Reversal of Cyanide Inhibition of Cytochrome c Oxidase by the Auxiliary Substrate Nitric Oxide

AN ENDOGENOUS ANTIDOTE TO CYANIDE POISONING?*

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Nitric oxide (NO) is shown to overcome the cyanide inhibition of cytochrome c oxidase in the presence of excess ferrocytochrome c and oxygen. Addition of NO to the partially reduced cyanide-inhibited form of the bovine enzyme is shown by electron paramagnetic resonance spectroscopy to result in substitution of cyanide at ferriheme $a_3$ by NO with reduction of the heme. The resulting nitrosylferroheme $a_3$ is a 5-coordinate structure, the proximal bond to histidine having been broken. NO does not simply act as a reversibly bound competitive inhibitor but is an auxiliary substrate consumed in a catalytic cycle along with ferrocytochrome c and oxygen. The implications of this observation with regard to estimates of steady-state NO levels in vivo is discussed. Given the multiple sources of NO available to mitochondria, the present results appear to explain in part some of the curious biomedical observations reported by other laboratories; for example, the kidneys of cyanide poisoning victims surprisingly exhibit no significant irreversible damage, and lethal doses of potassium cyanide are able to inhibit cytochrome c oxidase activity by only ~50% in brain mitochondria.

The reactions of cytochrome c oxidase with nitric oxide (NO) have recently been the subject of renewed scrutiny (1–6) because of their emerging physiological significance (7–12). Furthermore, the discovery and functional characterization of a mitochondrial NO synthase (9, 13, 14) raises a series of questions regarding the possible regulation of the electron transport chain by NO. Several catalytic cycles have been proposed (4, 6, 15–17) to explain the consumption of NO by the oxidase while turning over in the presence of cytochrome c and oxygen. Some of these cycles implicitly treat the NO as an auxiliary substrate rather than simply a competitive inhibitor, to account for the observed production of nitrite in addition to the slowing of electron transfer rate. In some cases an oxyferryl intermediate of heme $a_3$ is proposed and interaction of incoming NO with CuB, assumed (15, 16), whereas other schemes invoke the formation of a nitrosylheme $a_3$ intermediate (4, 6, 17). Consequently, clarification of which (if either) of the two centers of the binuclear pair, heme $a_3$ or CuB, preferentially reacts with NO to form detectable derivatives should be of some value in assessing the relative likelihood of one proposed scheme over another as conditions are varied.

When metalloenzymes have multiple binding sites for small molecular substrates and inhibitors, especially within the same active site domain, studies with mixed-ligand adducts can often reveal useful details of the coordination chemistry. Here we report the results of an investigation into the preferred site of NO reaction with bovine heart cytochrome c oxidase in the presence of the potent competitive inhibitor, cyanide, both under conditions of turnover and where the enzyme has been allowed to equilibrate in the presence of NO, cyanide, and reducing equivalents. The findings we report appear to be surprising in two respects. First, the inhibitory effects of NO plus cyanide toward the enzyme are not additive; NO actually eliminates cyanide inhibition. Second, contrary to the observations of earlier authors (18–21), we find that cytochrome c oxidase displays a marked tendency to form NO adducts of heme $a_3$ that are clearly 5-coordinate. The results have bearing on two related issues addressed under “Discussion.” These are the likely mechanism of NO oxidation by the enzyme under conditions of high electron flux and the apparent existence of a cyanide-insensitive “pool” of cytochrome c oxidase in vivo.

MATERIALS AND METHODS

Enzymes and Reagents—Cytochrome c oxidase (EC 1.9.3.1, complex IV) was isolated from beef heart pericardium without the preparation of Keilin-Hartree particles (requiring acidification to pH < 6) common to most procedures. Using intact mitochondria, complex IV was separated from the other components of the electron transport chain by deoxycholate extraction as described by Ragan et al. (22) and then finally purified by ammonium sulfate/cholate fractionation according to the procedure of Yonetani (23). This method (in which the pH is maintained in the range of 7.8 to 8.0 throughout) yields good activity preparations of the oxidase that exhibit a 424 nm Soret absorption maximum, do not exhibit a g = 12 EPR signal, rapidly bind cyanide, and rapidly react with NO, which are characteristics of the pulsed form of the enzyme (24). Moreover, the preparations usually persist in the pulsed form when stored overnight at 5–6 °C or for months (at least) in frozen (glassing) solution at 77 K. The enzyme was determined to be spectroscopically pure if the 444–424 nm ratio for the reduced enzyme was 2.2 or higher (25). Derivatives were prepared in 50 mM potassium phosphate, 1 mM in sodium EDTA, and 0.1% in lauryl maltoside, pH 7.4–7.8, to concentrations of 10–80 μM (in enzyme). Enzyme concentrations were determined as total heme $a$, using the differential (absorption) extinction coefficient of Δ$\varepsilon_{max} = 12$ mocm$^{-1}$ cm$^{-1}$ for the reduced minus oxidized spectrum of the enzyme (26). Concentrations throughout are given on a per enzyme concentration basis (not per [heme $a$]). Ferrocytochrome $c$ oxidoreductase activity was determined employing the high ionic strength method of Sinjogoro et al. (27). Using this assay, we obtain a turnover number with respect to cytochrome c of 340 (±30) s$^{-1}$ (0.1 M sodium phosphate, 0.1% lauryl maltoside, pH 7.4, 22 °C) similar to that of the bovine enzyme isolated from a variety of tissues by others (27).

* This work was supported by grants from the National Institutes of Health (HL61411 to J. P.) and the Medical Research Council of Canada (to B. C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ Received for publication, September 17, 2003, and in revised form, October 3, 2003. Published, JBC Papers in Press, October 8, 2003, DOI 10.1074/jbc.M310359200

This paper is available on line at http://www.jbc.org
or Sigma. Sodium dithionite, 87% minimum assay (prepared without adding NO. 0.26 mM (in total heme a) cytochrome oxi-
dition and under subdued light, was as follows. Approximately 2 ml of
initial oxygen concentrations were 50 nM and
0.5% lauryl maltoside, pH 7.4. Total cytochrome c oxidase and
ight oxygen concentrations were 50 nM and ~250 μM, respectively, for
each individual assay. Reactions were monitored spectrophotometrically
by following the disappearance of the ferrocytochrome c band at
550 nm. The broken line shows the expected result for the additive effect
of two concerted inhibitors.

A typical heme a extraction, maintaining nominally anaerobic condi-
tions and under subdued light, was as follows. Approximately 2 ml of
0.26 mM (in total heme a) cytochrome c oxidase solution was taken, and
8 drops of concentrated sulfuric acid were added to precipitate the
protein. After centrifugation (7000 × g, 22 °C, 2 min) the supernatant
was discarded and heme a extracted from the pellet (22 °C, 20 min, frequent vortexing) into 1 ml of dimethylformamide. The concentration
of heme a (80–100 μM) was determined using the extinction coefficient
εa 500 = 24 mm−1 cm−1 reported for the pyridine hemochrome (28). The
procedure also results in the extraction of copper from the enzyme
which appears in the final dimethylformamide solution as a stable
Cu(II) species that is not reducible by ascorbic acid. EPR signals arising
from this species have been removed from the nitrosylferroheme a spectra of Fig. 2, C and D, by subtracting the spectra of control samples
prepared without adding NO.

All reagents are ACS grade or better, used without further purifi-
cation, and unless stated to the contrary, were purchased from Aldrich
or Sigma. Sodium dithionate, 87% minimum assay (+H2O), was ob-
tained from EM Science. 14C sodium cyanide, 99%, was obtained from
Cambridge Isotope Laboratories. Argon and nitric oxide gases were
obtained from Matheson Incorporated. Nitric oxide was scrubbed with
water and KOH pellets prior to use and added to enzyme samples
volumetrically with gas-tight syringes. Buffers solutions never exhib-
ted any significant change of pH (i.e. <0.05 pH units) following NO
and/or sodium dithionate additions.

Instrumental Methods—Electronic absorption spectra were mea-
sured and photometric determinations made using a Shimadzu UV-
2501PC spectrophotometer. X-band EPR spectra were obtained using an
IBM ESP 300 spectrometer equipped with a Bruker B-E 25 electron-
magnet and Bruker ER4116DM resonant cavity. Cryogenic tempera-
tures were maintained using an Oxford Instruments ESR 910 liquid
helium flow cryostat in conjunction with a VC30 controller. Frequency
calibration was with reference to diphenylpicrylhydrazyl.

RESULTS

Enzyme Kinetics—The enzymatic activity (Fig. 1) of cyto-
chrome c oxidase (50 nm) was assayed photometrically by meas-
uring the rate of oxidation of reduced cytochrome c in the
presence of excess oxygen (control, ◦) as described previously
(17). The presence of a small excess of cyanide (100 nm) was
observed to inhibit the measured activity greatly (◆), and the
presence of NO (1 μM) was also observed to inhibit the activity,
but less so (▲). However, when NO was added to the assay
mixture in the presence of cyanide (♦), a net increase in the
enzyme activity was attained compared with the experiment
with cyanide alone. That is, with both “inhibitors” present, the
effect of NO was phenomemologically opposite to that of cy-
انيد، reversing the cyanide inhibition. This is a very surprising
result because the effects of two inhibitors competitive with
oxygen binding are expected to be additive and would be pre-
dicted to yield results as indicated by the broken line in Fig. 1.

In fact, we made the effort to verify, at least in qualitative
terms, that the net inhibition observed with 100 nM cyanide plus
1 μM carbon monoxide did indeed follow this simple ex-
pectation (not shown), although obtaining good quality linear
data at the resulting very high levels of inhibition was
challenging.

It should be noted additionally that under these particular
reaction conditions, biphasic kinetics were observed in the case
of the cyanide plus NO experiments (◆), with the initial rates
comparable with those obtained in the presence of NO only and
the later phase (not shown) identical to that observed with only
cyanide present. The results of these turnover experiments
strongly suggest that NO displaces cyanide during reaction
ycles with oxygen. Also, it is clear that the NO is not simply
ating as a reversibly bound competitive inhibitor, because if
were, the inhibitory effects of NO and cyanide would be additive.
Rather, NO seems to be consumed during the reaction, in
keeping with the earlier findings of several groups (2, 17, 29)
who have shown that the reaction between cytochrome c ox-
dase and NO leads to production of nitrite under a variety of
conditions. We have suggested previously (17) one way in
which this process can also support oxygen consumption (NO
oxidase activity, see “Discussion”). Cyanide is not consumed
during turnover, merely temporarily displaced, with the sys-
tem returning to cyanide-inhibited kinetics once the available
NO has been oxidized.

EPR Spectroscopy—A series of EPR experiments was under-
taken to further examine the ability of NO to displace cyanide
from the binuclear, oxygen-binding site of the enzyme. It has
been observed previously that NO undergoes a variety of reac-
tions with the resting oxidized and reduced forms of “dimeric
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cytochrome c oxidase (i.e. preparations containing enzyme ag-
gregates with >20 subunits, rather than the 13 minimally
required). The nitrosylferrocytochrome a2 (g = 2.09, 2.00, 1.97,
and 9-line hyperfine structure on g = 2.00, ANO = 21 G, AHis
= 7 G) resulting from the reduction of Hartzell-Beinert enzyme
preparations in T00n detergent incubated with NO have
been investigated intensely (19, 20). Studies using 15NO and
the yeast enzyme from cells grown in 15N-labeled histidine-rich
media (18, 21) demonstrated conclusively that (i) the NO was
bound to ferrocytochrome a2 and (ii) the EPR signal was fur-
ther split by the presence of the proximal histidine ligand of
the enzyme.

The EPR spectrum obtained immediately following the ad-
dition of NO (1 atm) to a reduced sample of the current pulsed
oxidase preparation is shown in Fig. 2A. Unlike the previously
reported EPR spectrum of nitrosylferrocytochrome a2 derived from
other preparations dispersed in Tween 80, the present
data consist of overlapping spectra containing both 3-line (A =
18 G) and 9-line (A = 20 G and A ~ 7 G) hyperfine features. The
two sets of signals clearly indicate a mixture of at least two
products; one is the 6-coordinate species described above and the
other (minority species) a new 5-coordinate derivative (see
below).

It has been shown repeatedly by magnetic circular dichroism
spectroscopy (30–33) that in the presence of excess reductant,
cyaniide-bound heme a2 is 6-coordinate and remains in the
ferric form, whereas the other metal ion centers become re-
duced. Almost certainly, this partially reduced cyanide adduct
is the major inhibited form in turnover experiments where
cyaniide is present. The EPR spectrum of the partially reduced
cyaniide adduct (10 mM in sodium cyanide plus excess sodium
dithionite) is broad but readily detectable at 15 K (Fig. 2B, broken line). Treatment of this inhibited species with NO (1 atm)
results in the quantitative formation of a new derivative
exhibiting a characteristic EPR signal containing a distinct

FIG. 1. Lineweaver-Burk plots showing inhibition of cyto-
ochrome c oxidase activity by 100 nm cyanide (●), a mixture of 100
nm cyanide plus 1 μM NO (♦), 1 μM NO only (▲), and a control
with no inhibitor present (○) at 22 °C, 0.1 M phosphate buffer,
0.05% lauryl maltoside, pH 7.4. Total cytochrome c oxidase and
ight oxygen concentrations were 50 nM and ~250 μM, respectively, for
each individual assay. Reactions were monitored spectrophotometrically
by following the disappearance of the ferrocytochrome c band at
550 nm. The broken line shows the expected result for the additive effect
of two concerted inhibitors.
[Figure 2: X-band EPR spectra of bovine cytochrome c oxidase and extracted heme a derivatives at 15 K (power = 0.2 milliwatt, modulation amplitude = 4 G, gain = 1 x 10^4). A, 60 μM oxidase in 0.1% lauryl maltoside, 25 mM phosphate, 1 mM EDTA, pH 7.4, reduced with excess sodium dithionite under 1 atm of NO. B, enzyme (buffering conditions as described in A) treated with 1 mM sodium cyanide and sodium dithionite reduced (broken line) following exposure of retawed sample to 1 atm of NO (solid line). C, extracted heme a (0.34 mM) in dimethylformamide reduced with ascorbic acid and 1 atm of NO introduced (solid line) compared with enzyme nitrosyl adduct from B (dotted line). D, heme a 15% in pyridine (other conditions as described in C).

3-line hyperfine pattern (A = 18 G) (Fig. 2B, solid line). This spectrum is predicted in the case of a single electron spin coupled to the three allowed orientations (M = −1, 0, +1) of a nucleus like 14N with nuclear spin I = 1 and is sometimes referred to as “superhyperfine” structure if it is known that the electron and nuclear spins are not mainly localized on the same atom. We have also obtained qualitatively similar results starting from preparations of the dimeric resting enzyme dispersed in Tween 80 detergent (not shown); but in this case, the derivative exhibiting the 3-line hyperfine signals appeared to be only one of at least two species formed. The nitrosyl complexes of ferrohemes have been shown to have 5-coordinate structures in the absence of other strong-field ligands by elemental analysis of pure crystalline model compounds (34–36) and a structure determination by x-ray crystallography (34). Moreover, complexes of this type (NO-bound, d7, 5-coordinate hemes) have been demonstrated unequivocally to exhibit distinct 3-line hyperfine EPR spectra like those shown in Fig. 2 (37, 38). To better illustrate the point, the EPR spectrum of the nitrosyl adduct of extracted ferroheme a is shown in Fig. 2C (solid line), and also the spectrum of the analogous enzyme derivative has been superimposed (broken line) for comparison. It is to be stressed that the 5-coordinate nature of the complex is unambiguously demonstrated by the nitrosylferroheme a EPR spectrum of Fig. 2C. If two axial nitric oxide ligands were bound, they would either couple antiferromagnetically, resulting in no EPR signal, or couple ferromagnetically, leading to a more complicated signature than the observed 3-line hyperfine. The effect of introducing a sixth nitrogenous ligand (e.g. pyridine) on the EPR spectrum of the extracted nitrosylferroheme a EPR spectrum is shown in Fig. 2D. Clearly, the spectrum of the nitrosyl enzyme derivative formed in the presence of cyanide does not look at all like the 6-coordinate case.

In summary, the EPR data convincingly demonstrate that substitution of cyanide by NO at heme a3 in the partially reduced cyanide adduct (starting with monomeric pulsed enzyme) renders the heme a3 site 5-coordinate (Fig. 2B). To verify that the enzyme spectrum of Fig. 2B (solid line) represents a species in which cyanide is no longer bound to heme a3 after NO addition, samples made with 13C-labeled cyanide were examined and found to contain no additional hyperfine features (not shown), confirming the displacement of the cyanide ion. The overall similarity of the 3-line hyperfine features of Fig. 2, A and B, indicates that, contrary to the earlier findings of others, a fraction of the heme a3 can also be pentacoordinate in the fully reduced derivative prepared in the absence of cyanide. These results suggest that the histidine ligand proximal to heme a3 is more labile than previously thought, indicating some conformational flexibility at the binuclear pair. In support of this observation, it is noteworthy that the proximal ligand-to-heme a3 bond length in the crystal structure of the Thermus thermophilus enzyme is significantly longer than in other reported structures (39).

Electronic Absorption Spectroscopy—In Fig. 3A the electronic absorption spectra of two NO adducts of cytochrome c oxidase are shown. The first is formed by the addition of NO to the dithionite-reduced enzyme (dashed line) and the second by the addition of NO to the partially reduced cyanide adduct (solid line). In the case of the second species, the order in which cyanide, NO, and reducing agent are added is not important, as the same derivative was always obtained (result confirmed by EPR spectroscopy; data not shown). These data appear to be in agreement with the EPR spectra of Fig. 2, A and B; that is, in the presence and absence of cyanide somewhat different NO adducts are obtained.

Because NO is converted to nitrite by cytochrome c oxidase (2, 17) it was important to establish whether any of the species formed in the presence of excess NO were nitrite adducts. As shown in Fig. 3B, the Soret maximum shifts from 424 nm in the pulsed enzyme (solid trace) to 419 nm (dotted trace) following the addition of excess sodium nitrite. Note that neither of these is the same Soret maximum found associated with the monomeric resting enzyme (421 nm, dashed line). Furthermore, we did not observe the appearance of a g = 12 EPR signal (not shown) upon formation of the nitrite adduct. In fact, when nitrite is added to the pulsed enzyme, some reduction of nitrite to NO occurs, and a very small amount of nitrosylcytochrome c a3 appears in the EPR spectrum. Superficially, the absorption spectrum of the nitrite adduct resembles that of the dimeric resting cytochrome c oxidase in Tween 80 (Soret maximum 417–418 nm, not shown), but these are clearly not the same species. More importantly, there is no evidence in the spectra of Fig. 3A, where the Soret features are at 431 and 442 nm, for a shoulder in the vicinity of 419 nm. Consequently, none of the species formed in the presence of excess NO seem to be simple nitrite adducts.

It is important to bear in mind that the Soret maxima of cytochrome c oxidase derivatives are subject to distortion by the presence of heme contaminants. Specifically, complex III of the electron transport chain can be an especially difficult im-
purity to eliminate (40). The pyridine hemochrome spectrum (28) of heme \(c\) extracted from the current enzyme preparations is shown in Fig. 3C. The presence of the visible region band at 588 nm establishes that the heme \(c\) macrocycle was not derivatized during the extraction procedure. More importantly, the absence of any peaks at 557 and 550 nm confirms that the preparations contained no significant amounts of heme \(b\) or heme \(c\), respectively (we estimate <3% of these relative to heme \(a\)). The Soret maxima we document herein have, of course, all been determined employing preparations essentially free of other heme-containing impurities.

**DISCUSSION**

**Mechanism of NO Turnover**—In conjunction with the findings of others, the present results have implications concerning the likely mechanism(s) of NO turnover by cytochrome \(c\) oxidase. Sarti et al. (4, 6) have recently proposed the existence of two competing mechanisms for the reaction of NO with the enzyme, one operating at low electron flux and the other at high electron flux. The experiments we report here all correspond to (or exceed in terms of excess reductant) the high electron flux conditions of the earlier authors (i.e. 50 mM enzyme and 18 \(\mu\)M cytochrome \(c\)). We have no basis in the current data for any opinion regarding the proposed low electron flux reaction, and so this need not concern us further.

Sarti et al. (4, 6) have demonstrated unequivocally that the rate-determining step in the high electron flux reaction of NO with cytochrome \(c\) oxidase is photosensitive; that is, dissociation of NO from ferroheme \(a_3\) is rate-limiting. However, the same authors assume (apparently) that the dissociated NO then diffuses out of the active-site pocket to be replaced by \(O_2\), and the uninhibited reaction proceeds. They did provide additional data showing that the presence of an NO scavenger further overcame NO inhibition of the enzyme, but this merely demonstrates the removal of free NO from solution, not its elimination from the active-site pocket. On the other hand, the present data (Fig. 1) show that at high electron flux and in the presence of cyanide, NO is not simply acting as a reversibly bound competitive inhibitor, because if it were, the inhibitory effects of NO and cyanide would be additive. Moreover, we have previously shown that cytochrome \(c\) oxidase out-competes oxy-myoglobin for available NO (17), consistent with the rapid formation of nitrosylferroheme \(a_3\) (4, 6) but leading to essentially quantitative production of nitrite (17).

In Fig. 4 we present an alternate reaction scheme for the high electron flux process that we think satisfactorily accounts for the observations from all laboratories. The present work and that of others clearly support a marked preference of NO for the heme site in variously prepared derivatives, and in transient kinetics experiments the inhibited state is thought to be associated with the formation of nitrosylferroheme \(a_3\) (5, 16). Therefore, in the chain of events where NO enters the active site pocket before \(O_2\), the available evidence strongly suggests that it will bind to heme \(a_3\) (1). We have previously shown that the nitrosylferroheme \(a_3\) derivative reacts with dioxygen in the presence of an additional electron donor to produce nitrite (17), perhaps via a peroxynitrite intermediate (41). Accordingly, any \(O_2\) entering the active site can reasonably be proposed to acquire an electron from \(C_u\) to form superoxide ion (\(O_2^-\)) (II). Following rate-limiting dissociation of the NO (but not its loss from the pocket (III)), the very rapid reaction between NO and \(O_2\) to form peroxynitrite (\(ONOO_2\)) can take place (IV). The \(ONOO_2\) formed at the active site will then quickly abstract two additional electrons from the enzyme, ejecting \(NO_2^-\) and water. Under appropriately controlled conditions *in vitro*, NO may certainly react with several of the intermediates in the normal catalytic cycle of cytochrome \(c\) oxidase (16). However, as the enzyme exhibits a marked tendency to react preferentially with NO compared with \(O_2\) (5), otherwise plausible inhibitory reaction sequences at high electron flux where \(O_2\) enters the active-site pocket before NO are likely minority reactions. We have previously shown in single cultured cardiomyocytes, the very rapid and essentially quantitative catastrophic conversion of en-
Inhibition of mitochondrial electron transport by the addition of cyanide is a standard protocol for establishing the basal O2 consumption in studies with respirometers (49). In practice, there is usually some residual O2 reduction measurable under these conditions. This observation can now be understood to be due, in part, to the endogenous NO production present in many samples. Also, there is a cyanide-resistant terminal oxidase known to be present in the mitochondria of all plants, many fungi, and some protozoa, but it is seemingly absent from metazoa (50). This alternate oxidase is certainly not a cyto-

dogenously generated NO to nitrite (17). Moreover, at endogenously generated levels of NO, there is no evidence for scavenging of NO by oxymyoglobin in cardiac tissue (42). These two observations are in keeping with a transient (not reversible) inhibition of cytochrome c oxidase, leading to quantitative production of nitrite in situ, with essentially zero release of NO from the active site of the enzyme. Also, as the oxygen concentration in mitochondria in vivo is a few micromolar at most (43), experiments at high electron flux (a large excess of reductant) are likely relevant to the in vivo situation. Therefore, on the basis of the presently available information, it is our opinion that the putative reaction scheme of Fig. 4 is a plausible candidate for that which operates in vivo when cytochrome c oxidase turns over in the presence of endogenously generated levels of NO.

**NO Binding to CuB2+**—The present data provide only evidence for NO bound to heme a3 and not CuB under reducing conditions (Fig. 2B). This requires some further comment, as NO is generally thought to be a good ligand for electron-rich metal ions such as both Fe2+ and Cu1+ rather than their common oxidized forms, Fe3+ and Cu2+. The fact that we were unable to readily make NO adducts of CuB in its reduced state is, therefore, intriguing. The tenacity with which NO seeks to displace cyanide from heme a3 in its Fe3+ state rather than bind CuB2+ both under turnover conditions (Fig. 1) and when NO is added to the partially reduced cyanide adduct (Fig. 2B) is even more remarkable. It seems that there is either some feature of the CuB site that blocks NO binding, or the strength of the interaction between heme a3 and NO results in CuB losing any competition for the ligand. Experiments with reduced forms of the dimeric bovine NO adduct (44, 45) have demonstrated that NO can be photodissociated from heme a3 and, in one case (45), transferred to CuB. However, there appears to be a consensual view among all authors that complexes of NO with reduced CuB do not form under non-photolytic ambient conditions. It has been suggested (46, 47) that reduced CuB may transfer an electron to a modified tyrosine close by in the active site. This would be expected to leave the nominally reduced CuB with considerable cupric character and might afford an explanation for the lack of reactivity with NO. Such a suggestion is not necessarily in conflict with the observation that in the oxidized enzyme CuB can seemingly exchange couple with NO (19, 20); because this simply requires the two spins to be in close proximity, there need be no bond between NO and the CuB2+. Although the available data indicate a marked preference for the addition of NO to reduced heme a3, this does not mean that electron transfer from CuB2+ to either NO, or O2 in the active site need necessarily be slow, as outer-sphere processes can be very fast over short distances.

**Cytochrome c Oxidase**—The presence in cells of an endogenous antidote to cyanide poisoning is probably of some ancient survival value. Inhalation of smoke from burning wood, other plant-derived fuels, and tobacco leads to the absorption of two particularly effective respiratory poisons, carbon monoxide and hydrogen cyanide. However, neither of these toxins inhibits cerebral O2 consumption when delivered at low levels (48). The bloodstream provides a significant barrier to carbon monoxide diffusing to the tissues by formation of carboxyhemoglobin. However, less than 1% of the total hemoglobin in blood is normally present as the cyanide-binding methemoglobin. Consequently, the bloodstream is less able to protect the tissues from cyanide exposure. The present observation of facile cyanide displacement from cytochrome c oxidase by NO would seem to have at least two additional practical consequences, the first of some possible experimental significance and the second relating to clinical issues.

Inhibition of mitochondrial electron transport by the addition of cyanide is a standard protocol for establishing the basal O2 consumption in studies with respirometers (49). In practice, there is usually some residual O2 reduction measurable under these conditions. This observation can now be understood to be due, in part, to the endogenous NO production present in many samples. Also, there is a cyanide-resistant terminal oxidase known to be present in the mitochondria of all plants, many fungi, and some protozoa, but it is seemingly absent from metazoa (50). This alternate oxidase is certainly not a cyto-

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**Fig. 4.** Plausible reaction sequence for transient NO inhibition of cytochrome c oxidase at high electron flux. For simplicity, only the reactions at the binuclear pair are explicitly considered. As NO is consumed and converted to nitrite, it should properly be thought of as an auxiliary substrate. In the absence of NO, turnover of the enzyme is limited by the rate of internal electron transfer to the binuclear pair. However, in the above scheme, NO dissociation from ferroheme (II → III) is suggested to be the rate-limiting step in keeping with the findings of others (4). The NO-inhibited structure (I) could be 5-coordinate, 6-coordinate, or a mixture, as indicated by the EPR spectra of Fig. 2. Conversion of peroxynitrite to nitrite and water (IV→) is a known facile reaction of the enzyme that appears to sustain the electron transport chain (41).
chrome and, based upon sequencing data, is thought to contain a di-iron active site (51), although crystallographic or spectroscopic verification of this assertion has proven elusive. In light of the activity data shown in Fig. 1 and the recently verified existence of mitochondrial nitric-oxide synthase (9), there is scope for erroneously ascribing any cyanide-resistant oxidase activity measured in mammalian mitochondria to the presence of an alternate di-iron oxidase if steps are not taken to inhibit NO production.

The standard treatment for cyanide poisoning involves the intravenous injection of sodium nitrite and sodium thiosulfate (52). The presumption has been that the nitrite oxidizes hemoglobin to methemoglobin, which may then strongly bind cyanide, whereas the thiosulfate promotes enzymatic conversion of cyanide to thiocyanate ion, a much less toxic compound, in the mitochondria. However, these arguments do not really explain how inhibition of cytochrome c oxidase by cyanide, an extremely facile reaction in vitro, is substantially avoided during acute poisoning. A study of murine brain mitochondria showed that lethal doses of potassium cyanide only inhibited cytochrome c oxidase activity by ~50%, and it was suggested that a large proportion of the oxidase activity may be a “functional reserve” (53). Moreover, the kidneys of cyanide poisoning victims have been shown to exhibit no significant irreversible damage and, therefore, have been suggested to be suitable for use as allografts (54, 55). The results shown in Figs. 1 and 2 strongly suggest that mitochondrial NO could function to protect cytochrome c oxidase activity, displacing any bound cyanide, which may then be relatively slowly converted to thiocyanate or may diffuse to the vasculature to be complexed by methemoglobin. Furthermore, it may also be the case that production of additional NO derived from the administered nitrite ion, boosting the availability of the auxiliary substrate for the terminal oxidase, is a previously unrecognized aspect of the standard therapy. These ideas would seem to clarify the otherwise puzzling observations that the benefits of nitrites in treating cyanide poisonings cannot be due solely to generation of methemoglobin and that vasodilatation is somehow involved in the mechanism (56).

Estimation of in Vivo NO Levels—Under physiologically relevant conditions, regulation of oxygen consumption by the normally functioning mitochondrial electron transport chain depends upon the prevailing \([\text{O}_2]/[\text{NO}]\) ratio (57). It is therefore necessary to know (or have reliable estimates of) the steady-state levels of both \(\text{O}_2\) and NO in tissue if reasonable assessments are to be made of the rate of mitochondrial respiration and the extent of secondary reactions producing reactive oxygen and/or nitrogen species in vivo. In the case of isolated rat heart and liver mitochondria, Boveris et al. (57) have shown that half-maximal inhibition of state 3 respiration occurs when the \([\text{O}_2]/[\text{NO}]\) ratio is 150. This seems to be in good agreement with our own data concerning purified beef heart cytochrome c oxidase (17), which suggest that half-maximal inhibition of the enzyme occurs for \([\text{O}_2]/[\text{NO}]\) ~ 100. In vivo the prevailing “free” \(\text{O}_2\) concentration of cardiomyocytes is thought to be ~4 \(\mu\text{M}\) (58), whereas the steady-state NO concentration in tissues has been suggested to be as high as 100 \(\text{nM}\) (57). One or both of these numbers must surely be in error, for if \([\text{O}_2]/[\text{NO}]\) = 40, mitochondrial respiration would be 80–90% inhibited. One measures sustained release of NO in cell cultures where inducible NO synthase has been up-regulated (mimicking inflammation) leading to steady-state NO concentrations of 100 \(\text{nM}\) or more (42). However, the cultures are usually maintained under 20% \(\text{O}_2\) at 37 °C, resulting in dissolved \(\text{O}_2\) concentrations of ~200 \(\mu\text{M}\) and hence \([\text{O}_2]/[\text{NO}]\) ratios on the order of \(10^3\), i.e. conditions under which cytochrome c oxidase is barely inhibited.

Consequently, although many mitochondria-rich cultured cell lines survive well at 100 \(\text{nM}\) NO, the same types of cell may well not survive in vivo at such high NO concentrations, and this probably contributes to the necrosis evident in inflamed tissue.

In the absence of any inducible NO synthase up-regulation, neither cultured cardiomyocytes (or other cell lines) nor intact endolymphate produce measurable levels of NO until a stimulus has been applied (9). The detection limit of the electrodes used for such measurements is ~1 \(\text{nM}\), and consequently, basal production of NO in all individual cell types studied thus far leads to NO concentrations of ~1 \(\text{nM}\). If these conditions hold in vivo, \([\text{O}_2]/[\text{NO}]\) ~ 10^4, and mitochondrial respiration will not be significantly inhibited. Following stimulation, NO levels can rapidly, but transiently, rise to several hundred nanomolar (9), where \([\text{O}_2]/[\text{NO}]\) ~ 10, and mitochondrial respiration will be more or less fully inhibited. It follows that the system appears to have evolved in such a way as to allow respiration to be turned off (or substantially inhibited) for ~1 s in a single cell and then recover. Alternately, at a reduced level of stimulation, NO release can probably be sustained for many seconds before a recovery period, but this will result in NO concentrations much closer to the basal level than the ~1 s duration transient maximum. The latter situation likely exists most of the time in vivo under normal physiological conditions. Accordingly, in cells/tissues of high mitochondrial content, where NO is efficiently catabolized, one can reasonably anticipate the steady state \([\text{O}_2]/[\text{NO}]\) ratio to be ~10^6.

The idea that cytochrome c oxidase catabolizes NO, which is strongly supported by the current findings, suggests that the functioning electron transport chain serves to limit nitrosative stress in mitochondria-rich tissues. It has been argued (43) that about 70% of the NO flux in liver mitochondria is converted to “free” peroxynitrite, a potentially damaging oxidizing and nitrating agent. However, a steady-state NO level of 50 \(\text{nM}\) was assumed, and the conversion of NO to nitrite by cytochrome c oxidase was not included in the analysis. In short, the amount of NO available for conversion to peroxynitrite was probably overestimated. Furthermore, we have previously shown (41) that cytochrome c oxidase is a reasonable peroxynitrite reducer (10^6 \text{M} s^{-1} \text{M}^{-1}) in the absence of excess \(\text{O}_2\) and/or NO. As the in vivo \(\text{O}_2\) consumption of mitochondria corresponds to about half-maximal activity of the terminal oxidase (58), peroxynitrite should be scavenged efficiently. In our opinion, if these additional observations are taken into account, it can plausibly be argued that the peroxynitrite output of functioning mitochondria under normal physiological conditions is virtually zero.

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