Cytochrome *c* oxidase rapidly metabolises nitric oxide to nitrite

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Abstract Previous studies have shown that the addition of nitric oxide to cytochrome *c* oxidase rapidly generates spectral changes compatible with the formation of nitrite at the binuclear haem:copper centre. Here we directly demonstrate nitrite release following nitric oxide addition to the enzyme. The nitrite complex is kinetically inactive and the off rate for nitrite was found to be slow (0.024 min^{-1}). However, the presence of reductants enhances the off rate and enables cytochrome oxidase to catalyse the rapid oxidation of nitric oxide to nitrite free in solution. This may play a major role in the mitochondrial metabolism of nitric oxide. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Cytochrome *c* oxidase; Nitrite; Nitric oxide; Inhibition; Metabolism; Cytochrome *c*

1. Introduction

Cytochrome *c* oxidase (ferrocytochrome *c* oxido-reductase, EC 1.9.3.1), CCO, is the terminal enzyme of the mitochondrial respiratory chain and catalyses the reduction of molecular oxygen to water [1]). Electrons enter the enzyme at the Cu_A site which is in rapid redox equilibrium with one of the haem irons, Fe_a. From here, electrons are transferred to the binuclear centre, comprising the magnetically coupled Cu_B and Fe_{a3} metals, where oxygen is reduced and water formed.

CCO is known to be reversibly inhibited by nitric oxide (NO), the final inhibited species being a ferrous nitrosyl complex formed between NO and ferrous Fe_{a3} [2–5]. However, a primary role in inhibition has also been attributed to the other metal in the binuclear centre, Cu_B. From an analysis of data obtained by optical spectroscopy, we have suggested that NO rapidly (in the ms time range) reduces Cu_B forming nitrite within the binuclear centre [6] (Fig. 1).

The scheme depicted in Fig. 1 predicts that on reaction with NO, one nitrite ion (or molecule of nitrous acid) is formed per enzyme molecule. In this paper, we report an experimental test of this hypothesis by measuring the nitrite released from the enzyme after reaction with NO and hence estimate the value of the stoichiometry of the reaction. In addition, we examine the effect of nitrite on the activity of the enzyme and enquire whether part of the inhibitory effect of NO may stem from the formation of nitrite within the enzyme's active site. Such an inhibitory effect may be expected if nitrite were to act in a manner similar to chloride. This latter anion is known to exert a powerful influence on the properties of the binuclear centre of CCO [7]. We have examined this possibility by measuring the rate constant for the oxidation of cytochrome *c* by CCO incubated with nitrite and comparing this to control experiments in which no nitrite was present. Our results provide evidence which supports the hypothesis that nitrite is formed on reaction of NO with oxidised CCO and demonstrate that this nitrite can act as an inhibitor of CCO. However, this inhibition is rapidly alleviated under reducing conditions, consistent with the observation that NO inhibition of CCO may be fully and rapidly reversed.

2. Materials and methods

2.1. Cytochrome oxidase preparation

CCO was prepared by the method of Soulimane and Buse [8]. As this preparation contains a fraction of the binuclear centre with chloride bound, the enzyme was 'pulsed' by reduction and reoxidation, as described previously [6]. This generated a fast form of the oxidised enzyme; this form most closely resembles the enzyme state in vivo and is able to interact rapidly with exogenous ligands at the binuclear centre, e.g. cyanide, NO [9]. The enzyme concentration was determined using $\Delta\varepsilon_{605-630} = 42\,000\ M^{-1}\ cm^{-1}$ for the reduced enzyme. Unless otherwise stated, the buffer used for all experiments was 0.1 M HEPES, 0.5% Tween 80, pH 7.4.

2.2. Measurement of nitrite release after addition of NO to oxidised 'O' CCO

The oxidised pulsed enzyme was briefly (< 1 min) incubated with 100 μM NO under anaerobic conditions. Excess NO and any breakdown products were removed by immediate passage through an air-equilibrated Sephadex G-25 column (10 cm by 1 cm) containing the same buffer. The time at which the enzyme sample from the column was collected was taken as 't_o'. Aliquots of 50 μl were taken at known times after passage through the column and centrifuged in 'Clear' Microcon microconcentrators, 30 000 MW cut-off in a microcentrifuge Sanyo Microcentaur at 13 000 rpm for 7 min. Typically, 30 μl was recovered after passage through the membrane. The filtrates were frozen immediately on dry ice and the nitrite concentration subsequently measured using the fluorescence of 2,3-diaminonaphthalene [10].

2.3. Formation of nitrite/CCO complex and assay of activity

Immediately after 'pulsing', CCO was incubated with either 100 μM NO or 10 mM sodium nitrite for 5 min. The excess of nitrite/NO was removed by passage through a Sephadex G-25 column equilibrated in the same buffer. The sample (2 μM enzyme), containing nitrite in the binuclear centre as judged by optical spectroscopy [6], was rapidly mixed under aerobic conditions with 30 μM ferrocytochrome *c* and 1.5 mM ascorbate in a stopped flow apparatus (model SX-18MV Applied Photophysics, Leatherhead, UK). Spectra were collected using a photodiode array detector with a time resolution of 3.3 ms. An initial burst phase of cytochrome *c* oxidation was seen as the electrons entered the haem *a* and Cu_A centres of the enzyme, followed by a slower exponential rate of cytochrome *c* oxidation. Global fitting of

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the data (Global analysis, Applied Photophysics) was used in order to obtain the rate constant for cytochrome *c* oxidation. The rates were compared to control enzyme, that had undergone the same procedure, except for the addition of NO/nitrite.

2.4. Steady state of cytochrome c

The steady state % reduction of cytochrome *c* following the addition of 20 mM ascorbate to 4 μM cytochrome *c* and 4 μM CCO was measured using the wavelength pair 550–540 nm in a Hewlett Packard diode array spectrophotometer, assuming that cytochrome *c* was fully oxidised prior to the addition of ascorbate and fully oxidised prior to the addition of ascorbate and fully reduced following anaerobiosis. The assay buffer used was 0.1 M K^+HEPES, 0.1% lauryl maltoside, pH 7.8.

All errors reported are ±1 S.D.

3. Results

3.1. Release of nitrite from the binuclear centre after reaction with NO

Addition of NO to species 'O', the fully oxidised 'pulsed' enzyme, led to the rapid reduction of a fraction of the cytochrome *a* (typically ~40%) and a spectral transition in the Soret region previously assigned to the formation of nitrite within the binuclear centre. These findings are in agreement with those previously reported [11]. If the enzyme (species 'O') was previously incubated with a binuclear centre ligand, such as azide or formate, then addition of NO led to no reduction of cytochrome *a* and no spectral changes indicative of nitrite binding within the binuclear centre.

In order to test further the hypothesis that nitrite is the product of the reaction of 'O' with NO, we carried out experiments in which we reacted this enzyme species with NO and, after passage through a gel filtration column to remove excess exogenous NO, nitrite and nitrate, we monitored the solution for the presence of nitrite. This methodology was chosen as it may be expected from comparison with other weak ligands such as chloride that nitrite may only very slowly leave the pocket in which the binuclear centre resides. Table 1 reports the results taken over the first 40 min of experiments in which 20 μM CCO was reacted with excess NO. A slow release of nitrite was observed, stoichiometric with the enzyme concentration. After 40 min, no further increase in nitrite concentration was observed. In fact the nitrite concentration slowly declined in a process itself dependent on the presence of CCO.

3.2. Effect of nitrite in the binuclear centre on the activity of CCO

3.2.1. Stopped flow experiments. CCO catalyses the oxidation of cytochrome *c* by molecular oxygen. On mixing ferrocytochrome *c* with aerobic CCO in a stopped flow apparatus, the absorbance at 550 nm, the α peak of ferrocytochrome *c*, is bleached as this protein is oxidised. The time course of this optical transition comprises a rapid 'burst' phase, denoting rapid electron entry into the Cu_A and Fe_a sites, followed by

Table 1
Time course of nitrite release into solution from cytochrome oxidase pre-incubated with NO

Time (min)	Nitrite concentration (μM)
0	0.1 ± 0.1
10	1.6 ± 0.1
12	4.5 ± 0.2
40	19.8 ± 3

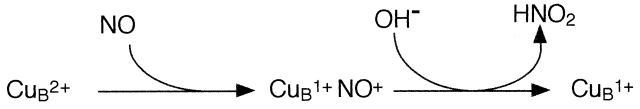

Fig. 1. Copper-catalysed NO oxidation by cytochrome oxidase.

an exponential decay as the remainder of the ferrocytochrome *c* is converted to the ferric form. The rate constant for this exponential process, at constant ferrocytochrome *c* and CCO concentrations, is a measure of the activity of the enzyme. Fig. 2 shows the results of experiments in which CCO species 'O' was reacted with NO (100 μM) and then rapidly passed through a gel filtration column (Sephadex G-25) to free it of excess NO. The enzyme was then mixed with ferrocytochrome *c* in a stopped flow spectrophotometer. No change was seen in the rapid burst phase, indicating that NO had not affected the haem *a* or Cu_A sites. However, inhibition was observed in the slower exponential phase of cytochrome *c* oxidation. The figure reports the rate constant for the exponential oxidation of ferrocytochrome *c* by this enzyme preparation as a fraction of that of the enzyme treated identically except for the omission of NO. It is seen that initially, i.e. approximately 10 min after the addition of NO, the enzyme expresses some 10% of the activity of the control. With the passage of time, the enzyme regained its activity such that after 2 h the rate constant was close to 70% of that of the control. Fitting the data to an exponential process yielded a rate constant of $3.9(\pm 1)\times 10^{-4}$ s^{-1}.

This experiment shows that on addition of NO to species 'O', the rapid reaction which leads to the reduction of cytochrome *a* leaves the enzyme in a partially inhibited state from which it slowly recovers. The time for recovery of activity is of the same order as that for the release of nitrite from the binuclear centre (Table 1) and we conclude therefore that nitrite formed within the active site, as described in Fig. 1,

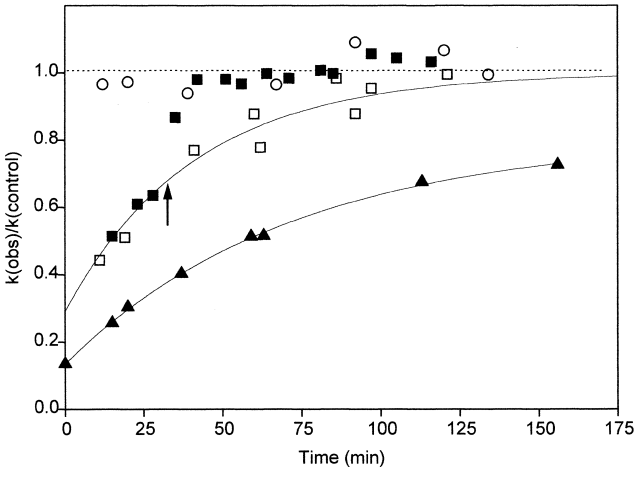

Fig. 2. Return of CCO activity following NO or nitrite treatment of the oxidised enzyme. Oxidised CCO was treated with excess nitrite, NO or neither (control), passed down a column and assayed for cytochrome *c* oxidation as described in Section 2. The time refers to the time post-removal from the Sephadex column. The rates are plotted as a fraction of the rate for the control enzyme at time zero. Control (open circles), nitrite (open squares), NO (filled triangles). The filled squares represent data from a sample treated with nitrite, but to which 1 mM ascorbate and 200 μM ruthenium hexamine chloride were added at a time indicated by the arrow.

impairs electron donation to the Fe_{a3} and/or Cu_B sites. This inhibition is lifted as nitrite dissociates from the enzyme.

In order to further test this hypothesis, the activity of the CCO/nitrite complex formed by incubating the enzyme with sodium nitrite was measured. This anion binds slowly to the binuclear centre over minutes and then only at mM concentrations (data not shown). Freshly prepared species 'O' was incubated with nitrite (5 mM) for 5 min and then freed from the excess anion by passage through a Sephadex G-25 column. The complex was then assayed through measurement of the rate constant for ferrocytochrome *c* oxidation as described above. Control experiments were performed in parallel in which nitrite was omitted from the incubation buffer. Fig. 2 shows the results and indicates a strong inhibitory effect of nitrite which is relieved slowly over approximately 1 h. During this time, the rate constant for ferrocytochrome *c* oxidation increased and approached that for the uninhibited control enzyme. The rate constant for the return of activity is $1.6(\pm 1)\times 10^{-4}$ s^{-1}, close to that found for the return of activity after the reaction with NO. We conclude that in both cases this rate constant is that of nitrite dissociation from the enzyme.

The only difference observed between the oxidised enzyme following excess nitrite treatment and that following excess NO treatment is that there is incomplete recovery of activity in the case of NO. Only 81% (±3%) of activity was restored; this suggests some irreversible damage accompanied the addition of 100 μM NO to the oxidised enzyme. Separate studies showed that this effect occurred immediately following NO addition and was due to the relatively high concentration of NO used. Adjusting the curves for this fraction of damaged enzyme showed them to be superimposable.

The slow alleviation of inhibition by nitrite poses a problem; if this anion is formed within the binuclear centre by reaction with NO then inhibition by NO should also be long lived. It is known, however, that inhibition by NO is rapidly and completely reversed [2,3]. In order to investigate this problem further, we injected electrons into the oxidised nitrite-inhibited enzyme via the addition of ascorbate and ruthenium hexamine. On addition of this redox system, inhibition is rapidly relieved; this suggests that entry of the negative electron can eject the negative nitrite anion from the binuclear centre and rapidly restore activity, consistent with the idea that the hydrophobic binuclear centre pocket favours electroneutral processes [12].

3.2.2. Steady state experiments. The experiments reported above were conducted under conditions in which the enzyme did not enter a true steady state. If the relief of inhibition by the addition of reductants is correct then the NO-incubated enzyme should become de-inhibited with time. In Fig. 3, we show the results of experiments in which a steady state is established by mixing the enzyme with cytochrome *c* in the presence of excess reductant (ascorbate) and oxygen. This figure reports the steady state level of reduction of cytochrome *c* while the enzyme is turning over. The steady state reduction is a function of how readily cytochrome *c* is reduced by ascorbate, and how fast it is oxidised by cytochrome oxidase. The former rate is fixed by the ascorbate concentration, so the lower the steady state level of *c* reduction, the faster the protein is being oxidised by the oxidase. During steady state, the control enzyme maintains cytochrome *c* as 20% reduced until the oxygen in solution becomes exhausted, at which point the excess ascorbate reduces it fully to ferrocytochrome *c*. Fig. 3 also shows the results obtained for enzyme treated with NO and passed through a gel filtration column. This enzyme was then allowed to age for ~30 min so that it regained some activity (see Fig. 2). This enzyme was initially unable to maintain the reduction level of cytochrome *c* at the low (20%) level seen with the control enzyme, indicating that it was unable to pass electrons so rapidly from ferrocytochrome *c* to oxygen and was thus partially inhibited. However with time, the enzyme regained activity as shown by the fall in the reduction level of cytochrome *c*. After 2 min, the enzyme had essentially the same activity as the control. The inhibition due to nitrite formation in the binuclear centre had been rapidly reversed by the presence of excess ferrocytochrome *c*.

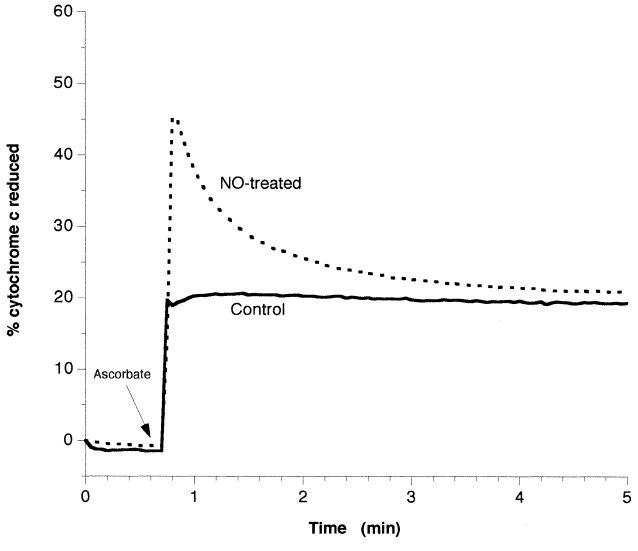

Fig. 3. Effect of NO pre-treatment on cytochrome *c* steady state. Oxidised cytochrome oxidase was used 30 min subsequent to NO treatment as in Fig. 2. At the indicated time, 20 mM ascorbate was added to the solution containing 4 μM cytochrome *c* and 4 μM cytochrome oxidase.

4. Discussion

CCO once reacted with NO and then freed from this reagent and its breakdown products slowly releases nitrite into solution. The time course of this release has a $t_{1/2}$ of approximately 20–25 min. Moreover, the maximum concentration of nitrite released was equivalent to the concentration of the enzyme. These data are consistent with Fig. 1 in which each NO reacts with the binuclear centre in a 1:1 stoichiometry to form nitrite. This anion once formed within the active site inhibits the enzyme (Fig. 2) and slowly dissociates from this site into solution. However, in the presence of reductants this relief of inhibition is greatly accelerated.

Mammalian haem enzymes must live in a world where NO is ever present. This is a particular problem for enzymes, like cytochrome oxidase, that must react with oxygen, given the difficulty in discriminating between NO and O_2 binding to ferrous haem metalloproteins [13]. Copper enzymes, like cytochrome oxidase, have the additional problem that NO can be oxidised by cupric copper to generate nitrite [14]. Both the ferrous haem nitrosyl complex and the ferric haem nitrite complex are potential long-term high affinity inhibitors of

cytochrome oxidase. The enzyme's active site has evolved to deal with both these difficulties by distinct mechanisms: in the first case, the haem a_3 nitrosyl complex has an unusually high intrinsic rate of dissociation [15]; in the latter case, the enzyme's substrate (electrons) effectively relieves the inhibition (this paper). It will be interesting to see how widespread these 'defence' effects are in other metalloproteins.

Finally, NO, although synthesised by a specific enzyme, seems to have multiple means of destruction in vivo [16]. Mitochondria have been demonstrated to be a major metaboliser of NO in the cell [17]. A significant fraction of this metabolism is cyanide sensitive and therefore likely to be associated with cytochrome oxidase. As shown in this paper, the oxidation of NO to nitrite by this enzyme results in a rapid means of metabolism of NO with a safe end-product.

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