Steady-state Nitric Oxide Concentrations during Denitrification*

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Three species of denitrifying bacteria, Paracoccus denitrificans, Pseudomonas stutzeri strain JM300, and Achromobacter cycloclastes, were allowed to reduce nitrate or nitrite in anaerobic, closed vials while the equilibration of gases between aqueous and gas phases was facilitated by vigorous stirring. The gas phase was sampled and analyzed for NO with use of a chemiluminescence detector calibrated against bottled NO standards or against NO produced by the nitrite-iodide reaction. [NO₃₋] was inferred from [NO₂⁻] and the solubility of NO. NO was detected only during denitrification in amounts that, once established, did not change with time, were independent of the initial concentration of nitrate or nitrite, and were largely independent of cell concentration, at least when nitrate was the oxidant. The usual level of NO was promptly re-established following the addition of exogenous NO or following the loss of NO by sparging. The aforementioned properties are expected for a steady-state intermediate in denitrification. Steady-state [NO₃₋] ranged between 1 and 65 nM depending on species and conditions. Similar results were also obtained in a related experiment in which P. stutzeri strain ZoBell respired nitrite under growth conditions.

The very low steady-state [NO₃₋] observed during denitrification imply that the maximum activity of nitric oxide reductase in vivo, if it could be realized, would be large relative to that for nitrite reductase. This circumstance allows NO to be an intermediate without reaching toxic steady-state levels.

Considerable evidence has appeared over the past few years that NO is the principal, possibly the only, intermediate between nitrite and N₂O during denitrification. For recent discussions see Ref. 1-3. There is little data, however, on extracellular [NO₃₋] during the steady-state of denitrification. A value of 2.2 nM was inferred for extracellular NO₃₋ from the kinetics of the complexation of NO by extracellular hemoglobin and 560 nM was estimated for intracellular NO₃₋ from the rate of denitrification and the diffusive mean residence time of NO₃₋ (1). The latter was a rough estimate. The use of a NO-electrode gave an upper limit on steady-state [NO₃₋] at 15 nM for several bacteria under a variety of conditions.

** Experimental Procedures

Cultures—Paracoccus denitrificans strain ATCC 19367, P. stutzeri strain JM300, Achromobacter cycloclastes strain ATCC 21921, and Escherichia coli strain JM101 were grown at 30–32 °C in either YP medium (2) or a defined SP medium (3, 6). Wolinella succinogenes was grown through ammonia-producing nitrate respiration as described previously (1). P. stutzeri strain ZoBell (form sp. P. perfectomarina (4)) ATCC 14405 was maintained on two marine media in which the principal carbon source was asparagine or tryptone plus yeast extract (TYSN) (7). Methylene blue indicator was omitted from all experiments involving NO. P. stutzeri strain JM300, W. succinogenes, and E. coli were supplied, respectively, by J. L. Ingraham (University of California, Davis), M. J. Wolin (Department of Health of New York State, Albany), and R. F. Schleif (Department of Biochemistry, Brandeis University). Maintenance, growth, and harvest of these bacteria have been described (1, 2). E. coli and, for certain experiments, denitrifying bacteria as well, were grown on air with sparging to prevent induction of the denitrification apparatus at low [O₂]. E. coli is not a denitrifier but can grow anaerobically by fermentation or nitrate respiration (nitrate → nitrite). Similarly W. succinogenes is not a denitrifier but can grow using an alternative nitrate respiration (nitrate → ammonia) or N₂O respiration (N₂O → N₂) (8). This organism lacks a NO-producing nitrite reductase (9).

Determination of [NO₃₋]—For short-term experiments, a denitrification system was contained in a 9-ml vial (Wheaton, 223739) sealed with a silicone septum (Wheaton, 224094), which had been previously treated with NO₂ to block subsequent reaction of the rubber with NO. The vial also contained a magnetic stirrer for stirring. Bacterial suspensions (5 ml) in either YP or SP medium were added to the vial and made anaerobic by sparging with N₂ and stirring. A small volume of an anaerobic solution of KNO₃ or KNO₂ was injected to initiate denitrification and vigorous stirring was resumed. After an appropriate delay to allow the steady-state to be established and gases to equilibrate between the gas and aqueous phases, 1 ml of the head space gas was removed with a gas-tight syringe (Unimetrics 4000 series) and was injected into the stream of intake gas (250 ml × min⁻¹) of a NO chemiluminescence detector (Monitor Laboratories, model 8840). The bell-shaped signal due to NO was integrated electronically (Hewlett-Packard integrator model HP3394A). For all experiments, two or more successive NO determinations were made to see if NO levels changed with time. Because no more than two successive 1-ml samples of head space gas were removed from any one vial, timed studies in excess of two points always involved the use of several identical systems.

NO standards were of two kinds. The one was a bottled mixture of extracellular [NO₃₋] during the steady-state of denitrification, and we now report values for several bacteria under a variety of conditions.

1 The abbreviations used are: YP, yeast extract plus peptone medium; SP, succinate phosphate minimal medium; TYSN, tryptone plus yeast extract medium.
50 ppm of NO in N₂ (Matheson) that was further diluted into N₂ by means of a pair of electronic mass flow controllers (Tylan). The other was NO produced through the quantitative reduction of 0.1–6 nmol of nitrite by KI in dilute acetic acid (10). The NO-generating system was identical to the one described above for denitrification, except that the concentration of 12 mM KI and 0.87 M acetic acid replaced the gas phase suspension. Because the solubility of NO is about 2 nM under 1 atm at room temperature (11), the ratio [NO₃⁻]/[NO] ≈ 20 at equilibrium. From this ratio and the head space and aqueous volumes in the vials (4 and 5 ml, respectively), the relationship at equilibrium between [NO₃⁻] in nanomolar and the total amount, X, of NO generated or nitrate reduced (in nanomol) is [NO₃⁻] = X/0.085. Expressed as [NO₃⁻], the limit of detection for the NO detector was about 0.1 nM; expressed as NO actually delivered to this detector, 2 pmol.

The concentrations of NO injected into the intake gas of the NO detector were always so low (≤40 ppm), the subsequent dilution so rapid, and the time between injection and detection so short (5 s) that we observed no difference between the use of N₂ or air as the intake gas. The ability to use air is a consequence of the fact that the NO–O₂ reaction is second order in NO₂ with k = 8 × 10⁻¹⁰ m³ mol⁻² × s⁻¹ (12), and becomes slow in air when NO₃ drops below 100 ppm. As measured by the transfer of NO₃⁻ to the gas phase, the equilibration of NO between gas and aqueous phases within a vial was essentially complete within a few min. For this reason, the earliest NO determination was made no less than 5, and generally 10, min after having initiated denitrification or after the chemical synthesis of NO for the standards. Subsequent determinations were generally at 10-min intervals.

Additional long term experiments were carried out in the laboratory of T. T. Packard (Bigelow Laboratory of Ocean Science, Boothbay Harbor, ME). The apparatus used was a closed system constructed almost entirely of glass and stainless steel to minimize reactivity with NO and allowed continuous recirculation of an atmosphere through the bacterial culture which had a volume of about 1 liter. Recirculation was driven by a stainless steel bellows pump and served to equilibrate gases between the gas and aqueous phases. Except for the recirculation of gas, the system resembled the single-pass sparged system described previously (3). The equilibrated head space gas was sampled at roughly 60-min intervals by use of 10- or 50-ml gas-tight glass or plastic syringes and analyzed immediately by chemiluminescence of gas, the system resembled the single-pass sparged system described previously (3). The equilibrated head space gas was sampled at roughly 60-min intervals by use of 10- or 50-ml gas-tight glass or plastic syringes and analyzed immediately by chemiluminescence procedures similar to those described above, but using a more sensitive home-built detector and photon counter. The detector was also equipped to carry out the selectivity test of Zafiriou and True (13) in order to distinguish NO from any NO-mimicking gases that might be present. Both nitrites and NO and N₂ were used to calibrate [NO] and of NO between gas and aqueous phases within a vial as described above. Following the appearance of NO₃⁻ after the injection of NO₃⁻, it was determined that the half-time for equilibration was <1.5 min. Anaerobic cultures containing nitrite as the N-oxide were started with sodium hypochlorite, which completely blocked nitrate uptake and brought [NO₃⁻] to undetectable levels. This sequence of events could be repeated closely by addition of 10 μM azide, which completely blocked nitrate uptake and brought [NO₃⁻] to undetectable levels. NO was determined colorimetrically (15). The time required for a denitrifying suspension of cells to exhaust the initial amount of nitrate or nitrite was taken to be the time at which nitrite could no longer be detected in the suspension (2). Typically 10 μl of the suspension was withdrawn for each nitrite assay.

RESULTS

Steady-state [NO₃⁻]—Standard curves for the relationship between integrator count and [NO₃⁻] were linear from 1 to 65 nM (Fig. 1). The two separate detector channels of the NO detector had slightly different sensitivities as indicated by the slopes for the two curves of Fig. 1. NO standards were run with every experiment, and the sensitivity of the detector was found to be very stable over a period of some 3 months over which the data were recorded.

Table I summarizes the short term steady-state [NO₃⁻] obtained for three denitrifying bacteria in two different media, at several cell concentrations, and with various initial concentrations of nitrate or nitrite. Several trends are evident from the data. First, [NO₃⁻] was nearly independent of the concentration of nitrate or nitrite, as long as they were great enough to saturate their respective N-oxide uptake system. Second, there appeared to be some differences among the three species of bacteria studied. P. putida J300 had the highest [NO₃⁻] when nitrite was oxidant, but P. denitrificans had the highest when nitrate was oxidant. Third, [NO₃⁻] tended to be greater (5–65 nM) with nitrite as oxidant than with nitrate (1–10 nM). With an initially equimolar mixture of nitrate and nitrite, [NO₃⁻] was similar to that expected for nitrite alone. Fourth, although there was little dependence of [NO₃⁻] on cell concentration when denitrification was supported by nitrate, there was a dependence when it was supported by nitrite. In that case [NO₃⁻] for P. denitrificans was observed to decrease some 5–10-fold when the cell concentration was lowered from 1 to 0.01 mg of protein X ml⁻¹.

The reason for this dependence is at present unknown. Incubation of P. denitrificans with 10 μM azide, which completely blocked nitrate uptake and brought [NO₃⁻] to undetectable levels, had no effect on the rate of nitrite uptake or the [NO₃⁻] generated from nitrite. This rules out the possibility that additional NO was being made from nitrite by a pathway involving the direct reduction of nitrite to NO by nitrite reductase, as can occur with enteric bacteria (16). The fact that YP and SP media gave very similar [NO₃⁻] values in the case of P. denitrificans, reducing nitrite tends to rule out explanations based on selective utilization of certain reducing substrates relative to others. The judgment that the [NO₃⁻] values of Table I represented the steady-state was based in part on their constancy over time, rapid recovery from perturbations, and reproducibility between two or more separate episodes of denitrification with the same lot of cells. NO₃ was always undetectable prior to the initiation of denitrification, reached some value within 5–10 min after addition of nitrate or nitrite, remained at this value until the N-oxide was exhausted and then returned to undetectable levels within the equilibration time of 5–10 min. This sequence of events could be repeated closely by addition of a second amount of nitrate or nitrite. Recovery from perturbation can be illustrated with the following examples. A system containing P. denitrificans (1 mg of protein X ml⁻¹) and reducing 10 mM nitrite showed a steady-state [NO₃⁻] of 36 nM. When the system was sparged with N₂ to lower NO concentrations of nitrate or nitrite.
levels, the apparent [NO₃⁻] immediately after sparging was 3 nM but rose to 40 nM at the next sampling 10 min later. In an analogous experiment, exogenous NO₂⁻ was injected to give a concentration of 130 nM, but the system returned to 39 nM within 15 min and remained at about that level until nitrate was exhausted. During the short term experiments, little or no increase in cell density occurred.

The results of long term experiments with P. stutzeri ZoBell are given in Table II. The time course for appearance and disappearance of nitrite, NO, and NO₂ qualitatively resembled those reported previously (3). [NO₂⁻] rose initially to rather stable plateau values which were maintained only as long as nitrite was present. These plateau values, expressed as [NO₂⁻], are reported in Table II. During the period over which data were taken, the cells grew with a doubling time of 3-4 h. Growth depended on nitrite respiration and was not fermentative. Initial cell concentrations were about 10⁶ cells X ml⁻¹ during the times that NO determinations were analyzed. (See text for details.)

In experiments with unincubated medium contained in the gas recirculation system, the progressive appearance of extremely low levels of NO (of the order of 1 nM for NO₂⁻ after some 8 h) was routinely observed. The chemistry leading to this production of NO is unknown. Scavenging of NO in the gas recirculation system used for longer term experiments was assessed by injecting NO₂⁻ into the head space of the system or with O₂ as the result of its slow diffusion into the NO₂⁻ pool due to denitrification. This flux can be approximated as the rate of nitrite uptake in the steady-state. At the

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**TABLE I**

| Organism          | Growth medium/assay medium | Initial [NO₃⁻]x mg protein X ml⁻¹ | Initial [NO₂⁻]x mg protein X ml⁻¹ | [NO₃⁻] at the indicated cell densities expressed as mg protein X ml⁻¹ |
|-------------------|-----------------------------|-----------------------------------|-----------------------------------|-------------------------------------------------------------------|
|                   |                             | mM                                | mM                                | [
| A. cycloclastes   | YP                          | 1.0-5.0 (R = 10)                   | 9                                 | 31 30                                                             |
| P. stutzeri JM300 | YP                          | 0.8-8 (R = 8)                      | 31                                | 57 62 02                                                          |
| Pa. denitrificans | YP                          | 0.08-8 (R = 8)                     | 5                                 | 3-5 (4) 7-11 (6) 17-18 (8) 17-21 (6) 23-27 (6) 27-49 (12)          |
|                   | SP                          | 0.00-0.8 (R = 8)                   | 5                                 | 5 11 14                                                           |
|                   |                             | 5                                 | 3 9 18 30                                                                       |
| A. cycloclastes   | YP                          | 0.10-8.0 (R = 10)                  | 0.7                               | 0.5 0.5 0.5                                                       |
| P. stutzeri JM300 | YP                          | 0.08-8 (R = 8)                     | 2                                 | 0.5 0.5 0.5                                                       |
| Pa. denitrificans | YP                          | 0.16-8 (R = 8)                     | 1-4 (8) 1-2 (8) 2-5 (6)           | 2-6 (6)                                                           |
|                   | SP                          | 0.16-4 (R = 8)                     | 1                                 | 2 3 4                                                             |
|                   |                             | SP                                 | 2                                 | 3 9                                                               |
|                   |                             | SP                                 | 4                                 | 4 10                                                              |
|                   |                             | YP                                 | 0.8-4 (R = 8)                      | 7 28                                                              |

* The designator, R = 8 or R = 10 following a range of values signifies that the ratio, R, of initial [nitrate] or [nitrite] (in millimolar) to the cell density (in milligrams of protein X ml⁻¹) was constant at 8 or 10. This situation allowed a running time for denitrification of 23-40 min.

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**TABLE II**

| Medium | Initial nitrate concentration* | Time over which gas samples were analyzed | [NO₃⁻] N² |
|--------|--------------------------------|-------------------------------------------|---------|
|        | mM                             | Range Median                             | nM      |
|        |                                |                                           |         |
| Asparagine | 0.45  3  19-28  25  3   |                                           |         |
| TYSN   | 2.3  6  19-34  27  7         |                                           |         |
| TYSN   | 2.3  7  31-34  33  7         |                                           |         |
| TYSN   | 0.090 7  23-32  30  8        |                                           |         |
| TYSN   | 0.045 3  18-24  23  3        |                                           |         |

* Nitrite was detectable throughout the time over which gas samples were analyzed.

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T. T. Packard (1986), personal communication.
highest cell densities used (1 mg of protein × ml\(^{-1}\)) the flux was about 300 nmol of N × min\(^{-1}\) × ml\(^{-1}\) or about 10\(^6\) times greater per min than the size of the steady-state pool of NO\(_aq\) which we shall take here to be 30 pmol × ml\(^{-1}\). But at a cell density of 1 μg of protein × ml\(^{-1}\), the flux should decrease to 300 pmol of N × min\(^{-1}\) × ml\(^{-1}\) or only some 10 times greater per min than the pool size. At some point, the size of the NO\(_aq\) pool will be diminished by competing reactions that scavenge NO. Although we have been unable satisfactorily to quantitate the instability of 10\(^{-8}\) M levels of NO\(_aq\) in the systems used for the short term experiments, it is clear that NO at low concentrations was not stable in these systems and decayed in a few minutes or tens of min if not replenished. On the other hand, NO appeared to be stable for at least 30 min in the KI/acetic acid mixtures used to generate the NO standards.

Because the scavenging of NO was negligible in the gas recirculation system used for the long term experiments, it was possible to use this system to obtain data at cell densities in the vicinity of 1 μg of protein × ml\(^{-1}\).

**DISCUSSION**

Methods based on the equilibration of NO between gas and aqueous phases allowed a determination of the extracellular [NO\(_aq\)] during denitrification for several bacteria under a variety of conditions. The properties of extracellular NO\(_aq\) inferred in these studies are, for the most part, those expected for a steady-state intermediate in denitrification. Because the methods used were at equilibrium, the extracellular [NO\(_aq\)] determined can be assumed also to hold for the intracellular systems used were at equilibrium, the extracellular [NO\(_aq\)] concentrations was not stable in these systems and decayed in a few minutes or tens of min if not replenished. This represents a simple but effective solution to a common evolutionary problem among denitrifiers. A second utility of a highly active enzyme for the reduction of NO is the interception of intracellular NO\(_aq\) before it can diffuse from the cell. Efficient interception and reduction would tend to maximize the energy yield of denitrification, at least at open systems as envisaged above. At least in open systems as envisaged above, and in systems as cited above, it is likely that a bacterial enzyme must itself be diffusion controlled if it is to compete against diffusive loss of a compound which faces no barrier to diffusion. Purified nitric oxide reductase from P. stutzeri ZoBell is reported to have a K\(_{m,NO} = 400\) nM, predict V\(_{NO}/V_{Ni} = 7\)–400. This value is smaller than the actual one because the levels of NO used partially inhibited NO uptake. The [NO\(_aq\)] of Tables I and II, when coupled with K\(_{m,NO} = 400\) nM, predict V\(_{NO}/V_{Ni} = 7\)–400.

These results overall suggest that nitric oxide reductase of denitrifying bacteria is a comparatively very active enzyme, probably the most active of the N-oxide reductases in vivo, and is capable of maintaining NO\(_aq\) at such low steady-state concentrations that the potential toxicity of NO (19, 20) is not realized. This represents a simple but effective solution to a common evolutionary problem among denitrifiers. A second utility of a highly active enzyme for the reduction of NO is the interception of intracellular NO\(_aq\) before it can diffuse from the cell. Efficient interception and reduction would tend to maximize the energy yield of denitrification, at least at open systems as envisaged above. At least in open systems as envisaged above, and in systems as cited above, it is likely that a bacterial enzyme must itself be diffusion controlled if it is to compete against diffusive loss of a compound which faces no barrier to diffusion. Purified nitric oxide reductase from P. stutzeri ZoBell is reported to have a K\(_{m,NO} = 400\) nM, predict V\(_{NO}/V_{Ni} = 7\)–400. This value is smaller than the actual one because the levels of NO used partially inhibited NO uptake. The [NO\(_aq\)] of Tables I and II, when coupled with K\(_{m,NO} = 400\) nM, predict V\(_{NO}/V_{Ni} = 7\)–400.

The lower values of [NO\(_aq\)] of Table I are within a factor of 10 or so of those obtained from the few observations extant on natural, anaerobic, aqueous environments where denitrification is thought to control [NO\(_aq\)]. [NO\(_aq\)] values as high as 0.5 nM and turnover times as short as a few minutes have been observed in these environments.

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