Published in final edited form as:

Nitric Oxide. 2009 May ; 20(3): 135–142. doi:10.1016/j.niox.2008.12.001.

The Resistance of Electron Transport Chain Fe-S Clusters to Oxidative Damage during the Reaction of Peroxynitrite with Mitochondrial Complex II and Rat Heart Pericardium

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Abstract

The effects of peroxynitrite and nitric oxide on the iron-sulfur clusters in complex II (succinate dehydrogenase) isolated from bovine heart have been studied primarily by EPR spectroscopy and no measurable damage to the constitutive 2Fe-2S, 3Fe-4S, or 4Fe-4S clusters was observed. The enzyme can be repeatedly oxidized with a slight excess of peroxynitrite and then quantitatively re-reduced with succinate. When added in large excess, peroxynitrite reacted with at least one tyrosine in each subunit of complex II to form 3-nitrotyrosines, but activity was barely compromised. Examination of rat-heart pericardium subjected to conditions leading to peroxynitrite production showed a small inhibition of complex II (16%) and a greater inhibition of aconitase (77%). In addition, experiments performed with excesses of sodium citrate and sodium succinate on rat-heart pericardium indicated that the “g ~2.01” EPR signal observed immediately following the beginning of conditions modeling oxidative/nitrosative stress, could be a consequence of both reversible oxidation of the constitutive 3Fe-4S cluster in complex II and degradation of the 4Fe-4S cluster in aconitase. However, the net signal envelope, which becomes apparent in less than one minute following the start of oxidative/nitrosative conditions, is dominated by the component arising from complex II. Taking into account the findings of a previous study concerning complexes I and III [L.L. Pearce, A.J. Kanai, M.W. Epperly, J. Peterson (2005) *Nitric Oxide* **13**, 254-63] it is now apparent that, with the exception of the cofactor in aconitase, mammalian (mitochondrial) iron-sulfur clusters are surprisingly resistant to degradation stemming from oxidative/nitrosative stress.

Keywords

aconitase; mitochondria; cardiomyocytes; complex II; EPR; iron-sulfur clusters; succinate dehydrogenase; nitric oxide; peroxynitrite

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INTRODUCTION

Iron-sulfur proteins have periodically been suggested to be critical targets of oxidative/nitrosative stress [1;2;3]. In mammals, these proteins are predominately found in mitochondria, with the exception of aconitase (containing a 4Fe-4S cluster) which is present in both mitochondrial and cytosolic forms [4]. Mitochondria have also been implicated in the production of oxidative/nitrosative stress via the formation of peroxynitrite [5;6;7] generated from the precursors superoxide and nitric oxide at diffusion-controlled rates [8]. Either directly, or through the action of one of its derivatives, the powerful oxidant peroxynitrite is known to modify biomolecules in several ways, including oxidizing iron-sulfur centers, generating thiyl radicals (which can decay to sulfenic acids) and reacting with protein tyrosines to form 3-nitrotyrosine [6;9;10;11]. The peroxynitrite anion (ONO_2^-) is actually quite stable in aqueous media, but will tend to become protonated at neutral pH forming peroxynitrous acid (HONO_2). It is almost certainly this more reactive molecular entity, or some other derivative such as carboxylate radical ($\text{CO}_3^{\bullet-}$) formed in the reaction between peroxynitrite and dissolved carbon dioxide, which are responsible for most reactions with biomolecules [8]. Herein, we do not attempt to distinguish between these possibilities and use the term “peroxynitrite” to describe the anion and its immediate short-lived derivatives, but specifically not the precursor nitric oxide.

Recent analysis of complex I (NADH dehydrogenase) and complex III (cytochrome *c* reductase) from bovine heart mitochondria showed that the cofactors contained in these enzymes, including the iron-sulfur centers, were quite resistant to oxidative/nitrosative stress [12]. Complex III contains only one 2Fe-2S center [13;14], while complex I contains multiple 2Fe-2S and 4Fe-4S clusters [15]. Oxidative damage to iron-sulfur proteins commonly involves loss of one iron atom from a 4Fe-4S core, leading to production of a 3Fe-4S cluster [16]. The fully oxidized forms of such products, $[\text{3Fe-4S}]^+$, formally containing 3 ferric ions, exhibit unique EPR signals with crossover *g*-values of 2.01 – 2.02 (the “*g* = 2.01 signal”) observable at liquid helium temperatures; whereas, the single-electron reduced forms $[\text{3Fe-4S}]^0$ are typically EPR silent [16]. We have previously observed loss of complex I and complex III activity in conjunction with the appearance of a *g* ~2.01 EPR signal in cultured cells and isolated mitochondria under conditions leading to the generation of peroxynitrite [12;17]. However, we subsequently showed that the addition of *bona fide* peroxynitrite to isolated complex I (and complex III), while clearly leading to loss of activity, does not result in the appearance of any *g* ~2.01 EPR signals. Thus, the origin of the oxidized $[\text{3Fe-4S}]^+$ cluster(s) responsible for this rapidly-developing (~1 minute) signal in mitochondria remains in doubt.

Two other mitochondrial enzymes are good candidates for this particular indicator of oxidative/nitrosative stress, aconitase and complex II (succinate dehydrogenase). Aconitase has been very carefully examined and shown to develop the 3Fe-4S center under a variety of conditions [18], but typically more slowly than the signals we describe here. Complex II contains a constitutive 3Fe-4S center which upon oxidation has an associated EPR *g*-value of ~2.01 [13;19]. Either, or both, of these enzymes could be responsible for this type of EPR signal that may be detected in mammalian tissues, cultured cells and isolated mitochondria under conditions of oxidative/nitrosative stress. We report here the results of an investigation into the identity of the particular $[\text{3Fe-4S}]^+$ cluster(s) responsible for the rapidly observed *g* ~2.01 signal in mitochondria-rich heart tissue.

MATERIALS AND METHODS

Chemical Reagents

Bovine cytochrome *c* (type III), dichloroindophenol, sodium cholate, ubiquinone-2 and sodium deoxycholate were obtained from Sigma/Aldrich. NaONO_2 was prepared according to the

method of Beckman *et al.* [20], using manganese dioxide to eliminate excess hydrogen peroxide. Sodium lauryl maltoside was obtained from Anatrace, sodium dithionite (87%) from E.M. Science, nitric oxide gas (99.5%) from Matheson, and all other reagents used were ACS grade and purchased from Sigma/Aldrich or Fisher.

Enzyme Purification

Complex II (*EC* 1.3.5.1., succinate dehydrogenase) was isolated from beef hearts (~15 hearts per preparation) obtained from a local slaughterhouse using the slightly modified method of Ragan *et al* [21]. Briefly, mitochondria were first extracted from the hearts in the presence of 10 μM CaCl_2 and then frozen at -20°C for no longer than 2 weeks before the complex II was isolated. A series of ammonium sulfate cuts in the presence of cholic acid, followed by ethanol and cyclohexane extractions to eliminate contamination by complex III, were used to purify the required complex II. The purity of the final product was determined by establishing the FAD content (~5 nmol/mg protein) and by SDS-polyacrylamide gel electrophoresis. Porcine heart aconitase (*EC* 4.2.1.3., aconitate hydratase; isocitrate hydrolyase) was obtained from Sigma/Aldrich and used without further purification since it was found to exhibit the EPR signal of interest (see Figure 5).

Enzyme Assays

Complex II activity was determined by the method of Hatefi and Stiggall using 20 mM sodium succinate, 50 μM (oxidized) ubiquinone-2, 75 μM 2,6-dichloroindophenol (DCIP) and 0.5 ng complex II in 50 mM potassium phosphate, pH 7.4, 1 mM EDTA [21]. The reaction was followed by monitoring the decrease in absorbance at 600 nm after first pre-incubating for 5 minutes at 37°C . Succinate dehydrogenase activity was calculated using the extinction coefficient $\epsilon_{600} = 21 \text{ mM}^{-1}\text{cm}^{-1}$ and expressed as μmol succinate/min/mg protein. Sodium cyanide was added (10 mM final concentration) to complex II assay mixtures in the case of tissue samples to inhibit Complex IV. Aconitase activity was assayed at 30°C in 50 mM Tris-HCl, pH 7.4, 30 mM sodium citrate, 0.6 mM MnCl_2 , 0.2 mM NADP^+ , and 1 unit of isocitrate dehydrogenase. The reaction was followed by measuring the increase in absorbance at 340 nm and the activity calculated as μmol citrate/min/mg protein using the extinction coefficient $\epsilon_{340} = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$.

Nitric oxide and peroxynitrite Additions

Protein samples were prepared in strongly buffered solution (M/10 sodium phosphate, 0.05% lauryl maltoside, pH 7.4). nitric oxide gas (99.5%) was bubbled through water and then passed over potassium hydroxide pellets to remove any acidic impurities before further experimental use. Nitric oxide additions to samples were made with gas-tight Hamilton syringes. Stock solutions of NaONO_2 in aqueous NaOH were further diluted in water to a final $[\text{OH}^-]$ of ~1 mM or lower before addition to protein solutions. Additions of NaONO_2 solutions to protein samples were made by quick expulsion through Teflon 'needles' from gas-tight Hamilton syringes with agitation to ensure rapid mixing. We have previously shown that, unlike slower 'bolus' additions, this rapid-mixing approach results in quantitative reduction of peroxynitrite by metalloenzymes that are able to donate at least two electrons [22]. Concentrations of NaONO_2 solutions were determined spectrophotometrically ($\epsilon_{302} = 1.67 \text{ mM}^{-1} \text{ cm}^{-1}$) [23]. Following addition of nitric oxide gas, or peroxynitrite solution, to protein samples the measured pH change was always < 0.05 .

Electrophoresis and Blots

Dot and Western blots were carried out using 15% pre-cast acrylamide gels, nitrocellulose membranes and electrophoresis/blotting apparatus from Bio-Rad, Richmond, CA and Chemiluminescence Reagent Plus from Perkin-Elmer Life Science, Boston, MA. Primary

rabbit anti-3-nitrotyrosine antibodies and secondary antibodies of goat anti-rabbit IgG conjugated with alkaline phosphatase (AP) from Upstate Biotechnology, Lake Placid, NY were used. Antiserum was diluted in 1% bovine serum albumin in 10 mM Tris-HCl, pH 7.4 and 0.9% NaCl (TBS). Bound conjugates were visualized by staining for enzymatic activity with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitro-blue tetrazolium (NBT) for alkaline phosphatase. Protein samples were denatured in 2% SDS at room temperature prior to electrophoresis.

Preparations of Rat Cardiac Tissue

Rat heart pericardium was minced and homogenized in an equal volume of buffer (5 mM potassium phosphate, 0.25 M sucrose, 5 mM KCl, pH 7.4) using a hand-held homogenizer just enough to enable introduction of the tissue slurry into EPR tubes. Previously, we have sectioned pericardium at 300 μ m intervals in two crossed directions using a tissue chopper [17] to ensure that the majority of cardiomyocytes in the samples remained uncut. However, as the results of EPR experiments using samples prepared by either method were essentially identical, we dispensed with the latter more time-consuming procedure. The pericardial tissue was used to prepare all samples within 10 minutes of sacrificing the animal. All samples were preserved by immersion into liquid nitrogen within 2 minutes of their rapid mixing and introduction to the EPR sample tubes. EPR spectra were subsequently recorded without the samples ever being thawed. Parallel samples for use in subsequent enzyme activity assays were cryogenically preserved at the same time. For purposes of the activity assays, it was convenient to use sub-cellular fractions concentrated in mitochondria. The cryogenically stored homogenized tissue samples were thawed and centrifuged at 500 g for 5 min, the supernatant decanted and, subsequently, spun at 10,000 g for 10 minutes. The mitochondria-enriched pellets were then re-suspended in 5 mM potassium phosphate, 0.25 M sucrose, 5 mM KCl, pH 7.4 buffer to 10 mg/mL for activity measurements. Protein determinations were made using the BCA method kit from Pierce, Rockford, IL.

Instrumental Methods

X-band (9.65 GHz) EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with a Bruker B-E 25 electromagnet and Bruker ER4116DM resonant cavity. Cryogenic temperatures were maintained with an Oxford Instruments ESR 910 cryostat in conjunction with a VC30 controller. Frequency calibration was with a microwave frequency counter and the magnetic field was calibrated with an NMR gaussmeter. The sample temperature was measured by means of a thermocouple calibrated using a Lakeshore carbon-glass resistor (CGR-1-1000). A modulation frequency of 100 kHz was used throughout and, except for the data of Figure 6, all EPR spectra were recorded under non-saturating conditions. Electronic absorption measurements were conducted with a Shimadzu UV-2501PC spectrophotometer and fluorescence spectra were recorded with a Shimadzu RF-5301 PC spectrofluorophotometer.

RESULTS

Reaction of Isolated Complex II with Peroxynitrite

Complex II of the electron-transport chain contains one *b*-type cytochrome, one 2Fe-2S, one 4Fe-4S, one 3Fe-4S and one flavin per enzyme [13]. While 3Fe-4S moieties are often formed as artifacts during the isolation of bacterial iron-sulfur proteins, the 3Fe-4S cluster of complex II is unusual since it is constitutive to the enzyme and functionally required [24;25]. The 15 K EPR spectra of isolated preparations of the bovine enzyme (fully oxidized form) exhibited a sharp signal centered at 3,430 gauss ($g \sim 2.01$) attributable to the $[3\text{Fe-4S}]^+$ core (Figure 1A). Following reduction of the enzyme with succinate, the initial EPR signal was found to have disappeared and another broader signal due to the $[2\text{Fe-2S}]^+$ center was observed (Figure 1B).

A small additional feature at 3450 gauss ($g = 2.00$) superimposed on the $[2\text{Fe-2S}]^+$ signal is due to a free radical, probably ubisemiquinone, which essentially disappeared upon further reduction of the enzyme with sodium dithionite (Figure 1C). At higher gain (Figure 1D) signals arising from the $[4\text{Fe-4S}]^+$ cluster were observed in the EPR spectra of dithionite-reduced samples. These findings are fully in keeping with the reported EPR characteristics of complex II [13] and thus verify the overall similarity of our preparations to those of other authors. Upon reaction of the succinate-reduced enzyme with excess peroxynitrite, the signals of the $[2\text{Fe-2S}]^+$ and $[4\text{Fe-4S}]^+$ centers vanished and the $g \sim 2.01$ EPR signal of the $[3\text{Fe-4S}]^+$ cluster reappeared (Figure 1E). As oxidized $[2\text{Fe-2S}]^{2+}$ and $[4\text{Fe-4S}]^{2+}$ cores are diamagnetic, they do not exhibit any EPR signals and, consequently, these results show that peroxynitrite was able to extract electrons from the iron-sulfur clusters with the cores changing between their normally accessible oxidation states. Re-oxidation of complex II by peroxynitrite, even at 1,000-fold excess, did not result in any change in the magnitude of the $g \sim 2.01$ signal compared to that obtained with the isolated enzyme, indicating that there was no decay of 3Fe-4S clusters, nor conversion of 4Fe-4S to 3Fe-4S. Furthermore, upon re-reduction of the enzyme with succinate, the EPR spectrum of the reduced $[2\text{Fe-2S}]^+$ core was found to quantitatively reappear along with the free radical signal at $g = 2.00$ (Figure 1F). This redox cycling of complex II with succinate and peroxynitrite could be repeated several times without either loss of activity, or the appearance of any additional EPR signals such as 'free' ferric species ($g = 4.3$). Consequently, the spectra of Figure 1 clearly demonstrate that the iron-sulfur clusters of bovine complex II are able to undergo facile redox chemistry with peroxynitrite without any apparent core degradation.

Functional Studies of Complex II

Neither excess nitric oxide (2 μM nitric oxide, 2 nM enzyme, 20 min at 22° C) nor excess peroxynitrite (20 μM NaONO_2 , 2 nM enzyme, 22° C) had any significant effect on the measured activity of purified complex II. In contrast, similar treatment with H_2O_2 was found to inhibit the enzyme by ~50% (Table I). This inhibitory reaction of H_2O_2 will be the subject of future studies – the result is included here to show that our failure to detect loss of complex II activity following exposure of the enzyme to nitric oxide and peroxynitrite was not simply due to a faulty activity assay. The lack of any significant reaction after exposure to 1,000-fold excesses of nitric oxide and peroxynitrite, was additionally confirmed by the observation that there were no apparent changes in the EPR spectra of either oxidized or reduced complex II samples. Furthermore, no changes in the oxidized heme, or in the FAD, were detected by electronic absorption spectroscopy following exposure of the enzyme to 1,000-fold excesses of nitric oxide, H_2O_2 , or peroxynitrite. The absence of any measurable activity loss and/or cofactor modification following the addition of 1,000-fold excess peroxynitrite to complex II is noteworthy because the protein is undoubtedly modified by this treatment, since 3-nitrotyrosine formation can readily be observed by Western blot and all four subunits of the enzyme contain tyrosine residues that are nitrated. In order to further verify the reliability of the sample manipulation procedures, we also examined the effects of nitric oxide, H_2O_2 and peroxynitrite on mitochondrial aconitase, which is known to be deactivated by oxidative degradation of its constitutive 4Fe-4S cluster to an inactive 3Fe-4S form. In keeping with the findings of others [18;26;27;28] we found that nitric oxide had negligible effect on aconitase activity at pH 7.4, while exposure to H_2O_2 and peroxynitrite clearly resulted in significant activity loss (Table I).

In order to compare these functional characteristics of isolated complex II with those of the *in situ* enzyme, we also undertook a set of activity assays on freshly excised and homogenized rat heart pericardium (Table II). Endogenous generators of nitric oxide and superoxide were stimulated to release the reactive species to avoid working at high levels in the tissue that would be physiologically unreasonable. It has previously been shown that topical application of

norepinephrine to cardiomyocytes elicits the intracellular production of nitric oxide, transiently (~1 s) reaching concentrations of several hundred nanomolar [29;30]. Inhibition of complex III with antimycin A is a convenient way of producing superoxide inside mitochondria of all cells – that is, significant levels result within the 2 minutes exposure time required in the present experiments. Note that alternate procedures such as the xanthine/xanthine oxidase method generate superoxide that does not efficiently enter the mitochondria of intact cells [31] and may only produce detectable effects in isolated mitochondria following incubation times in excess of 10 minutes [32]. Interestingly, the same barely significant degree of complex II inhibition in the cardiac tissue was observed whether nitric oxide production was stimulated by norepinephrine, superoxide generation was stimulated by antimycin A, or both norepinephrine and antimycin A were used together to give elevated peroxynitrite (Table II). Probably, one cannot avoid partial inhibition of the electron-transport chain at complex IV during elevated nitric oxide production, which will lead to some elevation in superoxide and H₂O₂ levels. Therefore, a plausible explanation for the just-detectable deactivation of complex II in the tissue is that it was due to inhibition of the enzyme by small amounts of H₂O₂ unavoidably formed in all three cases. Compared with the control, there was clearly significant loss of aconitase activity in the rat-heart pericardium following elevation of nitric oxide, superoxide and peroxynitrite (Table II) as is to be expected [28;31].

Studies with Rat-heart Pericardium Under Oxidative/Nitrosative Stress

We have previously reported the appearance of a *g* ~2.01 EPR signal in mitochondria-rich tissue under conditions where endogenous sources of superoxide and nitric oxide were stimulated to mimic oxidative/nitrosative stress. Compared to other cell types in the pericardium, the mitochondrial content of cardiomyocytes is very high, guaranteeing that the detected EPR signal arose from the latter only, any contributions from other sources being below the detection limit. The intensity of the EPR signal, indicating [3Fe-4S]⁺ cores, was greatest under those conditions where the production of peroxynitrite was maximized and, indeed, the addition of *bona fide* peroxynitrite to isolated mitochondria or tissue also leads to production of the same signal. It was further shown that the signal(s) in question were not associated with any cluster reorganization in complexes I or III [12]. In the present study, the 15 K EPR spectrum of minced rat-heart pericardium contains signals with average *g*-value below 2.0 (Figure 2A, solid trace) in keeping with the presence of one-electron reduced 2Fe-2S and 4Fe-4S clusters ([2Fe-2S]⁺ and [4Fe-4S]⁺ cores). Stimulation of the endogenous production of nitric oxide (2B, solid trace) superoxide (2C, solid trace) or both (2D, solid trace) led to the production of another EPR signal centered at *g* ~2.01 demonstrating the presence of a [3Fe-4S]⁺ core. The appearance of this signal unambiguously represents an oxidation of the center(s) in question and cannot be explained, for example, by stimulation of the citric acid cycle enzymes as this would result in a flux of reductants.

It should also be noted that following introduction to the bottom of an EPR tube, the finely divided pericardium becomes anaerobic within about 30 seconds – conversion of oxy-myoglobin to deoxy-myoglobin being readily apparent by observation of the color change from red-brown to darker red. The sample can be reoxygenated by inverting the tube and the change from aerobic to anaerobic conditions observed again – this process being routinely repeatable several times. Since the EPR samples, having undergone the color change described, were then aerated/mixed once in the tube before being cryogenically preserved, it is quite clear that all were prepared under conditions where reductive nutrients were not depleted. It has previously been shown that at the levels of NO achieved by stimulation of endogenous sources in pericardial tissue there is no measureable reaction with deoxy-myoglobin and/or oxy-myoglobin; that is, any signals arising from, respectively, formation of metmyoglobin and/or nitrosylmyoglobin remain below detection by EPR [33]. For example, in the present data set this is confirmed by the absence of any positive features arising from nitrosylmyoglobin at

< 3,300 gauss in the spectra of Figures 2 and 3. Similarly, there was no increase in the intensity of any metmyoglobin signals ~1,200 gauss following stimulation of the pericardial tissue to release NO (not shown). Therefore, as deoxymyoglobin and oxymyoglobin are themselves EPR silent, the presence of myoglobin in the tissue does not interfere with observation of the mitochondrial events of interest. Further to this point, at endogenously-generated levels of NO, the major product of NO catabolism in cardiomyocytes is nitrite, whereas reaction of NO with oxymyoglobin produces nitrate [30].

When the set of experiments mimicking oxidative/nitrosative stress was repeated in the presence of added succinate, the appearance of the $g \sim 2.01$ signal was suppressed (Figure 2B–2D, broken traces) strongly suggesting the $[3\text{Fe-4S}]^+$ core in question to arise from reversible redox chemistry of the constitutive $[3\text{Fe-4S}]^{0,+}$ -core cluster in complex II (succinate dehydrogenase). Confoundingly, however, the $g \sim 2.01$ EPR signal was also suppressed by the addition of citrate (Figure 3) in keeping with the findings of others working with isolated mitochondria [34]. The $[3\text{Fe-4S}]^{0,+}$ -core cluster in aconitase is known to undergo reconstitution into the active 4Fe-4S form upon turnover with substrate citrate [35]. Consequently, the $g \sim 2.01$ signal that develops under these conditions modeling oxidative/nitrosative stress may, in principle, be partly due to aconitase as well as complex II.

Not surprisingly, the addition of both citrate and succinate concomitantly to minced pericardium suppressed the EPR signal obtained following treatment with norepinephrine + antimycin A to a greater extent than either citrate or succinate alone, but we were unable to eliminate the $g \sim 2.01$ signal entirely (Figure 4, black dashes). Addition of *bona fide* peroxynitrite (in the form of pre-synthesized NaONO_2) to minced pericardium also resulted in the appearance of a $g \sim 2.01$ EPR signal (Figure 4, solid red trace) but at lower intensity than if peroxynitrite were generated inside the mitochondria using the norepinephrine + antimycin A procedure (Figure 4, solid black trace). Prior addition of citrate together with succinate lowered the intensity of the $g \sim 2.01$ signal obtained following the addition of peroxynitrite to minced pericardium, but again, the suppression was partial (Figure 4, red dots). In general, it was observed that pre-incubation of pericardial tissue with both citrate and succinate together (before treatment with either peroxynitrite, or norepinephrine + antimycin A) always suppressed the development of any $g \sim 2.01$ signal to a greater extent than either citrate, or succinate, alone (not shown). In the absence of peroxynitrite and/or norepinephrine + antimycin A, the addition of succinate and/or citrate led, as expected, to the appearance of characteristic signals of reduced iron-sulfur clusters only (Figure 4, blue trace). Unfortunately, these results remain equivocal, because as citrate is a precursor for succinate in the citric acid cycle, the addition of citrate must necessarily increase reduction of complex II in addition to turning over aconitase. That is, while the data clearly confirm that the $g \sim 2.01$ signal, a signature for the $[3\text{Fe-4S}]^+$ -core cluster, is formed in similar fashion either by the addition of *bona fide* peroxynitrite, or by norepinephrine + antimycin A, it does not reveal whether the signal arises principally from complex II, or it is derived from both aconitase and complex II. However, it should be noted that the sensitivity of the $g \sim 2.01$ signal to succinate does strongly suggest that the signature cannot be associated with aconitase alone.

In an effort to quantify the two potential contributions to the $g \sim 2.01$ in rat-heart pericardium (Figure 5, solid trace) we have additionally studied the characteristics of this signal in isolated (air-oxidized) aconitase (Figure 5, dotted trace) and isolated (air-oxidized) complex II (Figure 5, broken trace). Upon comparing the temperature dependence of the rat pericardium, porcine aconitase and bovine complex II signals, we found there to be no significant difference between them under non-saturating conditions (Figure 5, inset). (Note that much of the relevant early literature describes temperature-dependent EPR measurements performed under conditions of partially saturating power to distinguish between cluster types.) However, there was a readily detectable, difference between the power-saturation characteristics of the $g \sim 2.01$ in aconitase

and complex II at constant temperature (Figure 6, open squares and open triangles respectively). Moreover, the power-saturation characteristics of the rat pericardium (Figure 6, filled circles) could essentially be superimposed on the data obtained from complex II, indicating the signal to arise predominantly from this enzyme rather than aconitase.

DISCUSSION

It is sometimes argued that the breakdown of certain biological macromolecules such as iron-sulfur proteins, can lead to the liberation of “free iron” and subsequent problems associated with Fenton/Haber-Weiss chemistry. In order to obtain “free iron” from iron-sulfur clusters, 4Fe-4S, 3Fe-4S and/or 2Fe-2S must first be cleaved in some manner. The first step in degradation of many 4Fe-4S clusters has been shown to be the production of an oxidized [3Fe-4S]⁺ core with concomitant loss of labile iron [16]. In addition to our own group, others have shown that mitochondria, or mitochondria-rich cells, experiencing oxidative/nitrosative stress often exhibit an EPR signal with an associated g-value of ~2.01 [3;36]. This was once identified as stemming from a HiPiP-type iron-sulfur cluster but has more recently been shown to be a [3Fe-4S]⁺ center. Previously, we have demonstrated that exposure of complex I to near physiological levels of either peroxynitrite or nitric oxide does not result in formation of any [3Fe-4S]⁺ cores or appearance of detectable “labile iron” [12]. In this work we show that complex II, which contains a constitutive 3Fe-4S cluster, can be oxidized by peroxynitrite and subsequently re-reduced by succinate without any apparent cofactor degradation (Figure 1): that is, all three of the iron-sulfur cluster types (2Fe-2S, 3Fe-4S or 4Fe-4S) are resistant to oxidative damage. The results of further experiments with rat heart pericardium experiencing oxidative/nitrosative stress clearly demonstrate that the production of an oxidized [3Fe-4S]⁺-core cluster is significantly prevented by the pre-addition of succinate (Figure 2). We suggest that under physiological and non-inflammatory pathophysiological conditions, the iron-sulfur clusters of complexes I, II and III can be reversibly oxidized by peroxynitrite – that is, routinely be re-reduced without degradation and loss of labile iron.

While our work continues to suggest that low-level peroxynitrite generation (by implication, either in short bursts, or chronically) is of little consequence to mitochondria where the electron-transport chain is functioning and antioxidant metabolites such as glutathione are not depleted, this does not necessarily mean that peroxynitrite is never harmful. For example, in any situation where upregulation of inducible nitric oxide synthase accompanying inflammation is present, the resulting elevated level of peroxynitrite may be enough to overwhelm the capability of the (possibly impaired) electron-transport-chain complexes to deal with it. Work by Cammack *et al.* [3] showed that relatively high levels of authentic peroxynitrite (1mM) added to rat liver mitochondria diminished the amount of oxidized g ~2.01 EPR signal observed, suggesting 3Fe-4S cluster degradation. Also, the studies of Han *et al.* [34] clearly showed that very high levels of peroxynitrite resulted in destruction of the 3Fe-4S clusters in damaged aconitase. These results indicate that sufficiently elevated (extreme pathophysiological) peroxynitrite levels can damage and degrade the iron-sulfur cluster in aconitase at least. However, even at 1,000-fold excesses of peroxynitrite over enzyme, we observed no convincing evidence for this type of cluster destruction in complex II (Figure 1 and Table I).

It is now clear that, unlike their bacterial counterparts, mammalian iron-sulfur clusters are stable to oxidative degradation. The exception to this appears to be aconitase, the activity of which requires the presence of its constitutive 4Fe-4S cluster. The work of several other groups has clearly demonstrated that this enzyme is particularly vulnerable to oxidative/nitrosative stress, but inactivation of aconitase by formation of the 3Fe-4Fe cluster is entirely reversible provided the cysteine ligand to the fourth iron in the cluster is not derivatized [34;36]. We have confirmed that aconitase becomes inhibited under the particular conditions modeling oxidative/

nitrosative stress we employ here (Table II), but this does not necessarily involve the obligatory formation of a 3Fe-4S cluster [28]. However, we can only, with confidence, assert the $g \sim 2.01$ EPR signal to originate from complex II (Figures 2 and 6) – the argument that aconitase contributes at all being entirely circumstantial. In summary, we conclude that the oxidized $[3\text{Fe-4S}]^+$ -core cluster exhibiting the $g \sim 2.01$ EPR signal, formed rapidly following transient oxidant production, is the result of the reaction of the oxidant (most likely peroxynitrite) predominantly with complex II.

A remaining question is the connection between markers of oxidative stress, such as the oxidized 3Fe-4S clusters of complex II and aconitase, and cell injury or death. It would seem, based on this study, that complex II is not much compromised by the direct action of near physiological levels of nitric oxide or peroxynitrite (although irreversible damage by H_2O_2 is still possible). The observed reversible and succinate-dependent redox chemistry of the 3Fe-4S cluster in rat heart pericardial tissue (Figure 2) without significant loss of activity (Table II) provides further confirmation that the unusual 3Fe-4S center is constitutive in complex II [25;37]. While the $g \sim 2.01$ EPR signal is clearly a marker of oxidative/nitrosative stress, it is probably only an indirect indicator of compromised protein function under near physiological conditions. That is, other enzymes present in the mitochondria may be irreversibly damaged while complex II (and perhaps, aconitase) can remain active for some period of time during which a $g \sim 2.01$ signal may be evident. As further evidence that the iron-sulfur clusters in the complexes of the mammalian mitochondrial electron transport chain are resistant to oxidative damage, we note that these enzymes can be isolated aerobically without degradation of their constitutive clusters. In our laboratory, the sensitivity of the purified respiratory complexes containing iron-sulfur clusters to functional damage from peroxynitrite increases in the order: complex II < complex I < complex III. Consequently, as the most sensitive of these enzymes has the lowest iron-sulfur content (a single two-iron cluster in complex III) it is entirely reasonable that the mechanism(s) of irreversible inactivation must involve components other than the iron-sulfur clusters.

Acknowledgments

Supported by the National Institutes of Health (HL61411 to JP and LLP)

The authors would like to thank Michael P. Hendrich for access to the EPR facility in the Department of Chemistry at Carnegie Mellon University. This work was supported by a grant from the National Institute of Health (HL61411 to LLP & JP).

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Figure 1. X-band EPR spectra of isolated bovine complex II, demonstrating reversible oxidation and reduction of iron-sulfur clusters
 (Recording conditions: 15 K sample temperature; 4 mm o.d. sample tubes; 9.96 GHz; frequency; 40 μ W power; 10 G modulation amplitude; 100 kHz modulation frequency.)
 Dispersions of 20 μ M complex II in 0.1 M potassium phosphate buffer, 0.05% (w/v) in lauryl maltoside, pH 7.4. (A) Enzyme as isolated showing the signal arising from the oxidized [3Fe-4S] cluster at crossover g-value of 2.015. (B) Enzyme following addition of sodium succinate to 50 μ M, frozen after 10 minutes incubation. The main features have associated g-values of 2.01 and 1.94 and arise from the reduced [2Fe-2S] cluster. (C) Enzyme frozen immediately following the addition of sodium dithionite to $\sim$ 100 μ M. The main EPR features have g-values of 2.01 and 1.94 arising from the reduced [2Fe-2S] cluster and a sharp signal at

$g = 2.00$ due to a free radical. **(D)** Sample as in C, power $600 \mu\text{W}$, $\times 10$ gain increase. The central signals have been removed to highlight the outer g -values of 2.08 and 1.85 associated with the signal of the reduced [4Fe-4S] cluster. **(E)** Sample as in B thawed, then re-frozen immediately following the addition of sodium peroxynitrite to $20 \mu\text{M}$. **(F)** Sample as in E thawed, then re-frozen 10 minutes after the addition of sodium succinate to $50 \mu\text{M}$.

Figure 2. X-band EPR spectra of minced rat-heart pericardium demonstrating the effects of antimycin A, norepinephrine and succinate
 (Recording conditions: 15 K sample temperature; 4 mm o.d. sample tubes; 9.96 GHz; frequency; 40 μ W power; 10 G modulation amplitude; 100 kHz modulation frequency). Pericardium was prepared as described in Materials and Methods. All samples were frozen within 2 minutes of reagent additions and cryogenically preserved for subsequent introduction to the spectrometer without thawing. **Solid traces:** (A) Control sample. (B) Norepinephrine (cardiomyocyte stimulant for nitric oxide production) added to 1 μ M. (C) Antimycin A (specific complex III inhibitor leading to mitochondrial superoxide production) added to 100 μ M. (D) Antimycin A and norepinephrine added to 100 μ M and 1 μ M respectively. **Dashed**

traces: As above, but samples pre-incubated with sodium succinate (added to 100 μM) for five minutes prior to other additions.

Figure 3. X-band EPR spectra of minced rat heart pericardium demonstrating the effects of antimycin A, norepinephrine and citrate
 (Recording conditions: 15 K sample temperature; 4 mm o.d. sample tubes; 9.96 GHz; frequency; 40 μ W power; 10 G modulation amplitude; 100 kHz modulation frequency.) Pericardium was prepared as described in the materials and methods, frozen within 2 minutes of additions and then transferred to the spectrometer. **Solid traces:** (A) Control sample. (B) Norepinephrine (cardiomyocyte stimulant for nitric oxide production) added to 1 μ M. (C) Antimycin A (specific complex III inhibitor leading to mitochondrial superoxide production) added to 100 μ M. (D) Antimycin A and norepinephrine added to 100 μ M and 1 μ M respectively. **Dotted traces:** As above, but samples pre-incubated with sodium citrate (added to 1 mM) for five minutes prior to other additions.

Figure 4. X-band EPR spectra of minced rat-heart pericardium demonstrating the additive, but still partial, protective effects against peroxynitrite of both citrate and succinate (Recording conditions: 15 K sample temperature; 4 mm o.d. sample tubes; 9.96 GHz; frequency; 40 μW power; 10 G modulation amplitude; 100 kHz modulation frequency; $(?.? \times 10^x)$ gain.) Rat-heart pericardium stimulated with antimycin A and norepinephrine added to 100 μM and 1 μM final concentrations, respectively, then frozen within 2 minutes (solid black trace); pre-incubated with sodium citrate (1 mM in the medium) plus sodium succinate (100 μM in the medium) before stimulation with antimycin A and norepinephrine (black dashes); following addition of sodium peroxynitrite to 1 mM in the medium (solid red trace); pre-incubated with sodium citrate (1 mM in the medium) plus sodium succinate (100 μM in the medium) before addition of peroxynitrite (red dots); pre-incubated with sodium succinate only (100 μM in the medium) for 5 minutes prior to freezing (blue trace). The signals due to any $[2\text{Fe-2S}]^+$ and $[4\text{Fe-4S}]^+$ reduced clusters present in complex II at $> 3,700$ gauss have been truncated to provide an expanded view in the region of most interest.

Figure 5. Comparison of the X-band EPR spectra of rat-heart pericardium (black trace), isolated porcine aconitase (dotted trace) and isolated bovine complex II (dashed trace) (Recording conditions: 15 K sample temperature; 4 mm o.d. sample tubes; 9.96 GHz; frequency; 40 μ W power; 10 G modulation amplitude; 100 kHz modulation frequency). The minced rat-heart pericardium was treated with antimycin A (to 100 μ M in the medium) and norepinephrine (to 1 μ M in the medium) for 2 minutes at 22 $^{\circ}$ C prior to freezing in the EPR tube. The other samples were taken from preparations of the enzymes as isolated for introduction into EPR tubes. For ease of visual comparison, the intensities of the data sets have been arbitrarily scaled to match. **Inset: temperature dependence of the $g \sim 2.01$ components.** Intensity taken as the product of the peak height by its squared width. Aconitase ($\square$), complex II (∇) and rat heart pericardium ($\bullet$). See Materials and Methods for further details.

Figure 6. Power saturation curves of the $g \sim 2.01$ components of the x-band EPR spectra at 20 K of aconitase, complex II and rat-heart pericardium demonstrating that the signal arising from intact mitochondria is like that of complex II

(Recording conditions: 10 G modulation amplitude; 4 mm o.d. sample tubes; 9.96 GHz; frequency; 100 kHz modulation frequency. The magnitude of the instrument noise during these measurements was within the vertical dimensions of the symbols.) Same samples as for Figure 5. Isolated porcine aconitase ($\square$), isolated bovine complex II (∇) and minced rat-heart pericardium treated with antimycin A and norepinephrine, added to 100 μM and 1 μM in the medium, respectively ($\bullet$).

Table I

Effects of oxidative/nitrosative stress on the enzymatic activities of isolated complex II and aconitase

Conditions [*]	Complex II	Aconitase
Control	100 (±11) [†]	100(±10) [#]
Nitric oxide (2 µM)	90 (±8)	92 (±7)
H ₂ O ₂ (2 µM)	48 (±6)	70 (±6)
Peroxynitrite (20 µM)	95 (±8)	65 (±6)

^{*} Reagents added to enzyme solutions 20 minutes before dilution into assay mixtures containing substrates. NO exposure performed anaerobically.

[†] 25 µmol DCIP/min./mg protein (isolated from bovine heart)

[#] 5 µmol citrate/min./mg protein (porcine, Sigma)

Data are expressed as % relative to controls, numbers in parentheses are standard errors derived from at least 6 replicate measurements.

Table II

Effects of oxidative/nitrosative stress on the enzymatic activities of complex II and aconitase in rat-heart pericardium

Conditions [*]	Complex II	Aconitase
Control	100 (± 12) [†]	100 (± 8) [#]
+ norepinephrine	86 (± 3)	72 (± 7)
+ antimycin A	84 (± 4)	31 (± 2)
+ norepinephrine & antimycin A	84 (± 3)	23 (± 3)

^{*} Reagents added to homogenized tissue 2 minutes before freezing for storage prior to assay (see text).

[†] 9 μ mol DCIP/min/mg protein.

[#] 0.6 μ mol citrate/min/mg protein.

Data are expressed as % relative to controls, numbers in parentheses are standard errors derived from at least 6 replicate measurements.