Oxidation of Dithiothreitol During Turnover of Nitric Oxide Reductase: Evidence for Generation of Nitroxyl with the Enzyme from Paracoccus Denitrificans

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Summary. The stoichiometric relationship between thiol oxidized and NO reduced was studied for the reaction catalyzed by nitric oxide reductase from Paracoccus denitrificans. The reaction systems consisted of dithiothreitol, ascorbate, phenazine methosulfate, enzyme and NO, or that system minus ascorbate. The mole ratio of thiol groups oxidized to NO reduced was observed to be 2.3 to 1.5 over a range of NO from 0.09 to 0.35 μmol. A ratio of 1.0 was expected for the simple reduction of NO by 1-electron to N₂O. The oxidation of additional thiol is attributed to the trapping of nitrosyl hydride (nitroxyl, NO⁻/NOH) by thiol.

Nitric oxide reductase is one of the four reductases of the denitrification pathway in bacteria (1-4), and NO has been shown to be an obligatory intermediate in the pathway (5-8), at least for several bacteria examined. Nitric oxide reductase is associated with the cytoplasmic membrane and has been purified from Pseudomonas stutzeri (1) and Paracoccus denitrificans (3,4) following its solubilization by nonionic detergents. Structurally it appears to be a tight complex of a cytochrome b and a cytochrome c and to contain also some non-heme iron. Except for the fact the nitric oxide reductase reduces NO to N₂O with a 2:1 stoichiometry (2,3), little is known about its chemical mechanism. One possibility, which is attractive in its simplicity, is that the enzyme reduces NO by 1-electron to nitrosyl hydride (nitroxyl, NO⁻/NOH) and then this intermediate converts to N₂O as the result of its rapid dimerization and dehydration. The ability of chemically produced nitroxyl to convert to N₂O is well established (9-14) and the process appears to be nearly diffusion controlled (15). In addition, there is evidence that the reduction of NO by ferrous ion in weakly acidic solutions proceeds via nitroxyl as an intermediate (16,17). Whether nitric oxide reductase reduces NO by an analogous route involving nitroxyl has been the subject of two previous studies (5,18). In an isotope study (18), trioxodinitrate was allowed to decompose into nitroxyl and nitrite in the presence of denitrifying bacteria that were concomitantly reducing [¹⁵N]nitrite to N₂O. It was observed after short incubation times that the ¹⁴N/¹⁵N ratio in N₂O was much greater than that of

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the nitrite pool and much less than that of the trioxodinitrate pool, a result consistent with
dimerization of nitroxyl formed from trioxodinitrate with $[^{15}\text{N}]$nitroxyl formed from $[^{15}\text{N}]$nitrite via
the denitrification pathway. The $^{14}\text{N}$ and $^{15}\text{N}$ in $\text{N}_2\text{O}$ were randomized as required for mono-N
precursors. However, it could not be excluded that isotopically mixed $\text{N}_2\text{O}$ arose via the reaction
of nitroxyl with $^{15}\text{NO}$ (1:2) to yield nitrite and $\text{N}_2\text{O}$ (1:1). This reaction has been shown to be
significant in the case of nitroxyl produced by pulse radiolysis of NO solutions (19) and by
decomposition of trioxodinitrate in presence of NO (15). In a second study (5), an unsuccessful
attempt was made to trap extracellular nitroxyl with methemoglobin added to denitrifying bacteria.
However, in this and the previous study (18) with intact bacteria, questions concerning the
permeability of nitroxyl and its intracellular steady-state levels remained unanswered.

In the present study, we attempted to trap nitroxyl arising in the turnover of nitric oxide
reductase with a thiol (20). A cell-free system was used in which detergent solubilized nitric oxide
reductase served as the enzyme.

**EXPERIMENTAL PROCEDURES**

**Nitric oxide reductase.** Enzyme free of nitrite and nitrous oxide reductase activities was
prepared from denitrifying F. denitrificans according to the method of Dermastia et al. (4). "Crude"
enzyme was the membrane fraction solubilized between 0.1 and 0.8% octyl glucoside; "partly
purified" enzyme was a fraction eluted from the hydroxyapatite column toward the end of the
gradient elution step; "purified" enzyme was eluted with 1.5% octyl glucoside in a batch process
from hydroxyapatite and had an apparent purity of 90% or greater. Crude, partly purified, and
purified enzyme had specific activities of about 0.5, 2-3, and at least 6 $\mu$mol NO x min$^{-1}$ x mg$^{-1}$,
respectively, at pH 6.5 and 25°C in the NO-electrode assay system (4,21). These activities were
based simply on the time required to exhaust known quantities of NO and not on maximum rates
(4).

**Nitric oxide.** NO (Matheson) was passed through a 15 x 1.5 cm tube filled with NaOH
pellets in order to remove traces of NO$_2$ and then used to prepare saturated solutions of NO in
anaerobic 20 mM potassium phosphate buffer pH 6.5, 25-30°C. The concentration of NO was
taken from tables giving solubility of NO as a function of temperature (22).

**System to trap nitroxyl with thiol.** The system typically contained 10 $\mu$l of 1 M sodium
ascorbate freshly prepared in water, 10 $\mu$l of 0.01 M phenazine methosulfate (PMS) in water, 5 or
10 $\mu$l of 0.11 M dithiothreitol (DTT) in water, enzyme solution, and sufficient 20 mM potassium
phosphate buffer pH 6.5 to bring the final volume to 1 ml. In some experiments, ascorbate was
omitted. This mixture was placed in the chamber of a thermostated Clark-type O$_2$/NO-electrode
(Pt/Ag, AgCl; Hansatech model DW-1), and the removal of O$_2$ with use of an argon jet directed
at the surface of the solution was followed amperometrically. The nitric oxide reductase reaction
was initiated by the injection of 50-200 $\mu$l of buffered NO solution into the anaerobic mixture, and
the uptake of NO was followed amperometrically at 25° or 30°C. The amount of enzyme added,
typically 400 $\mu$g of crude, 100 $\mu$g of partially purified, or 25 $\mu$g of purified enzyme, was sufficient
to reduce 0.1 $\mu$mol of NO$_{aq}$ in about 0.5 min and 0.4 $\mu$mol in 4-5 min. This lack of proportionality
in reaction times was caused by a decrease in the rate of reduction of NO with increasing NO
concentrations due to the inhibition of the enzyme by NO (4,6).

Immediately after the exhaustion of NO, 100 $\mu$l of the reaction mixture was mixed with
100 $\mu$l of 6.6 mM 5,5'-dithiobis (2-nitrobenzoic acid)(Ellman's reagent (23)) and then diluted to
5 ml with 0.1 M sodium phosphate buffer pH 8.0. The absorbance at 412 nm due to the thiolate
of 2-nitro-5-thiobenzoic acid ($\epsilon_{412} = 1.36 \times 10^{4} \text{cm}^{-1} x \text{M}^{-1}$) was determined after 15 min. This
procedure assayed the amount of thiol in DTT after the reduction of NO. The amount of thiol oxidized as a result of the enzymatic reduction of NO was the amount present in an appropriate control system (minus enzyme) minus the amount assayed in the complete system.

The compositions of control systems are set forth in connection with Table 1. Controls lacking enzyme were allowed to incubate anaerobically in the electrode chamber for the same lengths of time as were required to exhaust the NO present in the complete enzymatic systems. Immediately thereafter, NO was removed by means of an argon jet and the assay for DTT thiol was carried out on the NO-free mixture. This procedure avoided the oxidation of NO by air to transient products which are known to oxidize thiols (24). Other controls were also incubated for periods of time corresponding to the time needed to exhaust NO enzymatically before DTT was assayed.

As reported by Doyle et al. (20), thiols can trap nitroxyl in a two-step reaction (Eq. 1) with production of disulfide and hydroxylamine 1:1. DTT was chosen for the thiol because its redox potential lies well below that of ascorbate at neutrality (25,26). DTT had the potential therefore to reduce dehydroascorbate which was expected to be formed in the initial 1-electron reduction of NO to nitroxyl.

**Assays.** Attempts to detect NH₂OH were undertaken with four colorimetric methods: The indooxine assay (27-29), the ferrous chelate method of Belanger et al. (30), the ferricyanide/phenolphthalein method of Shanine and Mahmoud (31), and the hydroxylamine chelate method of Kavlentis (32).

Ascorbate was oxidized after exhaustion or removal of NO by ascorbic acid oxidase (Calbiochem; 200 µmol ascorbate x min⁻¹ x mg⁻¹ at 25°C, pH 5.6). The oxidase (0.1 mg) was added to the reaction mixture in the electrode chamber and stirred under an O₂ jet. The level of dissolved O₂ was monitored and oxidation of ascorbate was judged to be complete when the O₂ concentration rose from a steady-state value close to zero to that corresponding to a solution in equilibrium with O₂ at 1 atm. The time required to oxidize ascorbate was typically 4 min or less. In the presence of PMS, most or all of the remaining DTT was also oxidized by this procedure, presumably via reduction of dehydroascorbate.

Protein was assayed by the bicinchoninic acid method (33) using reagents prepared by Pierce.

**RESULTS AND DISCUSSION.** The results of a single experiment by way of example are presented in Table 1. The experiment involved four levels of NO and five kinds of controls, four of which are included in Table 1 (columns A and B and as described in the footnote to Table 1). The observed levels of thiol were sufficiently similar between minus enzyme controls (column A) and minus NO controls (column B) so that the thiol levels for enzymically inactive systems were taken as the average of these two kinds of controls. The control lacking both enzyme and NO provided a value similar to those for the previous two kinds of controls. The similarity among controls lacking enzyme and/or NO suggests that the chemical reduction of NO was relatively unimportant. The control lacking enzyme, NO, ascorbate and PMS (containing only DTT) gave the highest final levels of thiol. The lower levels of thiol observed in systems containing ascorbate/PMS were caused by the presence of some contaminating dehydroascorbate which could oxidize DTT chemically. This ability of DTT to scavenge dehydroascorbate in presence of PMS simplified the studies, because oxidizing equivalents that may have been distributed between
TABLE 1. Oxidation of thiol groups of DTT during the reduction of nitric oxide in systems containing partially purified nitric oxide reductase, ascorbate, PMS and DTT.

<table>
<thead>
<tr>
<th>Initial Amount of NO</th>
<th>Differential oxidation of thiol groups (C minus D)</th>
<th>Average of A and B (C)</th>
<th>Complete System (D)</th>
<th>Average system (E)</th>
<th>Initial Thiol groups remaining in DTT for differential oxidation of NO enzyme (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol</td>
<td>µmol</td>
<td></td>
<td></td>
<td>µmol</td>
</tr>
<tr>
<td>0.092</td>
<td>2.01</td>
<td>2.01</td>
<td>1.78</td>
<td>0.23</td>
<td></td>
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<tr>
<td>0.182</td>
<td>1.91</td>
<td>1.95</td>
<td>1.68</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>0.274</td>
<td>1.86</td>
<td>1.89</td>
<td>1.52</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>0.365</td>
<td>1.92</td>
<td>1.86</td>
<td>1.39</td>
<td>0.47</td>
<td></td>
</tr>
</tbody>
</table>

The procedure was as described under Experimental Procedures and each complete system contained 100 µg of purified enzyme. The control lacking both enzyme and NO had a final content of thiol groups of 1.95 µmol; that lacking enzyme, NO, ascorbate and PMS (containing only DTT), 2.30 µmol. The temperature for equilibration of NO with buffer was 28°C; reaction temperature was 25°C. 

The fifth control (data not shown) contained only NO and served as a measure of loss of NO from the electrode chamber, presumably by way largely of gas phase diffusion through the narrow bore filling channel. The loss of NO was time dependent and increased with the initial concentration of NOaq. This kind of control allowed a more precise estimation of the amount of NO that was actually available for reduction. Corrections for the amount of available NO were made in the data of Table 2 but not of Table 1.

TABLE 2. Oxidation of thiol groups of DTT during the enzymatic reduction of nitric oxide over eight separate experiments.

<table>
<thead>
<tr>
<th>Available NOa or absence (-) of 10 µmol of ascorbate</th>
<th>Presence (+) or absence (-)</th>
<th>Enzymatic oxidation of thiol groups for systems with crude (C), partially purified (PP), or purified (P) enzyme in parenthesis</th>
<th>Overall Average of 10 µmol of available NO</th>
<th>Thiol oxidized divided by available NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol</td>
<td></td>
<td>Exp 1 2 3 4 5 Ave.</td>
<td>µmol</td>
<td></td>
</tr>
<tr>
<td>0.09</td>
<td>+</td>
<td>0.23(PP) 0.27(P) 0.22(C) 0.17(C) 0.18(PP) 0.21±0.04</td>
<td>0.21±0.04</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.24(PP) 0.16(C) 0.21(C)</td>
<td>0.20±0.04</td>
<td></td>
</tr>
<tr>
<td>0.17</td>
<td>+</td>
<td>0.27(PP) 0.32(P) 0.28(C) 0.19(C) 0.37(PP) 0.29±0.07</td>
<td>0.29±0.06</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.34(PP) 0.22(C) 0.30(C)</td>
<td>0.32±0.06</td>
<td></td>
</tr>
<tr>
<td>0.24</td>
<td>+</td>
<td>0.37(PP) 0.41(P) 0.30(C) 0.35(C) 0.50(PP) 0.39±0.08</td>
<td>0.38±0.11</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.29(PP) 0.24(C) 0.55(C)</td>
<td>0.36±0.17</td>
<td></td>
</tr>
<tr>
<td>0.31</td>
<td>+</td>
<td>0.47(PP) 0.32(P) 0.43(C) 0.43(C) 0.59(PP) 0.45±0.10</td>
<td>0.45±0.09</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.41(PP) 0.37(C) 0.58(C)</td>
<td>0.45±0.10</td>
<td></td>
</tr>
</tbody>
</table>

The procedure was as described under Experimental Procedures. Each experiment involved four different amounts of NO and included those controls described in Table 1 and the text. The temperature for equilibration of NO with buffer was 28-29°C; reaction temperature was 25 or 30°C.

aCorrected for physical loss of NO from the electrode chamber, as described in the text.

986
Corrections were about 15% when the initial amounts of NO were about 0.4 µmol and when the incubation time was equal to the time required for the enzymatic reduction of the NO. Such corrections were negligible when the initial amount of NO was about 0.1 µmol. As can be seen in Table 1, the enzymatic oxidation of thiol increased with increasing amounts of NO, and the ratio of thiol oxidized to NO added (column E/NO) was greater than one in each case.

Table 2 summarizes the results of a group of experiments for which the reducing system was DTT/ascorbate/PMS or DTT/PMS. The fact that little difference was observed between systems with and without ascorbate demonstrated that DTT/PMS can serve as a reductant for nitric oxide reductase. The ratio of thiol oxidized enzymatically to available NO ranged from about 2.3 at the lowest levels of NO used to 1.5 at the highest. Little difference was observed in these parameters between the crude and purified grades of enzyme, so long as the activity levels used were similar and sufficiently great to exhaust the NO within 4-5 min.

The 1-electron reduction of NO to nitroxyl would be expected to correspond to the disappearance of one mol of thiol per mol of available NO if the oxidizing equivalents appeared in DTT disulfide at the end of the reaction. If nitroxyl were a freely diffusible intermediate on the path to N₂O, it could partition into at least three different reactions: Trapping by DDT (Eq. 1), trapping by NO (Eq. 2) as described by Grätzel et al. (19), and dimerization/dehydration (Eq. 3)

\[
\text{NO}^- + 2\text{NO} \rightarrow \text{NO}_2^- + \text{N}_2\text{O} \tag{2}
\]

to yield the normal product, N₂O. If nitroxyl were trapped quantitatively by DTT, then the expected overall stoichiometry should be 3 mol of thiol per mol of available NO. If, instead, nitroxyl were exclusively trapped by NO or only underwent dimerization/dehydration, then the ratio should be 0.33 or 1, respectively. The observed ratios of 1.5-2.3 are consistent with the trapping of nitroxyl by DTT and therefore with a mechanism of reduction of NO by nitric oxide reductase involving at least some free nitroxyl. The observations that NO is reduced enzymatically to N₂O in a 2:1 ratio in absence of thiols (2,3), rather than a 3:1 ratio, suggests that the trapping of nitroxyl by NO (Eq. 2) cannot be very important relative to dimerization/dehydration in the enzymatic reaction. A ratio for NADH oxidized to NO consumed of 0.5 (instead of something approaching 0.17) with nitric oxide reductase containing membrane vesicles from P. denitrificans (2) also supports this conclusion.

Attempts to detect formation of NH₂OH in the range of 0.05-0.1 µmol, as expected from the data of Table 2 and Eq. 1, were defeated by interfering materials or an inherent lack of sensitivity, even after the reducing system had been exhaustively oxidized by ascorbic acid oxidase/O₂. In the case of the method of Kaventis (32), we were unable to reproduce the published results for assay of hydroxylamine.
Nitroxyl anion, which is isoelectronic with O₃, can apparently exist in two different states of reactivity that have been assigned singlet and triplet electronic states (34). The species formed from the spontaneous decomposition of trioxodinitrate is apparently in the singlet state (34) and can be trapped in part by thiols (20). This implies that the putative nitroxyl generated in the enzymatic reduction of NO is also probably a singlet state species.

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**REFERENCES**