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The reaction of reduced cytochromes *c* with nitrous oxide reductase of *Wolinella succinogenes*

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Kinetic studies were carried out on the oxidation of dithionite-reduced, monoheme cytochromes *c* by nitrous oxide reductase from *Wolinella succinogenes*. These reduced cytochrome *c*-N_2O oxidoreductase systems showed second-order kinetics, first-order each in reduced cytochrome *c* and enzyme, at concentrations of reduced cytochrome *c* between 1 and 10 μM. The second-order rate constant at 25°C and pH 6.8, k_2, was $3.1 \cdot 10^6$, $9.3 \cdot 10^4$ and about $1 \cdot 10^4$ M^{-1} s^{-1} for cytochrome *c* from *W. succinogenes*, horse heart and *Pseudomonas aeruginosa* (cytochrome *c*-551), respectively, at enzyme concentrations $\leqq 12$, $\leqq 3$ and $\leqq 11$ nM, respectively. With horse-heart cytochrome *c* and cytochrome *c*-551, k_2 diminished substantially at higher enzyme concentrations. Evidence for reaction via an E-S (Michaelis) complex was not obtained. Unlike systems for which the radical cation of benzyl viologen ($BV \cdot^+$) served as reducing agent, nitrous oxide reductase failed to show turnover-dependent inactivation when a reduced cytochrome *c* was the reductant. The system thus mimicked the ability of nitrous oxide reductase to turnover in vivo without inactivation. Cytochrome *c* oxidase activity of nitrous oxide reductase was not observed when O_2 replaced N_2O. Similarly, $BV \cdot^+$-CO_2 oxidoreductase activity was not detected with bicarbonate buffer (CO_2 is isoelectronic with N_2O). The values for M_r (9214), optical extinction coefficients and amino acid composition of the monoheme cytochrome *c* of *W. succinogenes* were found to be somewhat different from published values.

Introduction

Nitrous oxide reductase is an enzyme that catalyzes the reduction of N_2O to N_2 plus water in denitrifying and photodenitrifying bacteria [1–9] and in certain anaerobic or microaerophilic respiratory bacteria [10]. The enzyme contains about four Cu atoms per subunit and is purified as a dimer from most organisms. The enzyme from *Achromobacter cycloclastes* is monomeric [8] and that from *Wolinella succinogenes* contains a cytochrome *c* domain in addition to the Cu centers [10]. All are reported to be soluble enzymes except for that from *Flexibacter canadensis* [9] which is membrane-bound. The oxidized state of the enzyme from certain organisms contains spin coupled Cu(I)-Cu(II) binuclear (half-met) centers [3,6,11–15] and other Cu centers not detectable by electron paramagnetic resonance. These latter centers may consist of antiferromagnetically coupled cupric atoms [14–18]. Resonance Raman [19], electron spin echo [13] and paramagnetic resonance [20,21] spectrometric data imply the existence of a Cu center in nitrous oxide reductase analogous to the Cu_A center of cytochrome oxidase. This center may be identical to the half-met Cu_2 center [20]. Although some information is now available regarding redox state, spin couplings and ligands to Cu in nitrous oxide reductase [3,6,11–21], there is relatively little understanding of the catalytic mechanism or of how the enzyme interacts with the electron transport systems of denitrifying bacteria.

On the latter point, there is increasing evidence that a cytochrome, probably a cytochrome *c*, is the physiological electron donor to nitrous oxide reductase in bacteria [5,22–27]. The involvement of cytochrome c_2 in that role is particularly convincing in the case of *Rhodobacter capsulatus* [26] and cytochrome c_2 and *c*-550 have been implicated on the basis of indirect evidence in the case of *Rhodobacter sphaeroides* [24] and *Paracoccus denitrificans* [27], respectively. Both reduced cytochrome *c'* [5] and c_2 [26] of *R. capsulatus* have been shown to serve as reductants in vitro to the

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Abbreviations: BV^{2+} and $BV \cdot^+$, oxidized and semiquinone forms of benzyl viologen, respectively; $MV \cdot^+$, semiquinone form of methyl viologen; PPB, potassium phosphate buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

nitrous oxide reductase of that photodenitrifying bacterium, but kinetic details are lacking. Most kinetic data on nitrous oxide reductase have been obtained from systems in which the low potential reductants, $BV \cdot^+$ or $MV \cdot^+$, were used [1,3,6,10,28]. These systems are useful, but the enzyme suffers turnover-dependent inactivation in them, whereas the enzyme in vivo generally does not.

Studies of the reduced cytochrome c-N_2O oxidoreductase reaction are motivated by at least three considerations. The first is the practical one of finding a basis for a nitrous oxide reductase assay that avoids enzyme inactivation. The second is the general one of exploring the kinetics and mechanism of reactions that may illuminate the presumed analogous reactions in vivo. The third is the creation of a system with a non-autoxidizable reductant that would allow a test of whether O_2 is a substrate or inhibitor of nitrous oxide reductase. The enzyme from *W. succinogenes* [10,14,28] was chosen for these initial studies because of its high specific activity, good stability toward O_2 and dithionite and its ease of preparation. The three species of monoheme (class I) cytochrome c employed comprised a taxonomically and physically diverse set.

Experimental procedures

Purification and analysis of W. succinogenes cytochrome c

Wolinella succinogenes ATCC 29543 was grown anaerobically for 14 h at 37°C on N_2O and formate/succinate (100 mM/ 10 mM) in Yoshinari's modified broth [10,29]. Cells from 30 liters of culture were harvested by centrifugation at 4°C in a continuous flow DeLaval machine, washed once with 5 mM PPB pH 6.8 and suspended in 100 ml of this buffer supplemented with 5 μg ml^{-1} of DNase-I (Sigma). Cells were broken by use of a French press (Aminco) at 20.7 MPa (3000 psi). The resulting mixture was centrifuged briefly to remove remaining intact cells and heavy debris and then fractionated with ammonium sulfate at 60% saturation. The soluble fraction containing the cytochrome c was dialyzed against 5 mM PPB pH 6.8, and 80 ml of the resulting solution was applied to a CM-23 cation exchange column (2.5 × 15 cm, Whatman) previously equilibrated with this buffer. The column was washed with 80 ml of the buffer and cytochrome c was eluted with 240 ml of a 5–150 mM gradient in PPB pH 6.8 at a flow rate of 54 ml h^{-1}. Fractions with absorbance at 408 nm were collected, combined for a total volume of about 30 ml, dialyzed against 5 M PPB (pH 6.8), lyophilized, redissolved in 2 ml of the same buffer and fractionated by gel filtration chromatography on a Sephadex G-75 column (2.6 × 50 cm, Pharmacia) using 10 mM PPB. The flow rate of buffer was 12 ml h^{-1}. Samples were stored frozen.

The M_r of any one preparation of cytochrome c was estimated by SDS-PAGE [30] and gel filtration chromatography [31] in duplicate. SDS-PAGE was performed in slab gels basically by the method of Laemmli [32] using a mini-gel apparatus (Idea Scientific) at 30 V · cm^{-1}. Slabs were 0.8 mm thick and contained 4.4% total/2.6% crosslinking and 17.5% total/2.6% crosslinking acrylamide for the stacking and running gels, respectively. Gels were stained with Coomassie blue for protein [30] and with 3,3′,5,5′-tetramethylbenzidine/H_2O_2 for heme [33]. Gel filtration chromatography made use of a Sephadex G-75 column (1 × 78 cm). Sample volume was 0.5 ml and consisted of a mixture of proteins, each at about 1 mg ml^{-1} in 5 mM PPB pH 6.8 and the column was eluted with 20 mM PPB, same pH, at 9 ml h^{-1}. Fractions of 1.5 ml were collected and analyzed by absorbance at 280 and 408 nm and by SDS-PAGE. M_r standards included bovine serum albumin (66200), chicken ovalbumin (43000), bovine carbonic anhydrase (31000), soybean trypsin inhibitor (21500), chicken lysozyme (14400), horse-heart cytochrome c (12400), putidaredoxin (11420), truncated putidaredoxin (10816) and aprotinin (6500). The last cannot be used as a standard in the SDS-PAGE method. Putidaredoxin was cloned and expressed in *Escherichia coli* and obtained from Thomas Pochapsky, Brandeis University. Truncated putidaredoxin arose by the spontaneous hydrolysis of the C-terminal tetrapeptide, Asp-Arg-Gln-Trp. Densitometric data on gels after SDS-PAGE and staining with Coomassie blue were obtained with use of an ISCO gel scanner, model 1312. M_r of cytochrome c of *W. succinogenes* was also estimated by means of Electrospray mass spectroscopy [34] with use of a JEOL JMS-SX102A machine. The cytochrome c solution (30 μM) was dialyzed extensively with stirring against water, and to 50 μl of this solution was added 50 μl of methanol and 2 μl of glacial acetic acid. This mixture was drawn into a syringe and delivered into the Electrospray port at the rate of 1 μl min^{-1}.

Isoelectric focusing was performed at 4°C with use of a horizontal electrophoresis cell (Bio-Rad, Bio-Phoresis). The procedure was exactly according to the directions of the manufacturer and made use of standards (Bio-Rad IEF standard 161–0310) covering the pI range 4.65–9.60.

Amino acid analyses were carried out at the Protein Chemistry Facility of Tufts University School of Medicine with use of the PicoTag system (Waters). Protein was hydrolyzed by HCl gas in two analyses and by methanesulfonic acid (for tryptophan content) in another. Each analysis was in duplicate and included chicken lysozyme and horse-heart cytochrome c as external standards. In addition, tryptophan in *W. succinogenes* cytochrome c was determined semiquantitatively by a modification of the spectrophotometric

method of Edelhoch [35]. Absorbance values in the aromatic amino acid band for horse-heart cytochrome *c*, for which the content of tyrosine and tryptophan is known [36], were used to calculate an extinction coefficient for heme. This coefficient was then included in the equations used to solve for the tryptophan content of *W. succinogenes* cytochrome *c*.

Other preparations and materials

Nitrous oxide reductase was prepared from *W. succinogenes* by the method of Teraguchi and Hollocher [10]. The method of cell growth was as described above. Because both the monoheme cytochrome *c* and nitrous oxide reductase are basic proteins, they were retained by the CM-23 column and could be cleanly separated by elution under a linear gradient in phosphate concentration. By this means, a single lot of cells was occasionally used for purification of both proteins. Nitrous oxide reductase is a dimeric enzyme as isolated [10], but the enzyme concentration is expressed in the text and figures as the concentration of enzyme subunits. It is assumed that each subunit has one operational active site. This assumption is probably valid at the high N_2O concentrations used in these experiments [28].

Horse heart cytochrome *c* (type VI) and *Pseudomonas aeruginosa* cytochrome *c* (cytochrome *c*-551) were from Sigma.

Assays

Protein was assayed by the bicinchoninic acid method [37] using reagents from Pierce.

Nitrous oxide reductase was assayed spectrophotometrically at 600 nm in anaerobic cuvettes at 25°C by its $BV\cdot^+$-N_2O oxidoreductase activity [10,38], generally using photochemically generated $BV\cdot^+$. In certain experiments, dithionite (sodium hydrosulfite, $Na_2S_2O_4\cdot 2H_2O$) was used to generate $BV\cdot^+$. Final volume was 2.50–2.65 ml and the reaction was initiated by injection of enzyme in most cases and dithionite in others. Where $BV\cdot^+$ was produced photochemically, the system contained 10 mM PPB (pH 6.8), 40 mM triethanolamine, 4 μM proflavin, 0.1 mM BV^{2+} and 25 mM N_2O; where $BV\cdot^+$ was produced by dithionite, the composition of the mixture was the same, but the irradiation step was simply omitted.

The reduced cytochrome *c*-N_2O oxidoreductase activity of nitrous oxide reductase was assayed spectrophotometrically at 25°C and pH 6.8 in anaerobic cuvettes. Cytochrome *c* (1–10 μM) was reduced by a small (about 10%) electron excess of a freshly prepared solution of dithionite and the reoxidation of cytochrome *c* was followed at 416 or 550–552 nm, depending on the concentration of cytochrome *c*. N_2O concentration was generally 25 mM (saturated under 1 atm), and the reaction was initiated by the injection of enzyme in most cases and dithionite in others. N_2O at $\geqq 1$ mM saturated nitrous oxide reductase kinetically [10,28] and underwent only negligible decreases in concentration during the reoxidation of cytochrome *c*. Because the nitrous oxide reductase preparation contained about 500 mM PPB, the assay system was supplemented to a small extent with additional PPB from that source. When the enzyme concentration was 12 nM, the additional PPB was about 2.5 mM. Thus, most experiments were performed with PPB concentrations between 10 and 12.5 mM. The highest concentrations of PPB contributed by enzyme in any experiment was 10 mM.

The possibility that O_2 might be a substrate for nitrous oxide reductase was tested by attempts to measure reduced cytochrome *c* oxidase activity spectrophotometrically at 416 nm in systems containing enzyme from *W. succinogenes* (2.5 μg), dithionite-reduced cytochrome *c* from horse heart (5 μM) and O_2 (0.25 mM). Final volume, temperature and pH were 2.5 ml, 25°C and 6.8. The order of addition was: reduced cytochrome *c*, O_2 stock solution (1.25 mM), enzyme. Because horse-heart cytochrome *c* is oxidized only very slowly by O_2 at pH 6.8 (unlike *W. succinogenes* cytochrome *c* [39]), the putative enzymatic reaction could be followed for 1–2 h and compared with a control lacking enzyme. Cytochrome *c* was reduced by one- or slightly less than one-electron equivalent of dithionite under anaerobic conditions. In one experiment, reduced cytochrome *c* was subsequently sparged with air before injection of enzyme to guarantee removal of any possible excess of dithionite. O_2 reacts so rapidly with $BV\cdot^+$ or $MV\cdot^+$ that enzyme catalysis of the reaction cannot generally be observed.

The possibility that CO_2 might be an oxidizing substrate for nitrous oxide reductase was tested spectrophotometrically in a system containing enzyme from *W. succinogenes*, $BV\cdot^+$ and $NaHCO_3$ buffer pH 6.8. 2.5 milliliters of BV^{2+} mixture (0.1 mM in BV^{2+}) was irradiated anaerobically to generate about 80 μM $BV\cdot^+$ and then 0.1 ml of anaerobic 500 mM bicarbonate buffer and 10 μl (2 μg) of enzyme solution were injected in that order. Controls lacked enzyme. The above $BV\cdot^+$-CO_2 oxidoreductase system was analogous to the $BV\cdot^+$-N_2O oxidoreductase system except for the lack of N_2O and presence of bicarbonate buffer. Temperature and pH were also the same.

Dithionite stock solutions were freshly prepared in anaerobic 10 mM PPB pH 7 and standardized spectrophotometrically by the anaerobic titration of BV^{2+}. Stock solutions were about 25 mM for $BV\cdot^+$-N_2O oxidoreductase assays and about 2.5 mM for reduced cytochrome *c*-N_2O oxidoreductase reactions. Samples of dithionite were found to be 80–90% dithionite by weight, with the remainder presumed to be sodium sulfite.

Results

Reduced cytochrome c-N_2O oxidoreductase activity

Because nitrous oxide reductase of *W. succinogenes* is largely insensitive to inactivation by dithionite [10,14], this reductant could be used in small electron excess to reduce cytochrome *c*. The reoxidation of cytochrome *c* was initiated in presence of N_2O by the injection of enzyme. Spectrophotometric progress curves for the disappearance of reduced cytochrome *c* typically showed a short lag during which excess dithionite was being consumed, then a brief transition marking the exhaustion of dithionite and onset of oxidation of cytochrome *c* and finally a phase during which the rate of oxidation of cytochrome *c* decreased monotonically with time. This last kinetic phase is represented in Figs. 1, 2B and 3. Fig. 1 shows that there was no direct redox coupling between reduced cytochrome *c* and N_2O and that the rate of reaction of reduced cytochrome *c* with nitrous oxide reductase of *W. succinogenes* had the rank order, cytochrome *c* from *W. succinogenes* > horse-heart > *P. aeruginosa*. The reactivity of *P. aeruginosa* cytochrome *c* with the enzyme from *W. succinogenes* was so low as to allow only a semiquantitative determination of rate constants because of uncertainties in A_∞ and other technical problems.

The decreasing rate of cytochrome *c* oxidation in the final phase of reaction could have been due to any one of four processes: (a) inhibition by oxidized cytochrome *c* [40]; (b) an increasing state of oxidation of the heme or copper center of the enzyme in response to the increasing redox potential of the cytochrome *c* couple; (c) turnover-dependent inactivation, which all species of nitrous oxide reductase so far examined exhibit during catalysis of the $BV\cdot^+$- or $MV\cdot^+$-N_2O oxidoreductase reaction [1,6,7,9,10]; or (d) inherent first-order (or first-order-like) kinetic behavior. The first two possibilities were precluded by the observation that the progress curve for the oxidation of 1–10 μM reduced cytochrome *c* was unaffected by the presence or absence of 5 μM oxidized cytochrome *c*. The third possibility was precluded by experiments such as those depicted in Fig. 2. In Fig. 2A, catalysis of the $BV\cdot^+$-N_2O oxidoreductase reaction by the enzyme from *W. succinogenes* (open circles) was run two more times successively (filled circles, open squares) with the same reaction mixture simply by adding successive amounts of dithionite equal to the initial amount in order to regenerate $BV\cdot^+$. Dithionite was added each time in slight (about 10%) electron deficit relative to the BV^{2+}. There are clearly losses of activity between the first and second runs and between the second and third. These losses are due to turnover-dependent inactivation of nitrous oxide reductase. The analogous experiment (Fig. 2B), in which dithionite-reduced cytochrome *c* from *W. succinogenes* served as the donor

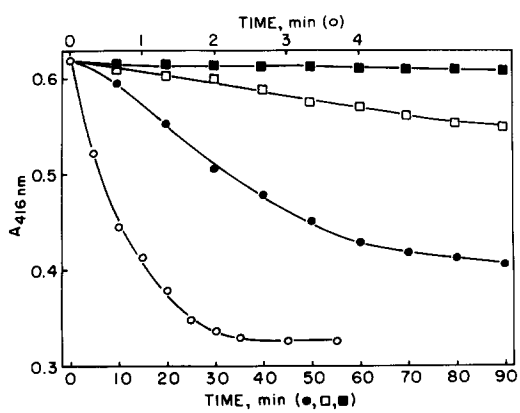

Fig. 1. Oxidation of reduced cytochromes *c* by purified nitrous oxide reductase of *W. succinogenes*. Open circles, filled circles and open squares, cytochrome *c* from *W. succinogenes*, horse heart and *P. aeruginosa*, respectively, in presence of 2.4 μg of nitrous oxide reductase. Filled squares, horse-heart cytochrome *c* in absence of nitrous oxide reductase. The reduced cytochrome *c* and N_2O concentrations were about 5 μM and 25 mM, respectively. Temperature, pH and buffer were 25°C, 6.8 and 10 mM PPB. The cuvettes were made anaerobic by sparging with N_2O, cytochrome *c* was reduced by a 5–10% electron excess of dithionite and reaction was initiated by injection of enzyme. Any lag phase in the progress curves and all or most of the transition phase were deleted, so that zero time marks the beginning approximately of the first-order phase.

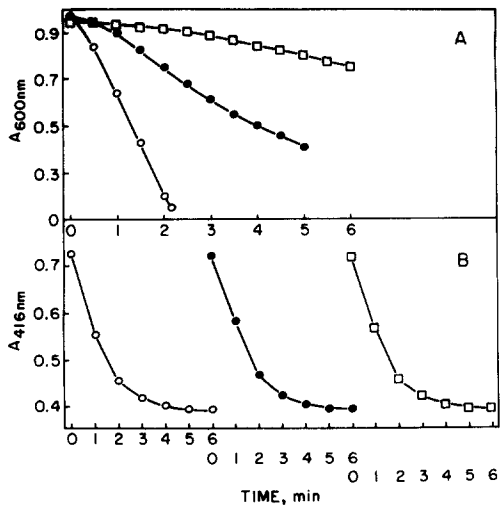

Fig. 2. Failure of nitrous oxide reductase to inactivate during oxidation of reduced cytochrome *c*. *A*, Oxidation of $BV\cdot^+$ by 0.5 μg of purified nitrous oxide at 25°C, pH 6.8 in 25 mM N_2O. *B*, Oxidation of reduced cytochrome *c* from *W. succinogenes* by 1.2 μg of purified nitrous oxide reductase under the same conditions of temperature, pH and N_2O concentration. Open circles, filled circles and open squares represent progress curves for the first, second and third cycles of reaction, respectively. BV^{2+} and oxidized cytochrome *c* were reduced by slightly substoichiometric amounts of dithionite to generate about 90 μM $BV\cdot^+$ (*A*) and 6 μM reduced cytochrome *c* (*B*) and reaction was initiated by injection of enzyme. The second and third cycles were initiated by injection of additional dithionite. It should be noted that although nitrous oxide reductase of *W. succinogenes* shows some direct dithionite-N_2O oxidoreductase activity (9,13), this reaction was sufficiently slow so that it never interfered with the reduction of cytochrome *c* or BV^{2+} by dithionite.

to nitrous oxide reductase, showed no significant loss of activity even after 4–5 sequential cycles of reaction. We conclude from Fig. 2 and related experiments that the decreasing rate with time of the reduced cytochrome c-N_2O oxidoreductase reaction is not caused by progressive enzyme inactivation. Semilogarithmic plots of the data established that the kinetics are first-order with respect to reduced cytochrome c from horse heart (Fig. 3A) and *W. succinogenes* (Fig. 3B). These plots were linear over at least two and generally three half-times. Small deviations from linearity that were observed on some occasions are attributed to small inaccuracies in the A_∞ value or to the placement of the first data point partly within the transition phase of the reaction. The kinetic order of the reaction of cytochrome c-551 is less clear, because these reactions were not monitored beyond one half-time (3–5 h); over this period, however, the kinetics also appeared to be first-order with respect to reduced cytochrome c.

Fig. 4 is a test of first-order dependency for nitrous oxide reductase (the enzyme) in the reduced cytochrome c-N_2O oxidoreductase reaction. The kinetics indicate first-order dependence when *W. succinogenes* cytochrome c was the reductant ($k_2 = 3.1 \cdot 10^6$ M^{-1} s^{-1} at 25°C, pH 6.8) at least up to 12 nM enzyme at which point the reaction became too rapid to monitor conveniently. With horse-heart cytochrome c, however, the second order rate constant, k_2, depended on enzyme concentration (Fig. 4). At enzyme concentrations of ≤ 3 and 12–46 nM, k_2 was $9.3 \cdot 10^4$ and $6 \cdot 10^3$ M^{-1} s^{-1}, respectively, for reduced horse-heart cytochrome c. Assuming that the reaction of cytochrome c-551 is in fact first order in the reduced species, then k_2 is calculated to be about $1 \cdot 10^4$ and $2 \cdot 10^3$ M^{-1} s^{-1} at enzyme concentrations of ≤ 11 nM and from 11–44 nM, respectively.

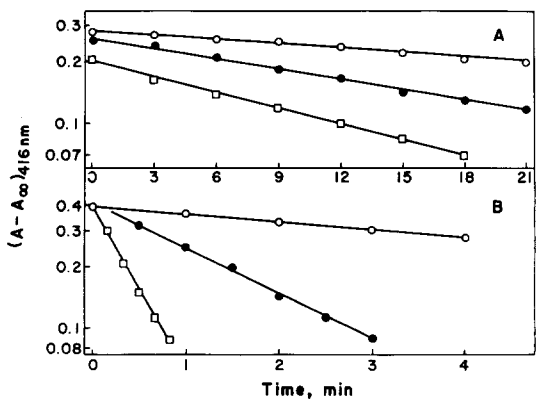

Fig. 3. Semilogarithmic plots of data for reduced cytochrome c-N_2O oxidoreductase systems. *A*, horse heart cytochrome c. Open circles, filled circles and open squares. 0.64, 2.6 and 10.2 μg of purified nitrous oxide reductase, respectively. *B, W. succinogenes* cytochrome c. Open circles, filled circles and open squares, 0.13, 0.64 and 2.6 μg of purified nitrous oxide reductase. Conditions were as described in Fig. 1, and the data refer to the final (first-order) phase of the reaction which applied after any slight excess of dithionite was exhausted. The lines represent the linear least-squares fit of the data.

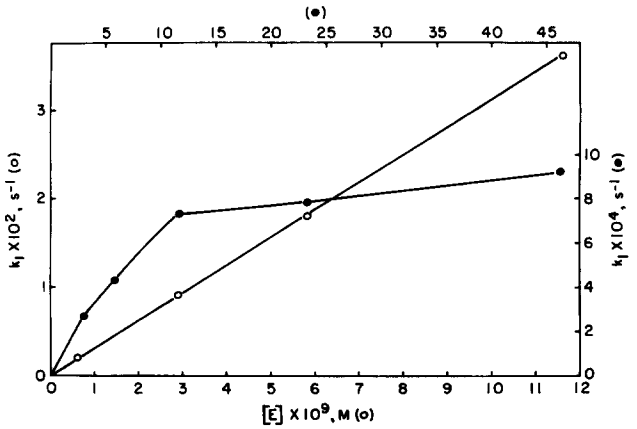

Fig. 4. k_1 vs. [E] for the reduced cytochrome c-N_2O oxidoreductase reaction. Open circles (left and bottom coordinates) and filled circles (right and top coordinates) refer to *W. succinogenes* and horse-heart cytochrome c, respectively. k_1 was calculated from first-order kinetic data of the kind represented in Fig. 3 using the slope method. k_2, the second order rate constant, is taken to be the slope of curves of k_1 vs. [E].

A kinetic scheme which is consistent with the data, at least at low concentrations of nitrous oxide reductase, is represented by Eqns. 1 and 2,

$$2\,(\text{red. cytochrome}\,c) + E_{ox} \rightarrow 2\,(\text{ox. cytochrome}\,c) + E_{red} \quad (1)$$

$$E_{red} + N_2O \rightarrow E_{ox} + N_2 + H_2O \quad (2)$$

where one of the two successive bimolecular redox steps implied by Eqn. 1 is the rate-limiting step. Eqn. 1 should contain the rate-limiting step, because the ratio of k_{cat} for N_2O reduction in the $BV \cdot {}^+$-N_2O oxidoreductase reaction [10,28] to k_2[reduced cytochrome c] in the cytochrome c-N_2O oxidoreductase reaction was 6.5 or more with 10 μM reduced cytochrome c from *W. succinogenes*. Thus, the processing of N_2O was never rate-limiting. Because reduced cytochromes c showed no sign, at least up to 10 μM, of having saturated nitrous oxide reductase (first- to zero-order transition), the occurrence of a Michaelis or intermediate complex between reduced cytochrome c and enzyme is omitted from Eqn. 1 and 2.

$BV \cdot {}^+$-N_2O oxidoreductase activity

This reaction was confirmed to be first-order in nitrous oxide reductase and zero-order in $BV \cdot {}^+$ at the concentrations employed [10], providing that the time of the reactions was sufficiently short so that secondary effects, such as the turnover-dependent inactivation of enzyme [1,6], had only small impact.

Reduced cytochrome c oxidase activity

The mean rate of oxidation of horse-heart cytochrome *c* by O_2 in presence of nitrous oxide reductase in four experiments was 2.1 ± 3.5 nM min^{-1} as compared with 160–200 nM min^{-1} (initial rate) for equivalent systems containing N_2O rather than O_2. The result was therefore not statistically significant for O_2. It was also shown that O_2 (0.25 mM) had no effect on the rate of the reduced cytochrome *c*-N_2O oxidoreductase reaction when $[N_2O] = 0.5$ mM. Thus, the presence of O_2 during turnover with N_2O seemed not to inhibit or inactivate the enzyme's ability to react with reduced cytochrome *c* or N_2O.

$BV\cdot^+$-CO_2 oxidoreductase activity

The rate of oxidation of $BV\cdot^+$ under anaerobic conditions in the presence of bicarbonate buffer was statistically insignificant and could not have been greater than 1% of the rate realized in comparable systems containing 24 mM N_2O and no bicarbonate buffer. The presence of 20 mM $NaHCO_3$ buffer (5–8 mM CO_2) did seem however to diminish the $BV\cdot^+$-N_2O oxidoreductase activity by about 2-fold. The basis for this effect has not been explored. Negative results were similarly obtained with azide (N_3^-), which is also isoelectronic with N_2O, but the fact that azide is a powerful inhibitor of the enzyme [1,10] makes this result moot.

Molecular properties of cytochrome c from W. succinogenes

The purification, certain molecular properties and ^{1}H-NMR parameters of the monoheme cytochrome *c* of *W. succinogenes* were reported by Moura et al. [39]. The method of purification used by these workers involved five column steps, four of which showed no retention of the cytochrome and one, only weak retention. Among the characteristics reported, the optical extinction coefficients were unusually low for a *c*-type cytochrome. We have developed a more simple method involving two columns for purification of this basic cytochrome *c*, as described in Experimental procedures. The procedure makes use of a cation exchange column to provide a positive retention step and routinely yielded cytochrome *c* with purity $\geq 96\%$ as judged by densitometry applied to Coomassie blue-stained gels after SDS-PAGE and by gel filtration chromatography on Sephadex G-75. Isoelectric focusing indicated that the p*I* of *W. succinogenes* cytochrome *c* is 8.5 ± 0.1. The determination of M_r by SDS-PAGE and gel filtration chromatography yielded values of 9800 ± 200 and $10\,300 \pm 200$, respectively. The cytochrome *c* is therefore monomeric in solution. The Electrospray mass spectrum showed two strong peaks at $m/z = 1317.20$ and 1536.52 for $(MH_7)^{7+}$ and $(MH_6)^{6+}$, respectively. Weaker lines were also observed for $(MH_6K)^{7+}$, $(MH_5K)^{6+}$, $(MH_4K_2)^{6+}$ and $(MH_3K_3)^{6+}$. The molecular weight consistent with all the data was 9213.5 ± 0.1, or more than 1000 greater than the value of 8200 estimated by Moura et al. [39] and some 600–1100 smaller than our determinations above. Because heme was covalently linked to protein on the basis of heme staining and because no other lines were observed in Electrospray mass spectrometry with inferred $M_r = 9214 \pm 618$ to imply protein containing or lacking heme *c*, the value of 9214 is taken to represent the M_r of protein with the heme attached. Using $M_r = 9214$ rather than 8200, it was found that the optical extinction coefficients at selected wavelengths (407 and 522 nm for oxidized protein and 416.5, 523 and 552 nm for reduced protein) for *W. succinogenes* cytochrome *c* were $14.4 \pm 5\%$ lower than those for horse-heart cytochrome *c* [41,42], rather than about 23% lower [39]. Although there was relatively good agreement between our data and those of Moura et al. [39] with regard to most amino acids (Table I), our data indicated values of 4, 2 and 1 residues of methionine, arginine and tryptophan, respectively, rather than 2, 1, and 0. The optical method indicated the presence of at least 1 but not more than 1.6 tryptophan residues.

TABLE I

Amino acid composition of cytochrome c from W. succinogenes

Amino acid	Residues per peptide of:	
	$M_r = 8596$ (this study) [a]	$M_r = 8200$ (Ref. 39) [b]
Asx	6.5 (6 or 7)	7
Glx	6.8 (7)	7
Ser	5.4 (5)	5
Gly	8.7 (9)	9
His	1.0 (1)	1
Arg	1.7 (2)	1
Thr	3.8 (4)	3
Ala	12.1 (12)	13
Pro	1.2 (1)	1
Tyr	2.9 (3)	3
Val	4.3 (4)	4
Met	3.7 (4)	2
Cys	1.6 (2)	2
Ile	2.4 (2)	2
Leu	6.1 (6)	6
Phe	1.5 (1 or 2)	1
Lys	10.5 (10 or 11)	11
Trp	0.9 (1)	0
Total	80.3	78

[a] The peptide M_r is taken to be 9214 minus the M_r of heme *c*, 618. The values to two significant figures represent the average of three duplicate determinations except for tryptophan, for which there was one duplicate determination. The error is believed to be about ± 0.20 for most amino acids and ± 0.25 for tryptophan. Nearest integers are in parenthesis.

[b] Data are from Ref. 39 which reports integer values. Apparently no correction was made for the contribution of heme *c* to M_r.

Discussion

Among the three monoheme cytochromes *c* studied, reactivities varied by a factor of about 300 based on maximum second-order rate constants for the reduced cytochrome *c*-N_2O oxidoreductase reaction. It would appear, therefore, that species differences can be important in the interaction between cytochrome *c* and nitrous oxide reductase. In all three cases, the oxidation of cytochrome *c* was actually or approximately first-order with respect to reduced cytochrome *c* and kinetic saturation indicative of a change in rate-limiting step, was not observed up to at least 10 μM. If a Michaelis or intermediate complex is relevant, the value of its dissociation constant must exceed 10 μM substantially. The lack of evidence for an intermediate complex and the large second-order rate constant ($k_{cat}/K_m \approx 10^8$ M^{-1} s^{-1}) for the half-reaction involving reduction of N_2O [28] make it likely that electron transfer from cytochrome *c* is the rate-limiting step. Although the participation of a kinetically significant intermediate complex can occur in redox reactions between redox proteins [43,44], e.g., the reaction of cytochrome *c* with yeast cytochrome *c* peroxidase [45] or cytochrome oxidase [46], there are numerous cases where such complexes are not indicated [44]. One well studied example, which may serve as a model for the cytochrome *c*-nitrous oxide reductase system, is electron exchange between cytochrome *c*-551 and azurin of *P. aeruginosa* [44,47,48]. On the basis of temperature-jump studies, Rosen and Pecht [47] concluded that the fast and slow relaxations in this reaction were due to second-order association/electron transfer steps and a protein isomerization, respectively, and complex formation was excluded as a thermodynamically or kinetically distinct step. Similarly fluorescence [49] and NMR [44] spectroscopy have failed to detect complex formation in this reaction. It is interesting to note that the second-order rate constants for the oxidation and reduction of cytochrome *c*-551 by azurin [47], $6.1 \cdot 10^6$ and $7.8 \cdot 10^6$ M^{-1} s^{-1} (25°C, pH 7.0), are only a factor of about 2 greater than k_2 for the reaction of reduced cytochrome *c* (*W. succinogenes*) with nitrous oxide reductase. A value of k_2 similar to the rate constant exhibited by this and other presumed outer sphere electron transfer reactions between redox proteins [44,48,50,51] suggests that the cytochrome *c*-nitrous oxide reductase reaction may also proceed by an outer sphere mechanism, at least in the most favorable cases. It should be emphasized in this regard that both the cytochrome *c* and nitrous oxide reductase of *W. succinogenes* are basic proteins [10]. Many if not all of the excess positive charges of the enzyme are probably carried on its cytochrome *c* domain [10]. If the primary site of reactions of reduced cytochrome *c* were at the cytochrome *c* domain of the enzyme, then electron transfer would be expected to have an appreciable electrostatic work function [52] and the rate would be expected to increase with increasing ionic strength. If the reaction site were at the acidic, negatively charged, copper domain [10], the effect of ionic strength might be reversed. Thus, the ionic strength dependency of the cytochrome *c*-nitrous oxide reductase reaction might provide a clue as to whether cytochrome *c* can react directly with the copper domain or via the cytochrome *c* domain. Such a study, which is now underway, would be assisted by surveying nitrous oxide reductases that lack a cytochrome *c* domain [1–9].

At the moment, we have no satisfactory explanation for why the second-order rate constants decreased with increasing concentration of nitrous oxide reductase in the case of horse heart and *P. aeruginosa* cytochrome *c*. Changes in the concentration of PPB (10–20 mM) over the full range of enzyme concentrations studied (3–46 nM) were too small to account for the effect observed. It is conceivable that the dimeric enzyme [10] underwent reversible dissociation into monomers at low enzyme concentrations and that monomers were much more active than dimers. However, the observation [28] that the specific activity of *W. succinogenes* nitrous oxide reductase was essentially constant in the $BV \cdot ^+$-N_2O oxidoreductase reaction from 0.08–0.40 μg ml^{-1} (0.9–4.5 nM) makes dissociation unlikely. In addition, the consequences of enzyme dissociation would be expected to be manifested to the same degree among the three cytochromes *c* tested, but this was not the case. The value of k_2 remained constant up to at least 12 nM enzyme in the case of *W. succinogenes* cytochrome *c* but began to diminish in the 3–6 nM range in the case of cytochrome *c* from horse heart and *P. aeruginosa*. The formation of inactive, unproductive complexes between cytochrome *c* and enzyme would not be expected to produce the kinetic effect observed as long as the concentration of cytochrome *c* greatly exceeded that of enzyme, as was the case throughout. Plots of k_1 vs. $[E]^{0.5}$ for horse heart and *P. aeruginosa* cytochrome *c* do not perfectly linearize the data, although the curvatures are substantially diminished for arithmetic reasons, if no other. Although we cannot rule out the possibility that the involvement of nitrous oxide reductase truly follows a one-half-order dependency in reaction with certain cytochromes *c*, the chemical significance of such a relationship escapes us so long as, as discussed above, it is unlikely that the enzyme dissociates from inactive dimers to active monomers. Finally, we can only say that the enzyme preparations were highly purified ($\geqq$ 95%) and exhibited no evidence for the presence of endogenous inhibitors in the $BV \cdot ^+$-N_2O oxidoreductase reaction.

Because nitrous oxide reductase experiences turnover-dependent inactivation when $BV \cdot ^+$ or $MV \cdot ^+$, but not reduced cytochrome *c*, serves as reduc-

tant, the inactivation would appear to be specifically related to the chemistry of viologen redox dyes and not an inherent property of the enzyme. The ability to avoid turnover-dependent inactivation may permit the reduced cytochrome c-N_2O oxidoreductase reaction to be developed into a standard assay for nitrous oxide reductase.

The apparent lack of reduced cytochrome c oxidase and $BV \cdot {}^{+}$-CO_2 oxidoreductase activities of nitrous oxide reductase supports the impression that the enzyme is highly specific for N_2O among reactive small molecules. No alternative oxidizing substrates or competitive inhibitors are known for the enzyme from *W. succinogenes* or any other denitrifying bacterium. Rejection of O_2 as a substrate or inhibitor is remarkable when one considers the reactivity and small size of O_2, the fact that O_2 is the substrate for a number of Cu-containing oxidases and precedence for reversible O_2 binding at the Cu_2 centers of hemocyanin. It should be stressed, however, that our data reject O_2 and CO_2 as substrates only at rates corresponding to about 10^{-2} of the corresponding rate afforded by N_2O. Whether O_2 and CO_2 will prove to be substrates in more sensitive tests remains to be determined.

The E'°_7 values and isolectric points for the monoheme cytochrome c from *W. succinogenes*, horse heart and *P. aeruginosa* are, respectively, 0.105 [39], 0.255 [53] and 0.259 V [54] and 8.5 (herein), 10.5–10.8 [55,56] and 4.1 [57]. Qualitatively, the rank order of reactivity among the three cytochromes c toward *W. succinogenes* nitrous oxide reductase follows that of redox potential to the extent at least that the strongest reductant showed the greatest rate constant. A 0.15 V difference in potential could support something up to a 345 fold difference in k_2. Reactivity does not correlate with isoelectric point and therefore with molecular net charge. Further speculation on structure-reactivity functions is unwarranted, because of the small sample and the absence of crystal structures for *W. succinogenes* cytochrome c and nitrous oxide reductase.

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References

1 Kristjansson, J.K. and Hollocher, T.C. (1980) J. Biol. Chem. 255, 704–707.
2 Kristjansson, J.K. and Hollocher, T.C. (1981) Cur. Microbiol. 6, 247–251.
3 Coyle, C.L., Zumft, W.G., Kroneck, P.M.H., Körner, H. and Jakob, W. (1985) Eur. J. Biochem. 153, 459–467.
4 Michalski, W.P., Hein, D.H. and Nicholas, D.J.D. (1986) Biochim. Biophys. Acta 872, 50–60.
5 McEwan, A.G., Greenfield, A.J., Wetzstein, H.G., Jackson, J.B. and Ferguson, S.J. (1985) J. Bacteriol. 164, 823–830.
6 Snyder, S.W. and Hollocher, T.C. (1987) J. Biol Chem. 262, 6515–6525.
7 SooHoo, C.K. and Hollocher, T.C. (1991) J. Biol Chem. 266, 2203–2209.
8 Hulse, C.L. and Averill, B.A. (1990) Biochem. Biophys. Res. Commun. 166, 729–735.
9 Jones, A.M., Hollocher, T.C. and Knowles, R. (1992) FEMS Microbiol. Lett. 92, 205–210.
10 Teraguchi, S. and Hollocher, T.C. (1989) J. Biol Chem. 264, 1972–1979.
11 Kroneck, P.M.H., Antholine, W.A., Riester, J. and Zumft, W.G. (1988) FEBS Lett. 242, 70–74.
12 Riester, J., Zumft, W.G. and Kroneck, P.M.H. (1989) Eur. J. Biochem. 178, 751–762.
13 Jin, H., Thomann, H., Coyle, C.L. and Zumft, W.G. (1989) J. Am. Chem. Soc. 111, 4262–4269.
14 Zhang, C.-S., Hollocher, T.C., Kolodziej, A.F. and Orme-Johnson, W.H. (1991) J. Biol. Chem. 266, 2197–2202.
15 SooHoo, C.K., Hollocher, T.C., Kolodziej, A.F. Orme-Johnson, W.H. and Bunker, G. (1991) J. Biol. Chem. 266, 2210–2218.
16 Farrar, J.A., Thomson, A.J., Cheesman, M.R., Dooley, D. M. and Zumft, W.G. (1991) FEBS Lett. 294, 11–15.
17 Dooley, D.M., Landin, J.A., Rosenzweig, A.C., Zumft, W. G. and Day, E.P. (1991) J. Am. Chem. Soc. 113, 8978–8980.
18 Dooley, D.M., McGuirl, M.A., Rosenzweig, A.C., Landin, J.A., Scott, R.A., Zumft, W.G., Devlin, F. and Stephens, P.J. (1991) Inorg. Chem. 30, 3006–3011.
19 Dooley, D.M., Moog, R.S. and Zumft, W.G. (1987) J. Am. Chem. Soc. 109, 6730–6735.
20 Kroneck, P.M.H., Antholine, W.A., Kastrau, D.H.W., Buse, G., Steffens, G.C.M. and Zumft, W.G. (1990) FEBS Lett. 268, 274–276.
21 Scott, R.A., Zumft, W.G., Coyle, C.L. and Dooley, D.M. (1989) Proc. Natl. Acad. Sci. U.S.A. 86 4082–4086.
22 Walter, B., Sidransky, E., Kristjansson, J.K. and Hollocher, T.C. (1978) Biochemistry 17, 3039–3045.
23 Boogerd, F.C., Van Verseveld, H.W. and Stouthamer, A.H. (1980) FEBS Lett. 113, 279–284.
24 Itoh, M., Matsuura, K. and Satoh, T. (1989) FEBS Lett. 251, 104–108.
25 Richardson, D.J., McEwan, A.G., Jackson, J.B. and Ferguson, S.J. (1989) Eur. J. Biochem. 185, 659–669.
26 Richardson, D.J., Bell, L.C., McEwan, A.G., Jackson, J. B. and Ferguson, S.J. (1991) Eur. J. Biochem. 199, 677–683.
27 Matchová, I. and Kucera, I. (1991) Biochim. Biophys. Acta 1058, 256–260.
28 Zhang, C., Jones, A.M. and Hollocher, T.C. (1992) Biochem. Biophys. Res. Commun. 187, 135–139.
29 Yoshinari, T. (1980) Appl. Environ. Microbiol. 39, 81–84.
30 Weber, K. and Osborn, M. (1969) J. Biol. Chem 224, 4406–4412.
31 Laurent, T.C. and Killander, J. (1964) J. Chromatogr. 14, 317–330.
32 Laemmli, U.K. (1970) Nature (London) 227, 680–685.
33 Goodhew, C.F., Brown, K.R. and Pettigrew, G.W. (1986) Biochim. Biophys. Acta 852, 288–294.
34 Jardine, I. (1990) Nature (London) 345, 747–748.
35 Edelhoch, H. (1967) Biochemistry 6, 1948–1954.
36 Margoliash, E., Smith, E.L., Kreil, G. and Tuppy, H. (1961) Nature (London) 192, 1125–1127.
37 Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A. K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
38 Snyder, S.W. and Hollocher, T.C. (1984) Biochem. Biophys. Res. Commun. 119, 588–592.

39 Moura, I., Liu, M.Y., Costa, C., Liu, M.C., Pai, G., Xavier, A.V., LeGall, J., Payne, W.J. and Moura, J.J.G. (1988) Eur. J. Biochem 177, 673–682.
40 Hollocher, T.C. (1964) Nature (London) 202, 1006–1007.
41 Margoliash, E. and Frohwirt, N. (1959) Biochem. J. 71, 570–572.
42 VanGelder, B.F. and Slater, E.C. (1962) Biochim. Biophys. Acta 58, 593–595.
43 Pettigrew, G.W. and Moore, G.R. (1987) Cytochromes *c* Biological Aspects, Chapter 2, Springer-Verlag, Berlin.
44 Moore, G.R. and Pettigrew, G.W. (1990) Cytochromes *c* Evolutionary, Structural and Physiochemical Aspects, Chapter 8, Springer-Verlag, Berlin.
45 Poulos, T.L. and Kraut, J. (1980) J. Biol. Chem. 255, 10322–10330.
46 Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E. (1976) J. Biol. Chem. 251, 1104–1115; (1978) J. Biol. Chem. 253, 149–159.
47 Rosen, P. and Pecht, I. (1976) Biochemistry 15, 775–786.
48 Wherland, S. and Pecht, I. (1978) Biochemistry 17, 2585–2591.
49 Corin, A.F., Bersohn, R. and Cole, P.E. (1983) Biochemistry 22, 2032–2038.
50 Wilson, M.T., Silvestrini, M.C., Morpurgo, L. and Brunori, M. (1979) J. Inorg. Biochem. 11, 95–100.
51 Wood, P.M. (1974) Biochim. Biophys. Acta 357, 370–379.
52 Marcus, R.A. (1964) Ann. Rev. Phys. Chem. 15, 155–196.
53 Henderson, R.W. and Rawlinson, W.A. (1956) Biochem. J. 62, 21–29.
54 Leitch, F.A., Moore, G.R. and Pettigrew, G.W. (1984) Biochemistry 23, 1831–1838.
55 Tint, H. and Reiss, W. (1950) J. Biol. Chem. 182, 385–396.
56 Flatmark, T. (1966) Acta Chem. Scan. 20, 1476–1486.
57 Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Kabai, M. and Okunuki, K. (1960) Biochem. J. 77, 194–201.