Reversible redox-dependent modulation of mitochondrial aconitase and proteolytic activity during *in vivo* cardiac ischemia/reperfusion

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Prooxidents can induce reversible inhibition or irreversible inactivation and degradation of the mitochondrial enzyme aconitase. Cardiac ischemia/reperfusion is associated with an increase in mitochondrial free radical production. In the current study, the effects of reperfusion-induced production of prooxidants on mitochondrial aconitase and proteolytic activity were determined to assess whether alterations represented a regulated response to changes in redox status or oxidative damage. Evidence is provided that ATP-dependent proteolytic activity increased during early reperfusion followed by a time-dependent reduction in activity to control levels. These alterations in proteolytic activity paralleled an increase and subsequent decrease in the level of oxidatively modified protein. *In vitro* data supports a role for prooxidants in the activation of ATP-dependent proteolytic activity. Despite inhibition during early periods of reperfusion, aconitase was not degraded under the conditions of these experiments. Aconitase activity exhibited a decline in activity followed by reactivation during cardiac reperfusion. Loss and regain in activity involved reversible sulfhydryl modification. Aconitase was found to associate with the iron binding protein frataxin exclusively during reperfusion. *In vitro*, frataxin has been shown to protect aconitase from $[4Fe\text{-}4S]^{2+}$ cluster disassembly, irreversible inactivation, and, potentially, degradation. Thus, the response of mitochondrial aconitase and ATP-dependent proteolytic activity to reperfusion-induced prooxidant production appears to be a regulated event that would be expected to reduce irreparable damage to the mitochondria.

Highly reactive oxygen derived free radicals, such as superoxide anion ($O_2^{\bullet-}$) and the prooxidant hydrogen peroxide (H_2O_2), can interact with a variety of cellular components, altering both structure and function (1). Although evidence for these reactions has long been sought as indication of free radical involvement in degenerative disorders, recent evidence indicates that these processes also participate in the regulation of cellular function (1, 2). This finding is exemplified by the discovery of enzymatic systems, such as thioredoxin reductase/thioredoxin (3), glutaredoxin (4), methione sulfoxide reductase (5), and sulfiredoxin (6), which catalyze reversal of oxidative modifications to protein and restoration of protein function. Additionally, receptor- and enzyme-mediated systems exist that catalyze the production of free radicals in response to changes in extracellular and intracellular factors (1, 2). It is therefore important that free radicals produced during physiological and pathophysiological conditions be investigated not simply for their potential to carry out damaging processes but also to induce appropriate alterations in response to changes in cellular homeostasis.

Reduction and/or cessation of blood flow to myocardial tissue, termed ischemia, occurs primarily as a result of formation of atherosclerotic lesions in the coronary arteries. Resolution of the ischemic event by reintroduction of blood flow is the sole means for preventing cell death and irreparable derangements in cardiac function (7–9). Nevertheless, reperfusion is associated with functional alterations and, in many cases, further loss in myocardial function (7–9). It has long been recognized that reoxygenation of myocardial tissue results in an increase in the production of oxygen-based free radical species (7–9). Based on their reactive properties, these species have been hypothesized to play a role in the loss of cardiac function during reperfusion. Mitochondria, a major subcellular source of oxygen radicals during reperfusion, exhibit decrements in activity attributed, in part, to oxidative modifications to key metabolic enzymes (10). Aconitase and α-ketoglutarate dehydrogenase, which are known to be susceptible to oxidative inactivation *in vitro* (11–19) have been reported to decline in activity during *in vivo* cardiac ischemia/reperfusion (20).

Loss in aconitase activity is commonly used as a biomarker of oxidative damage due to the susceptibility of the enzyme's $[4Fe\text{-}4S]^{2+}$ cubane cluster to oxidative disassembly (16–19). Nevertheless, we have recently shown that, when mitochondria are exposed to H_2O_2 *in vitro*, aconitase can undergo oxidative inhibition followed by reactivation upon resolution of the oxidative stress (15). If the oxidative stress is prolonged or of sufficient magnitude, reversible modulation of aconitase activity progresses to irreversible inactivation and ATP-dependent degradation (15). Thus aconitase is capable of participating in redox regulation and is itself a potential target of oxidative damage. The metabolic fate of aconitase depends on (*i*) the relative rates of mitochondrial free radical production and removal, (*ii*) the ability of components to reverse redox-dependent modifications; and (*iii*) mitochondrial capacity for protein degradation. In the present study, we investigated the effects of *in vivo* cardiac ischemia and varying durations of reperfusion on mitochondrial proteolytic capacity and the level and activity of aconitase to assess the progression of and relationships between specific oxidative events. We present evidence that mitochondrial aconitase and proteolytic activities are reversibly altered during cardiac reperfusion and discuss the potential regulatory role these processes play in the mitochondrial response to oxidative stress.

Materials and Methods

***In Vivo* Model of Coronary Occlusion/Reperfusion and Isolation of Cardiac Mitochondria.** As described ref. 20, Sprague–Dawley rats were anesthetized and ventilated, and, after midline thoracotomy and pericardiectomy, a ligature was placed around the left

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anterior descending coronary artery (LAD) close to its origin to induce coronary occlusion. Reflow was initiated by releasing the ligature. For each experimental condition tested, five rats were used. Experiments consisted of 0–90 min of sham-operated perfusion; 30 min of LAD occlusion; and 30 min of LAD occlusion, followed by 5, 15, 30, or 60 min of reperfusion. After each experimental protocol, hearts were removed and immediately rinsed in ice-cold homogenization buffer (210 mM mannitol/70 mM sucrose/1.0 mM EDTA/5.0 mM Mops, pH 7.4), and mitochondria were isolated by differential centrifugation as described in ref. 15.

Treatment of Isolated Mitochondria with a H_2O_2 Generating System. Isolated control mitochondria were diluted to 0.25 mg/ml in 125 mM KCl/5.0 mM KH_2PO_4, pH 7.25, and incubated with a H_2O_2 generating system for 30 min. As described in ref. 15, glucose/glucose oxidase was used to generate a steady-state level of 100 μM H_2O_2.

Assay of Aconitase. As described in ref. 15, mitochondria were diluted to 0.05 mg/ml in 25 mM KH_2PO_4, pH 7.25, containing 0.05% Triton X-100. Aconitase activity was assayed as the rate of $NADP^+$ reduction (340 nm) by isocitrate dehydrogenase upon addition of 1.0 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM $NADP^+$, and 1.0 unit/ml isocitrate dehydrogenase.

Measurement of Proteolytic Activity. Proteolytic activity was assayed as the rate of FITC-casein degradation (21, 22). Mitochondria were diluted to 5.0 mg/ml in assay buffer containing 10 mM $MgCl_2$, 1.0 mM DTT, 0.05% Triton X-100, and 50 mM Tris, pH 7.9, in the presence or absence of 8.0 mM ATP. Proteolysis of FITC-casein (5.0 μg) was then performed at 37°C. At incubation times of 0–60 min, a 20-μl aliquot was removed, and the protein was precipitated with 10% (wt/vol) TCA. The mixture was then centrifuged at 15,000 $\times$ *g* for 30 min at 4°C. The supernatant containing the peptide fragments was neutralized upon addition of 100 μl of 2.0 M potassium borate at pH 10. The level of peptide fragments was determined by spectrophotometric analysis (emission, 515 nm; excitation, 495 nm). Proteolytic activity was linear for 90 min under the assay conditions

Aconitase Immunopurification. Mitochondria were diluted to 5.0 mg/ml in 25 mM KH_2PO_4 at pH 7.25 containing 0.05% Triton X-100. Mitochondrial extracts (500 μg in 0.1 ml) were incubated with 10 μl of anti-aconitase polyclonal antibody at 4°C for 1 h. The mixture was then incubated with 50.0 μl of protein G Sepharose (Amersham Pharmacia Biosciences) for 16 h at 4°C. Samples were then centrifuged at 1,000 $\times$ *g* for 5.0 min, and the pellet was washed three times with PBS, pH 7.6, containing 1.0% Nonidet P-40. After resuspension in gel-loading buffer, SDS gel electrophoresis/Western blot analysis was performed.

Western Blot Analysis. Before analysis, samples were incubated in Laemmli sample buffer for 5.0 min at 100°C. Protein was then resolved on a 4–20% SDS/PAGE gel and electrotransferred onto a Hybond nitrocellulose membrane (Amersham Pharmacia Biosciences). Membrane-immobilized proteins were analyzed by using polyclonal antibodies specific to aconitase (15), Lon, and frataxin (G. Isaya, Mayo Clinic Foundation, Rochester, MN). Primary antibody binding was visualized by using peroxidase-conjugated secondary antibody and chemiluminescent substrate (SuperSignal West, Pierce).

Detection of Carbonylated Proteins. Carbonylated proteins were detected and analyzed after derivatization of protein carbonyl groups with a 2,4-dinitrophenylhydrazine OxyBlot kit (Chemicon). Western blot analyses were performed by using 5.0 μg of mitochondrial protein per lane and primary antibody against dinitrophenylhydrazone.

EPR Spectroscopy. Mitochondria isolated after each experimental protocol were placed in quartz EPR tubes (30 mg/ml) and frozen by immersion in liquid nitrogen. EPR spectra were recorded by using a Bruker ESP300 spectrometer operating at 9.45 GHz with a 10-G field modulation at 100 kHz. Measurements were carried out at 10 K by use of an Oxford liquid He flow cryostat. The microwave frequency was monitored by a frequency counter (model HP-5350, Hewlett–Packard) and the magnetic field strength was determined by an NMR gaussmeter (model ER-035M, Bruker, Billerica, MA). Each spectrum represents an average of 10 scans.

Results

Reperfusion-Induced Alterations in Mitochondrial Oxidatively Modified Protein and Protease Activity. Reperfusion is associated with a rapid and transient increase in the production of oxygen radicals (7–9). By using dinitrophenylhydrazine-reactive carbonyl groups as an index, evidence for oxidative modification to mitochondrial proteins was sought. As shown in Fig. 1*A*, the level of oxidatively modified protein increased during the first 5.0 min of reperfusion. With extended durations of reperfusion, protein-associated carbonyl groups returned to levels close to those observed in mitochondria isolated from instrumented control and ischemic hearts. As shown in Fig. 1*B*, mitochondrial ATP-dependent proteolytic activity increased $\approx$2-fold within the first 5.0 min of reperfusion and gradually returned to control values with longer periods of reperfusion (60 min). ATP-independent proteolytic activity in control mitochondria was $\approx$10-fold lower than ATP-dependent activity and exhibited no alterations during ischemia or reperfusion (data not shown). As shown in Fig. 1*C*, the gain and subsequent decline in proteolytic activity was not associated with a change in the level of Lon, a major ATP-stimulated mitochondrial protease. Treatment of isolated mitochondria with a H_2O_2 generating system as described in *Materials and Methods* resulted in a 1.6-fold (168 $\pm$ 34 to 275 $\pm$ 12 fluorescent units$\cdot$min$^{-1}\cdot$mg^{-1}) increase in ATP-dependent proteolytic activity. Oxidative stress encountered during myocardial reperfusion (7–9) may therefore serve to regulate mitochondrial proteolytic activity in a manner consistent with requirements for removal of oxidatively modified protein.

Alterations in Mitochondrial Aconitase Content, Activity, and Association with Frataxin As a Function of Cardiac Ischemia/Reperfusion. *In vitro* evidence indicates that aconitase is highly susceptible to prooxidant-induced inactivation (16–19). Additionally, oxidatively modified aconitase is a substrate of the Lon protease (23). Despite the transient increase in mitochondrial ATP-dependent proteolytic activity, the level of the aconitase did not change as a result of ischemia/reperfusion (Fig. 2*A*). In contrast, aconitase exhibited reversible modulation in activity in response to ischemia/reperfusion. At 5.0 min of reperfusion, aconitase activity was reduced to 65% of the value observed in mitochondria isolated from instrumented control animals (Fig. 2*B*). Extended periods of reperfusion resulted in restoration of enzyme activity (Fig. 2*B*). We have previously shown *in vitro* that when mitochondria are challenged with prooxidants, aconitase and the mitochondrial iron-binding protein frataxin interact. This interaction requires the enzyme's substrate citrate, protects the $[4Fe\text{-}4S]^{2+}$ cluster of aconitase from oxidant-induced disassembly, and is required for enzyme reactivation (24). Immunopurification of aconitase followed by Western blot analysis of frataxin revealed that the two proteins interacted exclusively during reperfusion (Fig.

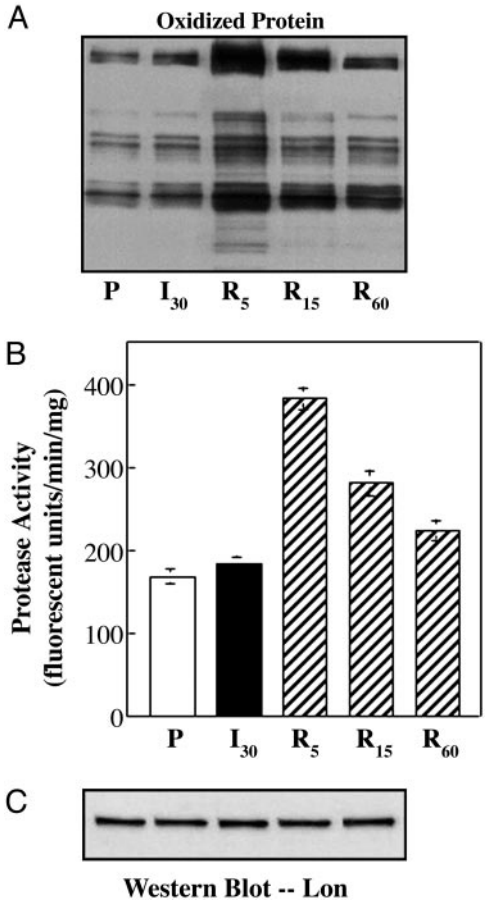

Fig. 1. Alterations in mitochondrial carbonylated proteins and proteolytic activity during coronary ischemia/reperfusion. Mitochondria were prepared from left ventricular free wall after 90 min of sham-operated perfusion (P), 30 min of occlusion (I_{30}), or 30 min of occlusion followed by 5, 15, or 60 min of reperfusion (R_5, R_{15}, and R_{60}, respectively). (*A*) Oxidatively modified proteins were detected upon treatment of mitochondrial extracts (5.0 μg of protein per lane) with 2,4-dinitrophenylhydrazine to derivatize protein carbonyl groups followed by Western blot analysis using polyclonal antibodies to the 2,4-dinitrophenyl moiety. (*B*) Mitochondrial proteolytic activity was measured as the rate of degradation of FITC-casein in the presence of 8.0 mM ATP. Values represent the mean ± SEM for five separate experiments. (*C*) The level of Lon protease was detected by Western blot analysis of mitochondrial protein (10.0 μg per lane) using a polyclonal antibody against Lon protease.

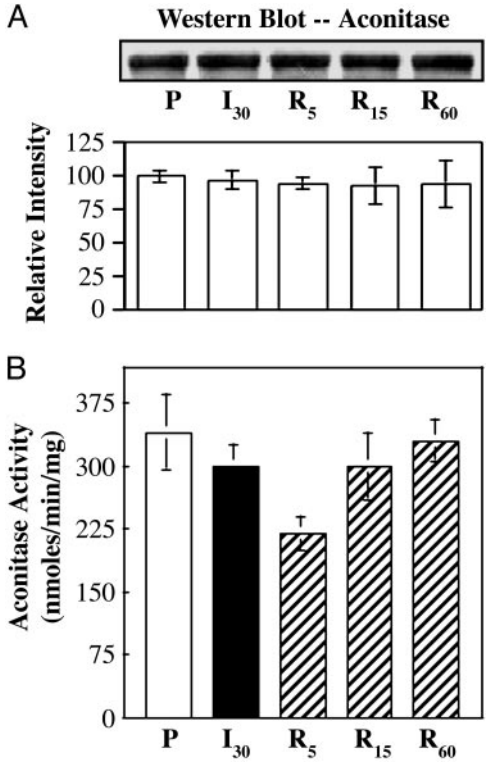

Fig. 2. Effect of coronary ischemia/reperfusion on mitochondrial aconitase content and activity. Mitochondria were prepared from left ventricular free wall after 90 min of sham-operated perfusion (P), 30 min of occlusion (I_{30}), or 30 min of occlusion followed by 5, 15, or 60 min of reperfusion (R_5, R_{15}, and R_{60}, respectively). (*A*) Mitochondrial protein (10.0 μg per lane) was subjected to Western blot analysis using a polyclonal antibody against aconitase. Densitometric analysis was performed (National Institutes of Health IMAGE software) on Western blots of mitochondrial protein isolated from four separate rats for each experimental protocol, and results are represented as mean ± SD with the mean for P intensity assigned a value of 100. Using a two-tailed *t* test, no statistically significant differences were observed between any of the experimental conditions indicated. (*B*) After each experimental protocol, mitochondria were disrupted and aconitase activity was determined as described in *Materials and Methods*. Values represent the mean ± SEM for five separate experiments.

3*A*). Based on previous *in vitro* results (24), the interaction between aconitase and frataxin upon reoxygenation likely protects aconitase from prooxidant-induced cluster disassembly, irreversible inactivation, and degradation.

Effect of Ischemia and Reperfusion on the $[4Fe\text{-}4S]^{2+}$ Cluster of Aconitase. Prooxidant-induced release of a labile α-Fe from the $[4Fe\text{-}4S]^{2+}$ cluster of aconitase results in loss in enzyme activity and in the formation of a $[3Fe\text{-}4S]^{1+}$ cluster that can be readily observed by EPR spectroscopy as a peak at $g = 2.02$ with a shoulder at $g = 2.014$ (10 K) (16, 17, 19). As shown in Fig. 3*B*, the relative loss in enzyme activity during the first 5.0 min of reperfusion did not correspond with a similar increase in the level of the $[3Fe\text{-}4S]^{1+}$ form of aconitase. As a comparison, we have previously reported that, in mitochondria (30 mg of protein per ml required for EPR analysis) treated for 40 s with 2.0 mM H_2O_2 in the presence of 2.0 mM citrate, ≈15% of aconitase is converted to the $[3Fe\text{-}4S]^{1+}$ cluster form of the enzyme (15). Ischemia/reperfusion failed to evoke a signal of comparable magnitude (Fig. 3*B*). Loss in activity does not therefore appear to be due to release of the labile iron from the enzyme's $[4Fe\text{-}4S]^{2+}$ cluster. This result is consistent with the potential for protection afforded by the enzyme's interaction with frataxin. It is interesting to note that ischemia results in the disappearance of the $[3Fe\text{-}4S]^{1+}$ cluster signal. Thus, a small fraction of aconitase appears to be in dynamic equilibrium between the $[3Fe\text{-}4S]^{1+}$ and $[4Fe\text{-}4S]^{2+}$ states of the protein.

Reversible Oxidation of Cysteine Residue on Aconitase Is Responsible for Modulation of the Enzyme Activity During Cardiac Reperfusion. The sulfhydryl moiety of cysteine readily interacts with prooxidants resulting in the oxidation to sulfenic, sulfinic, and sulfonic acid and in the formation of disulfide and mixed disulfide bonds (25–27). Of these modifications, sulfenic acids and disulfide bonds are reversible upon treatment with the reducing agent DTT. Treatment of solubilized mitochondria isolated from myocardial tissue that had undergone 30-min ischemia and 5.0 min of reperfusion with DTT resulted in full recovery in aconitase activity (Fig. 3*C*). In contrast, DTT had no significant effect on aconitase activity in mitochondria isolated from control, ischemic, or 15- and 60-min reperfused

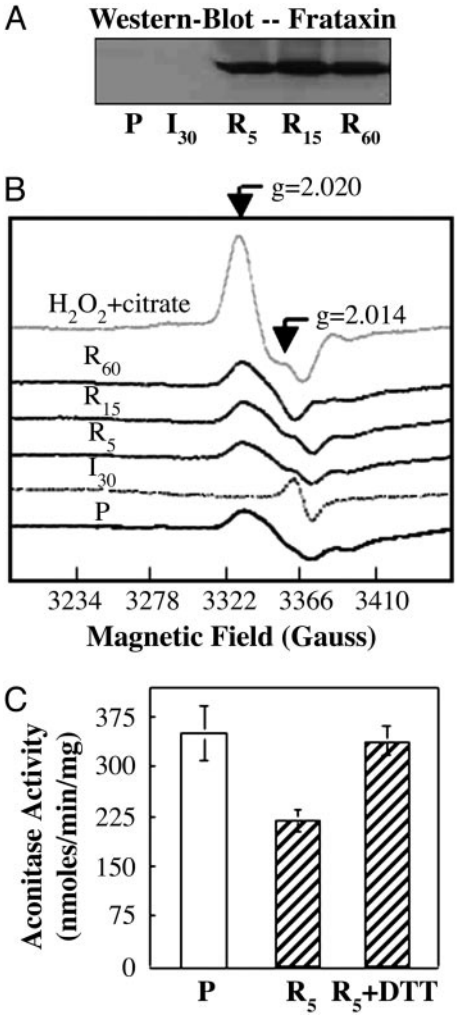

Fig. 3. Alterations in aconitase association with frataxin, EPR detectable $[3Fe\text{-}4S]^{+1}$ cluster, and oxidant-derived sulhydryl modifications during coronary ischemia/reperfusion. (*A*) Mitochondria were prepared from left ventricular free wall after 90 min of sham-operated perfusion (P), 30 min of occlusion (I_{30}), or 30 min of occlusion followed by 5, 15, or 60 min of reperfusion (R_5, R_{15}, and R_{60}, respectively). Mitochondria were disrupted in 0.05% Triton X-100, and immunoprecipitation was performed by using a specific antiserum raised against rat aconitase. Immunoprecipitated protein was then evaluated by Western blot analysis using polyclonal antibody specific to frataxin. (*B*) Mitochondria (30 mg/ml) (P, solid black line; I_{30}, dashed black line; and R_5, R_{15}, and R_{60}, solid black lines) were analyzed by EPR spectroscopy as described in *Materials and Methods*. To obtain a known EPR spectrum for the $[3Fe\text{-}4S]^{+1}$ cluster of aconitase, intact mitochondria (30 mg of protein per ml) were incubated with 2.0 mM H_2O_2 in the presence of 2.0 mM citrate for 40 s (solid gray line). (*C*) Mitochondria were prepared from left ventricular free wall after 90 min of sham-operated perfusion or 30 min of occlusion followed by 5 min of reperfusion. Mitochondria were then solubilized in 0.05% Triton X-100 and incubated with 5.0 mM DTT for 1.0 min before measurement of aconitase activity. Values represent the mean ± SEM for five separate experiments.

tissue (data not shown). Reperfusion induced inhibition and subsequent reactivation of aconitase therefore involves reversible oxidative modification of a critical sulfhydryl residue(s) on the protein. It has previously been shown that modification of a cysteine residue at or near the active site with the sulfhydryl reactive compound *N*-ethylmaleimide inactivates purified mitochondrial aconitase (28–30). This residue is therefore a likely candidate for reversible redox-dependent modulation in enzyme activity.

Discussion

Investigations into molecular events involving an increase in the level of free radical species, particularly in pathophysiological settings, such as ischemia/reperfusion, have long been focused on identifying oxidative damage. Links have been sought, with varying success, by using antioxidants to diminish functional alterations (7–9). The unique aspect of the current findings is that ischemia/reperfusion-induced alterations in the redox state of the mitochondria differentially modulate certain mitochondrial components. The potential for regulation is indicated not simply by the reversibility of these processes but also by a response consistent with the cellular and mitochondrial environment. Mitochondrial proteolytic activity increased during reperfusion coincident with the rapid and transient burst in oxygen free radical production. Treatment of isolated mitochondria with a H_2O_2 generating system resulted in the stimulation of proteolytic activity implicating prooxidants as potential activating species during reperfusion. The appearance and subsequent disappearance of oxidatively modified protein during cardiac reperfusion paralleled the increase and decrease in ATP-dependent proteolytic activity. Therefore, prooxidant-induced activation of mitochondrial proteolytic activity during reperfusion could represent a regulated response to remove altered forms of protein (31–33).

In contrast to proteolytic activity, aconitase exhibited a loss and regain in activity during cardiac reperfusion. Reversible oxidation of a cysteine residue(s) is implicated in this process based on the fact that DTT reactivated reperfusion-inhibited aconitase. Loss in aconitase activity could reduce NADH production, limiting electron flow and thus oxygen radical production by a compromised electron transport chain (34). Diminished aconitase activity may also reduce the proton gradient and, thus, the Ca^{2+} overload and/or reflect a reduction in energy demand. Reactivation would occur when electron transport deficits and, thus, oxygen radical production are diminished. The potential beneficial roles of redox regulation may be easily overlooked if the duration of reperfusion is short or if prolonged ischemia results in the progression to irreversible alterations in function. These considerations are not without precedence. Preconditioning, a series of brief periods of ischemia and reperfusion, protects the myocardium from deleterious events associated with extended durations of ischemia and reperfusion (7–9). Cardioprotection afforded by preconditioning appears mediated, in part, by the production of oxygen radicals (35–40) and is associated with reductions in Ca^{2+} overload (7, 41–43) and free radical production (44) during extended periods of ischemia/reperfusion.

In vitro studies with purified protein have demonstrated that aconitase can be inactivated through oxidant-induced release of a labile α-Fe from the enzyme's $[4Fe\text{-}4S]^{2+}$ cluster (16–19). Additionally, the Lon protease was shown to degrade aconitase that had undergone extensive oxidative modification (23). These events are likely terminal in the dynamic response of aconitase to alterations in redox status. We have shown *in vitro* that aconitase can undergo reversible oxidative inhibition in the absence of alterations in the enzyme's $[4Fe\text{-}4S]^{2+}$ cluster (15). Reversible inhibition appears dependent on the interaction of aconitase with the mitochondrial iron-binding protein frataxin. *In vitro* evidence indicates that frataxin can act as a chaperone protein, protecting aconitase from prooxidant-induced disassembly of the $[4Fe\text{-}4S]^{2+}$ cluster, and that frataxin is required for reversible modulation of enzyme activity (24). Nevertheless, depending on the magnitude and duration of the oxidative stress, reversible inhibition can progress to irreversible inactivation through $[4Fe\text{-}4S]^{2+}$ cluster disassembly, carbonylation, and ATP-dependent degradation (15). In the *in vivo* scenario of ischemia/reperfusion, there are likely numerous variables, such

as duration of ischemia, nutritional status, seasonal alterations, and age, that affect the balance between reversible and irreparable loss of mitochondrial function. The interaction of frataxin with aconitase and the degree of oxidative stress resulting from the conditions of ischemia/reperfusion used in the current study are likely factors favoring reversible modulation of aconitase activity rather than the progression to irreversible inactivation and degradation, despite an increase in ATP-dependent proteolytic activity.

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