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Original Contribution

Nitrogen dioxide oxidizes mitochondrial cytochrome *c*

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ABSTRACT

We previously reported that high micromolar concentrations of nitric oxide were able to oxidize mitochondrial cytochrome *c* at physiological pH, producing nitroxyl anion (Sharpe and Cooper, 1998 Biochem. J. 332, 9–19). However, the subsequent re-evaluation of the redox potential of the NO/NO^- couple suggests that this reaction is thermodynamically unfavored. We now show that the oxidation is oxygen-concentration dependent and non stoichiometric. We conclude that the effect is due to an oxidant species produced during the aerobic decay of nitric oxide to nitrite and nitrate. The species is most probably nitrogen dioxide, $NO_2^{\bullet}$ a well-known biologically active oxidant. A simple kinetic model of NO autoxidation is able to explain the extent of cytochrome *c* oxidation assuming a rate constant of $3\times10^6\ M^{-1}\ s^{-1}$ for the reaction of $NO_2^{\bullet}$ with ferrocytochrome *c*. The importance of $NO_2^{\bullet}$ was confirmed by the addition of scavengers such as urate and ferrocyanide. These convert $NO_2^{\bullet}$ into products (urate radical and ferricyanide) that rapidly oxidize cytochrome *c* and hence greatly enhance the extent of oxidation observed. The present study does not support the previous hypothesis that NO and cytochrome *c* can generate appreciable amounts of nitroxyl ions (NO^- or HNO) or of peroxynitrite.

Introduction

The intercellular messenger nitric oxide has several target molecules in a typical cell. Most but perhaps not all are heme proteins. The main target, guanylate cyclase, is a heme protein whose enzymatic functioning is controlled by NO binding to a heme group in its ferrous state [1]. Another possible target, cytochrome *c* oxidase, can bind NO in both reduced and oxidized states [2], affecting cell respiration in both obvious [3] and more subtle [4] ways. Catalase is unusual in binding NO primarily in its ferric state [5] and suffering consequent inhibition [6]. Cytochrome *c* is another multifunctional cellular heme protein. It is a key component of the mitochondrial respiratory chain, cycling reversibly between ferrous and ferric states; in its ferric [7] but not ferrous [8] state it is also a trigger for programmed cell death (apoptosis). In the ferric state cytochrome *c* also binds NO; the corresponding ferrous–NO complex can be formed only by inducing major structural changes in the molecule, classically by high pH [8] and more recently by binding to cardiolipin [9,10]. Some of these processes, such as those binding to ferrous heme in guanylate cyclase and cytochrome *c* oxidase, and ferric heme in catalase and cytochrome *c*, are reversible. The bound NO can dissociate back to give the original unmodified heme and free NO in solution. Others, including the NO interactions with oxidized cytochrome *c* oxidase, are irreversible. The bound NO is oxidized to nitrite and one of the associated redox centers in the NO binding site is reduced [11].

In 1998 Sharpe and Cooper [12] reexamined the reactions of NO with cytochrome *c*. They were able to confirm the reversible binding of NO to the native protein in the ferric state, with a dissociation constant of about 20 μM. But they also observed a previously undocumented effect of NO on the ferrous cytochrome at neutral pH and in its native state, contrasting with classical observations that NO binding to ferrocytochrome *c* occurs only at extreme pH. They were also able to show a ferrocytochrome *c*-catalyzed increase in the decay rate (autoxidation) of aqueous NO. They interpreted these results in terms of nitroxyl (NO^-/HNO) production from NO followed by nitroxyl decay.

More recently, however, the redox potential of the $NO^{\bullet}/NO^-$ couple, previously calculated as more positive than the cytochrome *c* potential [13], has been reanalyzed by two research groups [14,15]. Both agree that the E_0' for this couple is far more negative than originally estimated. The most accessible couple is NO, $H^+/{}^1HNO$. Shafirovich and Lymar [14] estimate a reduction potential of −0.55 V at pH 7. The reduction to the triplet state, ^3HNO, necessary to form peroxynitrite by reaction with oxygen, is even less favorable.

If these new estimates are correct the $NO^{\bullet}/NO^-$ couple is highly unlikely to be involved in the NO-induced oxidation of cytochrome *c*. Furthermore in the absence of oxygen, HNO should form N_2O either via reacting with NO or via dehydrative dimerization. However, attempts to detect N_2O by adding NO anaerobically to ferrocytochrome *c* were unsuccessful (N. Hogg, Medical College of Wisconsin, Milwaukee, WI, USA, personal communication).

The results of Sharpe and Cooper thus call for a mechanistic reinterpretation. One possible complication is that the observed oxidation could reflect the catalytic activity of a small population of modified

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cytochrome *c* molecules, possibly polymeric forms [16], which then oxidize the major cytochrome *c* fraction. The second complication is that experiments were done aerobically. This was deliberate, as the action of NO on cytochrome *c* was being compared with the effects of cytochrome *c* on aerobic NO decay and the resultant consequences for the inhibitory effects of NO on cytochrome *c* oxidase activity. However, this creates the possibility that the oxidant is not NO itself but a reactive species derived from NO autoxidation.

Although there is evidence for NO^- (HNO) formation in the presence of some heme proteins, the source is typically a strong reductant such as hydroxyurea or cyanamide [17]. Ferricytochrome *c* reacts with HNO to give NO and ferrous cytochrome *c*[18], suggesting the driving force in this system acts in the direction of reduction of cytochrome *c*, not its oxidation. We will show that the most likely cytochrome *c* oxidant is nitrogen dioxide ($NO_2^{\bullet}$), formed in the autoxidation of NO in the aerobic system used in the assay.

Materials and methods

Horse heart cytochrome *c* (Sigma; type VI, prepared without the use of trichloroacetic acid) was repurified by cation column chromatography, and the resulting fractions were tested for CO binding and for ascorbate and dithionite reducibility to determine the amounts of any modified fractions of the protein. Cytochrome *c* (100 mg) was dissolved in 1 ml of 100 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM diethylenetriamine pentaacetic acid (DTPA). The cytochrome *c* was passed down a CM52 cation-exchange column equilibrated with 85 mM potassium phosphate, pH 7.4, buffer. Fractions were collected automatically (60 fractions, 10 ml each) and analyzed by optical spectroscopy for the extent of reduction of the ferric form by ascorbate and dithionite. The major repurified cytochrome *c* fraction showed negligible spectrophotometric differences between ascorbate and dithionite reducibility and negligible CO binding to the reduced state. The estimated polymer concentration (from the difference between the A_{550nm} and the A_{540nm} values for dithionite and ascorbate reductions) was less than 1% of the total. Unless otherwise stated the repurified polymer-free samples were used in all experiments.

Ferrocytochrome *c* was made by the addition of excess ascorbate (50 mM) and the solutions were incubated for 20 min at room temperature. To remove ascorbate the samples were then passed down a Sephadex G-25 column equilibrated with 100 mM potassium phosphate buffer, 0.1 mM DTPA, pH 7.0. The cytochrome *c* concentrations were calculated using a reduced (Fe^{2+}) minus oxidized (Fe^{3+}) extinction coefficient ($A_{550nm} - A_{540nm}$) of 21.2 $mM^{-1}\,cm^{-1}$.

Sodium proliNONOate (proliNO) from Aventis Chemicals was used as a rapid-release nitric oxide donor (pure NO gas had similar effects). The concentration of proliNO in solution was determined in 20 mM NaOH using an extinction coefficient at 248 nm (A_{248nm}) of 8.6 $mM^{-1}\,cm^{-1}$. The ratio of [proliNO] to [NO] released was obtained by titration with oxyhemoglobin ($A_{557nm} - A_{630nm}$) using a difference extinction coefficient of 14.4 $mM^{-1}\,cm^{-1}$. The stock proliNO sample gave a [proliNO]:[NO]ratio of 1:1.94.

Experiments carried out under anaerobic conditions were achieved using either extensive vacuum degassing or the addition of a glucose/glucose oxidase/catalase mixture. Decay products of proliNO (control) were produced by allowing proliNO to decay in 100 mM potassium phosphate buffer, 0.1 mM DTPA, for 45 min.

Cytochrome *c* reduction was followed using the change in absorbance at 550–540 nm. To convert this to percentage reduction, fully oxidized and fully reduced cytochrome *c* absorbance values were measured before the termination of each experiment by addition of ferricyanide (10 μM $K_3Fe(CN)_6$) and dithionite (10 μl of a 10% solution in 3 ml), respectively. All spectrophotometric data were collected via a Cary 5E optical spectrophotometer (Varian) and analyzed using Microsoft Excel with data-fitting in Kaleidagraph®.

DTPA was used as a metal chelator in all experiments to prevent unwanted redox side reactions from contaminating metals such as iron. DTPA was used at 0.1 mM for the studies illustrated, but varying the concentration from 0.01 to 0.1 mM had no effect on the extent of cytochrome *c* oxidation observed.

Concentrations of dissolved oxygen and nitric oxide were measured in a glass chamber connected to an oxygen electrode with a Digital Model 10 controller (Rank Brothers, Cambridge, UK). The chamber was fitted with a specially adapted flowthrough plunger to accommodate an ISO-NO NO electrode connected to an ISO-NO MK2 NO meter (World Precision Instruments, Stevenage, UK). The NO electrode was calibrated with proliNO under anaerobic conditions (achieved by a glucose/glucose oxidase/catalase mixture). The oxygen and NO electrode systems were connected to a MacLab 8E data acquisition system (AD Instruments) and the stored data analyzed with Microsoft Excel. Optical spectra were collected simultaneously with electrode data using an Ocean Optics CCD-based fiber-optic spectrophotometer in a custom-built spectroelectrode system [19].

Kinetic modeling of differential equations used the Rosenbrock stiff algorithm in the Macintosh computer software Berkeley Madonna (version 8.3.18; copyright Robert I. Macey and George F. Oster); the minimum step size was set to 1×10^{-6} s and the maximum to 1 s, with a tolerance of 0.01.

Unless otherwise stated the term "significant" in the text refers to unpaired *t* tests, with the significance level set at $p<0.05$.

Results

As is the case with NO gas [12], the addition of the fast-releasing NO donor proliNO oxidizes horse heart ferrocytochrome to produce ferricytochrome *c* (Fig. 1). Repurification of the commercial cytochrome sample to remove the small fraction of NO-binding polymers (cf. Materials and methods) had no significant effect on the amount of ferrocytochrome *c* remaining after 30 min (84.0 ± 2.4% for unpurified and 84.7 ± 1.6% for purified; mean ± SD, $n=4$). Catalysis by polymers is thus not responsible for cytochrome *c* oxidation by NO solutions.

It was therefore important to study the effect of oxygen and medium composition. Fig. 2 summarizes the spectral changes occurring under

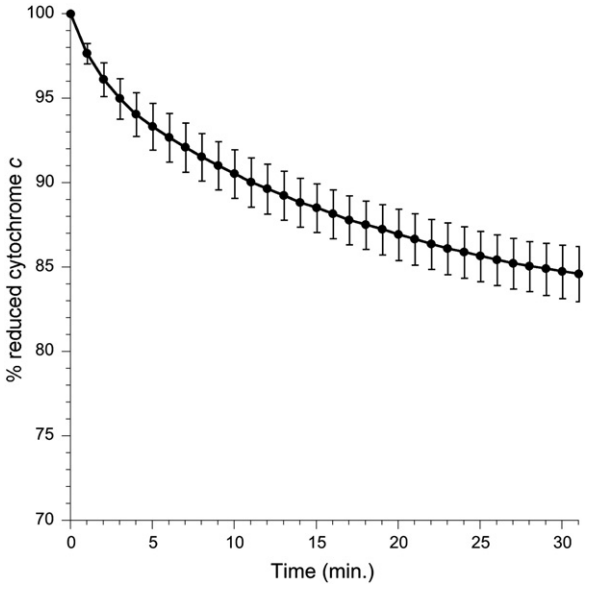

Fig. 1. Cytochrome *c* oxidation by proliNO under aerobic conditions. 40 μM proliNO was added to 10 μM ferrocytochrome *c* at a temperature of 30 °C and a pH of 7.4, buffered with 100 mM potassium phosphate, 100 μM DTPA. Cytochrome *c* reduction was calculated as described under Materials and methods. Means ± SD ($n=4$).