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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 "$g \sim 2.01$" 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 1 min 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, Nitrosative stress results in irreversible inhibition of purified mitochondrial complexes I and III without modification of cofactors, Nitric Oxide 13 (2005) 254–263) 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.

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Introduction

Iron–sulfur proteins have periodically been suggested to be critical targets of oxidative/nitrosative stress [1–3]. 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 [5–7] 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 [6,9–11]. 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 ($CO_3^{\bullet -}$) 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

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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, $[3Fe–4S]^+$, 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 $[3Fe–4S]^0$ are typically EPR silent [16]. We have previously observed loss of complex I and complex III activity in conjunction with the appearance of a $g \sim 2.01$ 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 $g \sim 2.01$ EPR signals. Thus, the origin of the oxidized $[3Fe–4S]^+$ cluster(s) responsible for this rapidly-developing (∼1 min) 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 $[3Fe–4S]^+$ cluster(s) responsible for the rapidly observed $g \sim 2.01$ 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 Fig. 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 min at 37 °C. Succinate dehydrogenase activity was calculated using the extinction coefficient $\varepsilon_{600} = 21\ mM^{-1}\ cm^{-1}$ 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 $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$.

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 $[OH^-]$ 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 ($\varepsilon_{302} = 1.67\ mM^{-1}\ cm^{-1}$) [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 μm intervals in two crossed directions using a tissue chopper [17] to ensure that the majority of cardiomyocytes in the samples remained uncut.

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