidative stress may further damage the already jeopardized heart muscle [4]. Recent studies from this laboratory demonstrate that oxidative stress adversely affects cardiac glycolysis, mitochondrial function and nucleotide levels [5–7]. As metabolic competency and energy balance are prime determinants of cardiac functional recovery in the post-ischaemic period [1,8], identification of oxidant-sensitive molecular targets in myocardial metabolism may have significant mechanistic and therapeutic implications [9].

In this regard, we [6,7,9] and others [10] have begun to identify specific myocardial proteins affected by oxidative stress. For some enzymes, the oxidant sensitivity reflects redox changes in critical thiol moieties [7,10]. Oxidation reactions involving a transition metal (usually iron in biological systems), an oxidant

(e.g. H_2O_2) and a reductant cause oxidative enzyme damage *in vivo* [11]. The inhibition of at least some non-cardiac AMPD isoforms by thiol-blocking reagents [12,13] and the potential importance of the cardiac AMPD isoform to heart muscle nucleotide catabolism in disease states with an increasingly recognized oxidative-injury component [1,3] led us to examine the oxidant sensitivity of myocardial AMPD. The present work directly demonstrates that oxidative stress compromises the thiol status of cardiac AMPD, modifying the activity profile and kinetic and allosteric properties of the enzyme. Ultimately, irreversible oxidative inactivation of cardiac AMPD results.

EXPERIMENTAL

Materials

4-(2-Pyridylazo)resorcinol (PAR), *NNN'*-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), phenylarsine oxide (PAO), 2,3-dimercapto-propanol, GSH, GSSG and trypsin (treated with TosPheCHCl₂; specific radioactivity 12000 units/mg) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). *NN'*-Diphenyl-1,4-phenylenediamine (DPPD) was from Aldrich (Milwaukee, WI, U.S.A.). Superoxide dismutase (EC 1.15.1.1) (SOD) and catalase (EC 1.11.1.6) were from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). NaB^3H_4 (specific radioactivity 100 mCi/mmol), ^{125}I -Protein G (specific radioactivity 20 μ Ci/ μ g) and [^{35}S]cystine (specific radioactivity 576 Ci/mmol)

Abbreviations used: AMPD, adenylate deaminase (EC 3.5.4.6); DPPD, *NN'*-diphenyl-1,4-phenylenediamine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PAO, phenylarsine oxide; PAR, 4-(2-pyridylazo)resorcinol; SOD, superoxide dismutase (EC 1.15.1.1); TPEN, *NNN'*-tetrakis-(2-pyridylmethyl)-ethylenediamine.

* To whom correspondence should be addressed at: NitroMed Inc., 801 Albany Street, Boston, MA 02118, U.S.A.

† Present address: Medical Education and Publications, Ciba-Geigy Corp., Summit, NJ 07901, U.S.A.

were from New England Nuclear (Boston, MA, U.S.A.). Other reagents and supplies were from previously identified commercial sources and were of the highest available grade [1,2,5–7,9,14].

AMPD Isolation

AMPD was isolated from left ventricular rabbit myocardium [2]. In the reported experiments, enzyme preparations had activity at saturating substrate concentrations of $6.0 \pm 0.5 \mu\text{mol/min per mg}$ (mean $\pm$ S.D., $n = 10$) under standard assay conditions (below). This value equals the 100 % (control) activity designation in the Tables and Figures. Enzyme solutions were concentrated with a Centriprep-30 microconcentrator (Amicon, Beverly, MA, U.S.A.). Apoenzyme (i.e. with the active-site Zn^{2+} removed) was formed by dialysing cardiac AMPD for 16 h at 4°C against several changes of $54 \text{ mM KH}_2\text{PO}_4/35 \text{ mM K}_2\text{HPO}_4$ (pH 6.5) containing 2 mM EDTA (final concns.) [12]. Aged AMPD was generated by dialysis for 16 h at 4°C against the same buffer without EDTA and storage at 4°C for 1 month [2].

Enzyme assay and determination of kinetic constants

The AMPD reaction was conducted in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl and substrate (AMP) at concentrations specified in the Tables and Figures [2]. Enzyme activity was quantified as NH_3 formation with phenol/hypochlorite [2]. Apparent kinetic constants were derived by regression analysis from Lineweaver–Burk transformations of AMPD reaction velocity as a function of substrate concentration [15].

Electrophoretic, immunoblot and liquid chromatographic analyses

SDS/PAGE was performed under reducing conditions in 10 % polyacrylamide slab gels calibrated with standards of known molecular mass [2]. Gels were subsequently stained with Coomassie Brilliant Blue G-250 and dried for peptide visualization. Alternatively, for immunoblotting, proteins were electrophoretically transferred from the gel to nitrocellulose membranes. 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 AMPD at a 1:1000 volume dilution in PBS. The blots were then washed with PBS, incubated with ^{125}I -protein G for 1 h, rewashed, and dried before autoradiography [14]. Size-exclusion h.p.l.c. was performed under non-denaturing conditions with a calibrated gel-filtration column [2].

Oxidative-stress conditions

Cardiac AMPD (routinely $25 \mu\text{g/ml}$) was subjected to oxidative stress in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl by incubation at 37°C with $1 \text{ mM H}_2\text{O}_2 + 1 \text{ mM ascorbate} + 20 \mu\text{M FeCl}_2$ (final concns.). In selected cases, 1 mM GSH was substituted for the ascorbate, or various combinations of H_2O_2 , ascorbate, FeCl_2 and/or FeCl_3 were used. The oxidative-stress period was terminated by adding either 50 units/ml catalase (to dismutate the H_2O_2 [9]) or $250 \mu\text{M EDTA}$ (to chelate the iron) (final concns.), as appropriate. Enzyme activity was then assayed.

Inhibitor studies

Agents tested as potential inhibitors of oxidative injury to cardiac AMPD were mixed with the enzyme ($25 \mu\text{g/ml}$) in 50 mM

imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl before the imposition of oxidative stress by the addition of $1 \text{ mM H}_2\text{O}_2 + 1 \text{ mM ascorbate} + 20 \mu\text{M FeCl}_2$ (final concns.) and incubation at 37°C for 5 min. The oxidative-stress period was terminated by adding catalase (50 unit/ml , final concn.), and AMPD activity was then assessed [2]. Enzyme samples not oxidatively stressed (control activity) or stressed without test agent were incubated in parallel. The degree to which a test agent preserved the activity of oxidatively stressed AMPD relative to full control activity was taken as the extent of inhibition of the oxidative AMPD damage. Preliminary experiments verified that the test agents *per se* did not affect AMPD activity (results not shown).

Controlled proteolysis studies

AMPD ($150 \mu\text{g/ml}$) was incubated at 37°C for 1 h with $20 \mu\text{g/ml}$ trypsin (final concn.) in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl , as detailed [16]. Samples from the trypsinolysis reaction were then subjected to SDS/PAGE under reducing conditions [2]. The gels were either stained with Coomassie Blue for peptide visualization or processed for immunoblot analysis.

Protein carbonyl assay

The carbonyl content of AMPD was quantified radiometrically by enzyme reduction with NaB^3H_4 [17]. Final AMPD concentration in the reaction was $150 \mu\text{g/ml}$. The net difference in borohydride reactivity between identical amounts of non-injured and oxidatively stressed AMPD was taken as the extent of protein oxidation to form carbonyl moieties.

Assay of Zn^{2+} mobilization

The potential for oxidative stress to release active-site Zn^{2+} from cardiac AMPD was assessed chemically with the metallochromic indicator PAR [18]. Parallel AMPD samples (1 mg/ml , final concn.) were assayed, one non-injured, the other oxidatively stressed by incubation for up to 60 min at 37°C with $1 \text{ mM H}_2\text{O}_2 + 1 \text{ mM ascorbate} + 20 \mu\text{M FeCl}_2$. The extent of oxidant-induced Zn^{2+} mobilization was taken as the TPEN-inhibitable absorbance increase at 500 nm displayed by oxidatively stressed AMPD relative to the non-stressed enzyme [18].

Thiol-modification studies

AMPD used in the studies with thiol-modifying reagents was dialysed for 16 h at 4°C against $54 \text{ mM KH}_2\text{PO}_4/35 \text{ mM K}_2\text{HPO}_4$ (pH 6.5) containing 0.1 M KCl to ensure elimination of any residual 2-mercaptoethanol from the enzyme-isolation buffers [2]. The reaction mixture for DTNB modification was derived from Raggi et al. [12] and consisted of rabbit heart AMPD ($750 \mu\text{g/ml}$), 0.5 mM DTNB , 0.45 M KCl , and, where indicated, 3 M urea in 0.1 M potassium phosphate buffer (pH 7.8). The thiol-group titration was conducted at 37°C and was initiated on addition of DTNB. Formation of the thio-nitrobenzoate ion was monitored at 412 nm [19]. Corresponding reaction mixtures not containing enzyme were run in parallel as blanks for background absorbance correction. The mol-equiv. of DTNB-reactive thiol groups per mol of enzyme were calculated using the absorption coefficient $13.3 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [19] and the apparent holoenzyme molecular mass of 320 kDa [14]. In some cases during the course of the reaction, samples were withdrawn and assayed for AMPD activity. PAO (0.5 mM) and GSSG (up to 10 mM at various GSH/GSSG ratios) were allowed to react

with the dialysed enzyme (125 $\mu\text{g/ml}$) at 37 °C in the same buffer as used for the DTNB titration.

Re-activation of aged, oxidatively injured or chemically modified enzyme

Cardiac AMPD which had been aged, oxidatively stressed for up to 60 min with 1 mM H_2O_2 + 1 mM ascorbate + 20 μM FeCl_2 , or allowed to react with 0.5 mM DTNB for up to 30 min (above) was dialysed for 24 h at 4 °C against several changes of 54 mM KH_2PO_4 /35 mM K_2HPO_4 (pH 6.5) containing 0.1 M KCl and either 15 mM 2-mercaptoethanol or 15 mM 2,3-dimercapto-propanol. Parallel enzyme samples treated identically but dialysed against the same buffer without reducing agent were the controls. In each case, AMPD before and after dialysis was assayed for activity.

Assay of S-thiolation

Parallel AMPD samples (200 $\mu\text{g/ml}$) were incubated at 37 °C with 1 mM [^{35}S]cystine in 50 mM imidazole/HCl buffer containing 0.1 M KCl, one of which also contained 1 mM H_2O_2 + 1 mM ascorbate + 20 μM FeCl_2 to establish oxidative stress. After 60 min, the enzyme was precipitated by adding ice-cold trichloroacetic acid (9 % v/v, final concn.) followed by centrifugation in a Microfuge. The enzyme pellet was washed three times with ice-cold 9 % trichloroacetic acid, solubilized in 5 M NaOH, and analysed by liquid-scintillation spectrometry for incorporation of ^{35}S label as the index of mixed-disulphide formation [20].

Protein determination

Protein was quantified colorimetrically with IgG standard [2].

RESULTS

Influence of oxidative-stress systems on cardiac AMPD activity

Exposure of rabbit heart AMPD to an H_2O_2 /ascorbate/iron (as either FeCl_2 or FeCl_3) oxidation system compromised catalytic activity (Table 1). The complete oxidative-stress system elicited about 80 % deactivation of AMPD within 10 min. Incomplete oxidation systems or the individual reactants had comparatively little, if any, effect, ruling out non-specific effects of the system components used to establish oxidative stress.

The adverse influence of the oxidation system on cardiac AMPD activity depended on the concentration of each reactant (Figure 1). For example, a 5 min oxidative-stress period with 1 mM H_2O_2 + 1 mM ascorbate + 50 μM FeCl_2 and 25 $\mu\text{g/ml}$ AMPD elicited a virtually complete loss of enzyme activity, whereas the identical oxidation system with 2 μM FeCl_2 was a markedly less efficient injury stimulus. The data in Figure 1 led us to select as our standard oxidative-stress system 1 mM H_2O_2 + 1 mM ascorbate + 20 μM FeCl_2 .

AMPD deactivation kinetics could be well resolved. Virtually total loss of enzyme activity occurred within about 15 min, but only in the presence of the complete oxidation system (Figure 2). The activity loss was rapid (about 60 % within the initial 2 min of oxidative stress) and continued thereafter at a lower rate. Substitution of GSH for ascorbate in the reaction elicited a more gradual deactivation. A fourfold increase in AMPD concentration likewise attenuated the rate and extent of oxidative AMPD injury.

Table 1 Effect of oxidative-stress systems on cardiac AMPD activity

Rabbit heart AMPD (25 $\mu\text{g/ml}$) was incubated at 37 °C for 10 min in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl and a specified addition, whereon either EDTA (250 μM) or, in the case of the H_2O_2 -treated enzyme, catalase (50 units/ml) was added. AMP (5 mM) was next introduced, and the incubation was continued for an additional 5 min. AMPD activity was then assayed and expressed relative to that of a contemporaneous control enzyme reaction without addition. The final concentration of each addition was: H_2O_2 , 1 mM; ascorbate, 1 mM; FeCl_2 or FeCl_3 , 20 μM . Data are means of at least three independent determinations; the range about each mean is < 10 %.

Addition	AMPD activity (% of control)
None (control)	100
H_2O_2 + ascorbate + FeCl_2	20
H_2O_2 + ascorbate + FeCl_3	22
Ascorbate + FeCl_2	88
H_2O_2	80
Ascorbate	100
FeCl_2 or FeCl_3	100

Kinetic and regulatory properties of oxidatively stressed cardiac AMPD

Exposure of rabbit heart AMPD to oxidative stress profoundly altered the kinetic and allosteric properties of the enzyme. Relating catalytic activity to substrate (AMP) concentration by the Lineweaver–Burk method [15] yielded a linear double-reciprocal plot for both oxidatively stressed and non-injured rabbit heart AMPD (Figure 3). In both cases, the linear-regression coefficient was ≥ 0.98 , and the apparent K_m values did not differ (oxidatively stressed, 7.6 mM AMP; non-injured, 7.5 mM AMP). However, the apparent V_{max} of oxidatively stressed cardiac AMPD (1.4 $\mu\text{mol/min per mg}$) was some 7-fold less than that (10.3 $\mu\text{mol/min per mg}$) of the non-injured enzyme.

During oxidative stress, cardiac AMPD lost its responsiveness to nucleotide effectors. For example, the injured enzyme was no longer allosterically activated by ATP and retained the initial relationship to increasing non-saturating substrate levels on a Michaelis plot as did non-stressed AMPD in the absence of ATP (Figure 3). Similar results were obtained with ADP as allosteric activator of GTP as inhibitor [2] (results not shown). The presence of a nucleotide effector (ATP, ADP or GTP) at the start of the oxidative-stress period significantly attenuated oxidative AMPD deactivation (Table 2). However, this protection was unrelated to the effector property of the nucleotide. ATP and GTP afforded the same degree of protection against oxidative AMPD deactivation at a concentration at which the former is an allosteric activator of cardiac AMPD and the latter is an inhibitor [2,14]. The non-specificity may also be appreciated from the protection exerted by uridine nucleotides, which do not modulate rabbit heart AMPD [2] (Table 2). It is likely that the apparent protection of AMPD by nucleotides reflects an antioxidant effect as a result of iron chelation from the mixed-function oxidation reaction [21].

Inhibition of oxidative injury to rabbit heart AMPD

Several agents with antioxidant properties [5–7,9] were tested for their ability to ameliorate the oxidative loss of cardiac AMPD activity (Table 3). The metal chelators EDTA and desferrioxamine afforded complete protection against oxidative injury, whereas low-molecular-mass mercaptans (e.g. cysteine)

Figure 1 Influence of the concentrations of oxidation-system reactants on cardiac AMPD injury

Samples of rabbit heart AMPD (25 μg/ml) were incubated at 37 °C for 5 min in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl and one indicated concentration of H₂O₂ + 1 mM ascorbate + 20 μM FeCl₂ (a), 1 mM H₂O₂ + one indicated concentration of ascorbate + 20 μM FeCl₂ (b) or 1 mM H₂O₂ + 1 mM ascorbate + one indicated concentration of FeCl₂ (c). The oxidative-stress period was terminated by adding 50 units/ml catalase. AMPD activity was then determined and expressed relative to that of a contemporaneous control enzyme sample not oxidatively stressed. Data are means of three independent experiments performed in triplicate; the range about each mean is < 10%.

Figure 2 Kinetics of AMPD activity loss caused by oxidative stress

Sample of rabbit heart AMPD were incubated at 37 °C for up to 30 min in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl and one of the following: □, 1 mM H₂O₂; Δ, 1 mM H₂O₂ + 20 μM FeCl₂; ○, 1 mM ascorbate + 1 mM H₂O₂; ▲, 1 mM H₂O₂ + 1 mM GSH + 20 μM FeCl₂; ■ and ●, 1 mM H₂O₂ + 1 mM ascorbate + 20 μM FeCl₂. The final AMPD concentration was 25 μg/ml in all cases except ■, where it was 100 μg/ml. Samples were removed from each reaction mixture at the indicated times, 50 units/ml catalase or 250 μM EDTA was added, and the samples were assayed for AMPD activity. Activity in each case was expressed relative to a control enzyme sample incubated contemporaneously without oxidative-stress components. Data are means of six independent experiments performed in triplicate; the range about each mean is < 10%.

were less protective. Scavengers of hydroxyl radicals (e.g. mannitol), chain-breaking antiperoxidative agents (e.g. DPPD or Trolox) and SOD were not protective.

Molecular properties of oxidatively stressed cardiac AMPD

A series of experiments was conducted to evaluate whether exposure of rabbit heart AMPD to the oxidation system elicited molecular alterations detrimental to the catalytic and allosteric properties of the enzyme. Combined SDS/PAGE and size-exclusion h.p.l.c. analysis demonstrated that the enzyme, when oxidatively stressed to negligible catalytic activity, behaved as a tetramer with a subunit molecular mass of ~ 81 kDa and a holoenzyme molecular mass of ~ 320 kDa, identical with the non-injured enzyme (results not shown). Neither the immunoreactivity nor the response of the enzyme to controlled proteolysis was affected by oxidative stress (results not shown). Oxidative stress did not lead to the net introduction of NaB³H₄-reducible carbonyl groups into the amino acid residues of the enzyme, nor did it liberate the active-site Zn²⁺ (results not shown).

Enzyme thiol status and oxidative AMPD inactivation

Altered thiol status is responsible for the oxidative inactivation of selected enzymes [7,22], and the activity of some non-cardiac forms of AMPD is sensitive to the thiol-modifying reagent DTNB [12,13]. Consequently we studied whether exposure of cardiac AMPD to the H₂O₂/ascorbate/iron oxidation system altered the thiol complement of the enzyme. Non-injured rabbit heart AMPD exhibited about 9 mol of DTNB-reactive thiol groups per mol of denatured enzyme and about 6 mol of DTNB-reactive thiol groups per mol of non-denatured enzyme (Figure 4). Oxidatively stressed inactive AMPD displayed 3 less DTNB-reactive thiol groups, even when denatured. The denatured apoenzyme had a total of 12 mol of titratable thiol groups/mol.

To gain insight into the chemical nature of the thiol-group loss occurring during oxidative stress, modifying reagents were used. Both thiol alkylation with DTNB and oxidative stress reduced the catalytic activity of cardiac AMPD by at least 90% in

Figure 3 Kinetic and allosteric properties of oxidatively stressed rabbit heart AMPD

(a) Enzyme samples (25 $\mu\text{g/ml}$) in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl were either oxidatively stressed by incubation at 37 °C for 5 min with 1 mM H_2O_2 + 1 mM ascorbate + 20 μM FeCl_2 (○) or not (●). The oxidative-stress period was terminated by adding 250 μM EDTA, and portions of oxidatively stressed and non-injured enzyme were allowed to react with various concentrations of substrate (AMP) for up to 10 min at 37 °C. AMPD activity was then assessed, and the data were expressed as the Lineweaver-Burk transforms shown. (b) Enzyme samples oxidatively stressed (●) or non-injured (○, ■) (as in a) were allowed to react with various concentrations of substrate (AMP) for up to 10 min at 37 °C in the absence (○) or presence (■, ●) of 1 mM ATP. AMPD activity is presented in a Michaelis plot as a function of AMP concentration. Data are means of four experiments performed in triplicate; the range about each mean is < 10%.

30 min. However, the deactivation kinetics were distinct in each case (Figure 5). After 10 min of oxidative stress, for example, the apparent AMPD activity was 1.1 ± 0.1 $\mu\text{mol/min per mg}$, whereas it was 2.5 ± 0.2 $\mu\text{mol/min per mg}$ after a 10 min incubation with DTNB (means $\pm$ S.D.; $n = 9$). Higher concentrations of DTNB did not alter the deactivation profile in the latter case (results not shown). AMPD activity lost on either long-term storage of the enzyme in solution [2] or reaction with DTNB was fully recoverable on dialysis against 2-mercaptoethanol, which failed to re-activate the oxidatively injured enzyme (Table 4). Again in contrast with oxidative AMPD deactivation, prolonged enzyme exposure to PAO, a reagent specific for vicinal dithiols [23], only modestly (by 30 % at most) inhibited cardiac

Table 2 Influence of nucleotide effectors on oxidative injury to cardiac AMPD

Rabbit AMPD (25 $\mu\text{g/ml}$) was incubated at 37 °C for 20 min in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl and 1 mM nucleotide, as indicated. Oxidative stress was then imposed by adding 1 mM H_2O_2 + 1 mM ascorbate + 20 μM FeCl_2 and incubating for 5 min, whereupon the oxidative-stress period was terminated by adding catalase (50 units/ml). Substrate (5 mM AMP) was next added, and the incubation was continued for 5 min. AMPD activity was then assessed and expressed as residual activity relative to the appropriate contemporaneous enzyme reaction containing nucleotide, but not oxidatively stressed. Data are means of three independent determinations; the range about each mean is < 10%.

Nucleotide	Effector action	AMPD activity (% of residual)
None		25
ATP	Allosteric activator	80
ADP	Allosteric activator	53
GTP	Inhibitor	78
UTP	None	80
UDP	None	60
UMP	None	41

Table 3 Inhibition of oxidative injury to cardiac AMPD

Rabbit heart AMPD (25 $\mu\text{g/ml}$) in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl and one of the test agents indicated was oxidatively stressed by incubation with 1 mM H_2O_2 + 1 mM ascorbate + 20 μM FeCl_2 for 5 min at 37 °C. The oxidative-stress period was terminated by adding catalase (50 units/ml), and the incubation was continued for 5 min in the presence of AMP (5 mM). AMPD activity was then assessed and expressed relative to the respective contemporaneous control enzyme reaction which had not been oxidatively stressed. Data are means of three independent determinations; the range about each mean is < 10%.

Test agent	AMPD activity (% of control)
None	25
EDTA (250 μM)	100
Desferrioxamine (100 μM)	100
Cysteine (5 μM)	47
Mannitol (100 μM)	29
DPPD or Trolox (50 μM)	20
SOD (10 units/ml)	17

AMPD, and the activity was fully restored by 2,3-dimercaptopropanol (Table 4).

Exposure of rabbit heart AMPD to GSSG at various GSH/GSSG redox ratios reduced enzyme activity (Figure 6), and full activity was restored by 2,3-dimercaptopropanol (Table 4), suggesting that the enzyme is S-thiolatable via thio-disulphide exchange [20]. Formation of protein thiyl radicals from single thiol groups in response to oxidative stress is a well-recognized mechanism promoting S-thiolation of at least some oxidant-sensitive target proteins to generate mixed disulphides [20]. However, inactivation of rabbit heart AMPD by a metal-catalysed oxidation system was not accompanied by mixed-disulphide formation with [^{35}S]cysteine beyond a basal level of 0.4 nmol of protein-bound cystine per mol of holoenzyme.

DISCUSSION

Identification of tissue injury targets at molecular resolution is essential to understand the pathobiology of oxidative stress and design appropriate therapeutic modalities [24]. The present work constitutes the initial identification of AMPD, a soluble protein

Figure 4 Quantification of thiol groups in native, denatured and oxidatively stressed cardiac AMPD

Parallel samples of rabbit heart AMPD (25 μ g/ml) were either oxidatively stressed by incubation at 37 °C for 30 min with 1 mM H_2O_2 + 1 mM ascorbate + 20 μ M $FeCl_2$ (●) or not (■, ▲). Enzyme in each sample was then concentrated by microdialysis to 750 μ g/ml and allowed to react with 0.5 mM DTNB in the absence (▲) or presence (■, ●) of 3 M urea. The DTNB reaction was monitored to apparent completion in a kinetic spectrophotometer. Absorbance values in each case were converted into mol of thiol groups/mol of enzyme using an absorption coefficient at 412 nm of $13.3 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the DTNB–thiol complex and a holoenzyme molecular mass of 320 kDa.

Figure 5 Differential kinetics of the loss of cardiac AMPD activity caused by either oxidative stress or DTNB modification

Parallel enzyme samples (25 μ g/ml) at 37 °C in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.1 M KCl were either oxidatively stressed by incubation with 1 mM H_2O_2 + 1 mM ascorbate + 20 μ M $FeCl_2$ (●) or allowed to react with 0.5 mM DTNB (○). At the times indicated, samples were withdrawn from each reaction mixture, 250 μ M EDTA was added, and the samples were immediately assayed for enzyme activity. The activity was expressed relative to control AMPD which was neither oxidatively stressed nor modified with DTNB. Data are means of three independent experiments performed in triplicate; the range about each mean is < 10%.

Table 4 Re-activation of aged, chemically modified or oxidatively stressed cardiac AMPD

Samples of rabbit heart AMPD dialysed for 16 h at 4 °C against 54 mM KH_2PO_4 /35 mM K_2HPO_4 (pH 6.5) containing 0.1 M KCl were treated as indicated, after which a portion of each was taken for activity determination. The remainder was dialysed for 24 h at 4 °C against the same buffer containing either 15 mM 2-mercaptoethanol or 2,3-dimercaptopropanol, and the activity was reassessed. Enzyme activity is expressed relative to a contemporaneous control AMPD sample subjected to enzyme assay only. Data are means of three independent determinations. The range about each mean is < 10%. n.d., not determined.

Treatment	AMPD activity (% of control)		
	After treatment	After treatment and dialysis	
		2-Mercapto-ethanol	2,3-Dimercapto-propanol
None	100	100	100
Aged*	25	100	n.d.
DTNB†	9	100	n.d.
Oxidative stress‡	12	10	12
PAO§	70	n.d.	100
GSSG¶	38	n.d.	100

* Stored at 4 °C for 1 month.

† Allowed to react with 0.5 mM DTNB for 60 min, 37 °C.

‡ Incubated with 1 mM H_2O_2 + 1 mM ascorbate + 20 μ M $FeCl_2$ for 60 min, 37 °C.

§ Allowed to react with 0.5 mM PAO for 60 min, 37 °C.

¶ Allowed to react with 5 mM GSSG for 60 min, 37 °C.

Figure 6 Response of cardiac AMPD to various GSH/GSSG redox ratios

Enzyme (125 μ g/ml) was incubated for 60 min at 30 °C in 0.1 M potassium phosphate buffer containing 0.45 M KCl and 1 mM (●), 5 mM (■) or 10 mM (▲) total [GSH + GSSG] at the [GSSG]/[GSH + GSSG] ratios indicated. The samples were then immediately assayed for enzyme activity relative to control AMPD incubated in the absence of GSH + GSSG. Data are means of three independent experiments performed in duplicate; the range about each mean is < 10%.

found ubiquitously in eukaryotes [25], as an oxidant-sensitive S-thiolatable enzyme. Specifically, oxidative stress imposed by an H_2O_2 /ascorbate/iron system acutely compromised the overall catalytic activity of rabbit heart AMPD, abolished its allosterism toward nucleotide effectors and ultimately led to its irreversible inactivation.

The precise chemistry underlying the effects of oxidative stress on any given protein is probably complex [11]. Nonetheless, the injury profile of a specific target enzyme can be used to gain mechanistic insight into the oxidative damage [26]. The lack of inhibition of oxidative AMPD inactivation by radical scavengers or chain-breaking antioxidants argues against a Fenton-type mechanism [27] with superoxide as intermediate to generate hydroxyl radicals which attack the enzyme, forming protein peroxy radicals. Additional evidence against such a mechanism is the lack of AMPD fragmentation during oxidative stress, which would have occurred consequent on peroxy radical decomposition [28]. A radical-based injury mechanism is also inconsistent with the finding that the mixed-function oxidation reaction did not potentiate AMPD S-thiolation, which would have occurred through protein thiyl radicals [20].

Oxidative inactivation of rabbit heart AMPD did not alter the molecular mass, tetrameric nature, immunoreactivity or trypsinolytic pattern of the enzyme. Nor was the oxidative injury manifested as carbonyl formation or Zn^{2+} release. Such an injury profile implies that subtle changes in discrete amino acids/functional moieties essential to catalytic and regulatory competency had occurred in response to oxidative stress. Cardiac AMPD sensitivity to S-thiolation and the monothiol-reactive reagent DTNB (Figure 5), the irreversible nature of the oxidative inactivation of the enzyme (Table 4), and the net thiol-group loss on oxidative inactivation (Figure 4) strongly suggest that essential SH groups were converted into stable higher-oxidation states such as $-\text{SO}_2\text{H}$ (sulphinic acid) or $-\text{SO}_3\text{H}$ (sulphonic acid) on oxidant insult [29]. These data also offer compelling evidence that thiol status is a determinant of the catalytic competency of the cardiac AMPD isoform. A role has been proposed for AMPD thiol groups in maintenance of conformation for efficient catalysis [12,13]. If a conformational change caused by altered thiol redox status were at the basis of the oxidative AMPD inactivation detailed here, it did not affect recognition of AMPD by either polyclonal antibodies or trypsin. Subtle redox-linked conformational changes in certain proteins have been documented [30].

Neither the thiol content of an enzyme nor its susceptibility to oxidation *in vitro* are absolute predictors of its *in situ* response to oxidative stress [7]. Oxidative protein injury is considered an important biologically relevant process [11,29] which may contribute to disease states having an oxidative-stress component, such as myocardial ischaemia-reperfusion [4,24]. Therefore our identification of rabbit heart AMPD as an oxidant-sensitive enzyme forms a biochemical basis for the study of the modulation of cardiac AMPD activity *in vivo* in response to oxidative stress/cardiac ischaemia. It remains to be determined whether

other tissue changes consequent on myocardial ischaemia-reperfusion (e.g. nucleotide catabolism [1]) have the ability to modulate cardiac AMPD activity *in situ*. These possibilities have important implications because of both the number of disease states potentially involving oxidative stress to cardiac muscle [4,24] and the critical role of myocardial adenylate charge and adenosine production from 5'-nucleotidase in the recovery of pump function after ischaemic injury [1,7].

REFERENCES

- Sandhu, G. S., Burrier, A. C. and Janero, D. R. (1993) *Am. J. Physiol.* **264**, H1249–H1256
- Thakkar, J. K., Janero, D. R., Yarwood, C., Sharif, H. M. and Hreniuk, D. (1993) *Biochem. J.* **290**, 335–341
- Van Belle, H., Wynants, J. and Goossens, F. (1988) *Basic Res. Cardiol.* **80**, 653–660
- Janero, D. R. (1991) *Free Radical Biol. Med.* **10**, 315–324
- Janero, D. R., Hreniuk, D. and Sharif, H. M. (1993) *J. Cell. Physiol.* **155**, 494–504
- Janero, D. R., Hreniuk, D., Sharif, H. M. and Prout, K. C. (1993) *Am. J. Physiol.* **264**, C1401–C1410
- Janero, D. R., Hreniuk, D. and Sharif, H. M. (1994) *Am. J. Physiol.* **266**, C179–C188
- Bak, M. I. and Ingwall, J. S. (1994) *J. Clin. Invest.* **93**, 40–49
- Janero, D. R., Hreniuk, D. and Sharif, H. M. (1991) *J. Cell. Physiol.* **149**, 347–364
- Lesnefsky, E. J., Dauber, T. M. and Horwitz, L. D. (1991) *Circ. Res.* **68**, 605–613
- Stadtman, E. R. (1993) *Annu. Rev. Biochem.* **62**, 797–821
- Raggi, A., Ranieri, M., Ronca, G. and Rossi, C. A. (1972) *Biochim. Biophys. Acta* **271**, 102–113
- Nagel-Starczynowska, C. and Kaletha, K. (1993) *Biochim. Biophys. Acta* **1164**, 261–267
- Thakkar, J. K., Janero, D. R., Yarwood, C. and Sharif, H. M. (1993) *Biochem. J.* **291**, 523–527
- Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
- Thakkar, J. K., Janero, D. R., Sharif, H. M., Hreniuk, D. and Yarwood, C. (1994) *Biochem. J.* **300**, 359–363
- Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S. and Stadtman, E. R. (1990) *Methods Enzymol.* **186**, 464–478
- Fliss, H. and Menard, M. (1991) *Arch. Biochem. Biophys.* **287**, 175–179
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Gilbert, H. F. (1984) *Methods Enzymol.* **107**, 330–351
- Tadolini, B. (1989) *Free Rad. Res. Commun.* **5**, 237–243
- Gopalakrishna, R., Chen, Z. H. and Gundimeda, U. (1993) *J. Biol. Chem.* **268**, 27180–27185
- Stoken, L. A. and Thompson, R. H. S. (1946) *Biochem. J.* **40**, 529–535
- Janero, D. R. (1994) in *Natural Antioxidants in Human Health and Disease* (Frei, B., ed.), pp. 411–445, Academic Press, San Diego
- Ogasawara, N., Goto, H. and Watanabe, T. (1975) *Biochim. Biophys. Acta* **403**, 530–537
- Pacifici, R. E. and Davies, K. J. A. (1990) *Methods Enzymol.* **186**, 485–502
- Miura, T., Muraoka, S. and Ogiso, T. (1993) *Biochem. Mol. Biol. Int.* **31**, 125–133
- Davies, K. J. A. (1987) *J. Biol. Chem.* **262**, 9895–9901
- Finch, J. W., Crouch, R. K., Knapp, D. R. and Schey, K. L. (1993) *Arch. Biochem. Biophys.* **305**, 595–599
- Mantele, W. (1990) *Eur. J. Biochem.* **187**, 565–572