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Dimethylsulphoxide and trimethylamine-*N*-oxide as bacterial electron transport acceptors: use of nuclear magnetic resonance to assay and characterise the reductase system in *Rhodobacter capsulatus*

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Abstract. Nuclear magnetic resonance is established as a sensitive and specific method for following the reduction of dimethylsulphoxide and trimethylamine-N-oxide by bacteria. Using this method it has been shown that cells of *Rhodobacter capsulatus* reduce both dimethylsulphoxide and trimethylamine-N-oxide at linear rates at all concentrations of these acceptors that can be conveniently detected during a continuous assay. The rate of reduction of trimethylamine-N-oxide was eightfold higher than the rate of dimethylsulphoxide reduction. An upper limit of approximately 0.1 mM may be placed upon the apparent K_m value for each acceptor, but the value for dimethylsulphoxide is deduced to be lower than that for trimethylamine-N-oxide on the basis of the strong inhibitory effect of the former on the reduction of the latter. Reduction of trimethylamine-N-oxide by *Rb. capsulatus* was inhibited by illumination and by oxygen, but only the former effect was relieved following dissipation of the proton electrochemical gradient across the cytoplasmic membrane. Rotenone inhibited the reduction of trimethylamine-N-oxide whereas myxothiazol did not, consistent with a pathway of electrons to the reductase from NADH dehydrogenase that does not involve the cytochrome bc_1 complex.

Key words: *Rhodobacter capsulatus* – Nuclear magnetic resonance assay – Dimethyl sulphoxide – Dimethyl sulphide – Trimethylamine-*N*-oxide – Trimethylamine – Electron transport

The E^{01} values for reduction of trimethylamine-N-oxide (TMAO) to trimethylamine (TMA) and dimethylsulphoxide (DMSO) to dimethylsulphide (DMS) are 130 mV and 160 mV, respectively (Castell 1950; Wood 1981). This means that the energetics of reduction of TMAO or DMSO by NADH in cells are such that a proton translocating electron transport system can function to generate sizeable proton electrochemical gradients (McEwan et al. 1983). Thus, many bacteria (Barrett and Kwan 1985; Zinder and Brock 1978), including *Escherichia coli* (Bilous and Weiner 1985; Yamamoto and Ishimoto 1977) and *Rhodobacter capsulatus* (formerly *Rhodopseudomonas capsulata*) (Yen and Marrs 1977; Madigan and Gest 1978) are able to use these two electron acceptors to support growth under anaerobic conditions. In *Rb. capsulatus* a single enzyme, located in the periplasmic space, is responsible for the reduction of both TMAO and DMSO (McEwan et al. 1985b, 1987).

Available assays for TMAO and DMSO, or their reduction products, TMA and DMS, are not very convenient. TMA can be measured, but not at high sensitivity or without interference, either by complexation with picric acid (Dyer 1959) or by use of a modified ammonia electrode with solutions of pH greater than 9 (Chang et al. 1976). A third method for following the conversion of TMAO to TMA involves measurement of changes in solution conductance (Easter et al. 1982). The calculation of rates from such measurements can, however, be complicated (Owens et al. 1985). DMS can be measured by analysing with gas chromatography the gas space above a solution (Dickenson and Martin 1978).

In recent work we have shown that the rate of reduction of DMSO by *Rb. capsulatus* can be estimated from the duration of the membrane potential that is generated following addition of this electron acceptor to an anaerobic suspension of cells (Richardson et al. 1986). The membrane potential was monitored using the electrochromic shift of the endogenous carotenoid pigments. Such a method is clearly restricted to the photosynthetic bacteria, although in principle a specific cytochrome oxidation could serve the same purpose in other bacteria. Here we show that nuclear magnetic resonance (NMR) provides a direct and continuous method for assay of DMSO and TMAO reduction by cells of *Rb. capsulatus*. Use of this method has allowed several additional features of these reductions to be recognised.

Methods

Growth and harvesting procedures

Rb. capsulatus strain AD2 which possesses TMAO/DMSO reductase activity was obtained from Professor J. H. Klemme, Institut für Mikrobiologie der Universität Bonn, and used for all experiments. Precultures were routinely grown photoheterotrophically in 30 ml screw-capped McCartney bottles on RCV medium (Weaver et al. 1975) as described previously (Richardson et al. 1986). Cells for experimental use were grown photoheterotrophically on RCV medium supplemented with 20 mM TMAO and harvested at a late exponential phase of growth. Harvesting

Abbreviations. DMS, dimethyl sulphide; DMSO, dimethyl sulphoxide; DSS, 3-(trimethylsilyl)-1-propane-sulphonic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; TMA, trimethylamine; TMAO, trimethylamine-N-oxide

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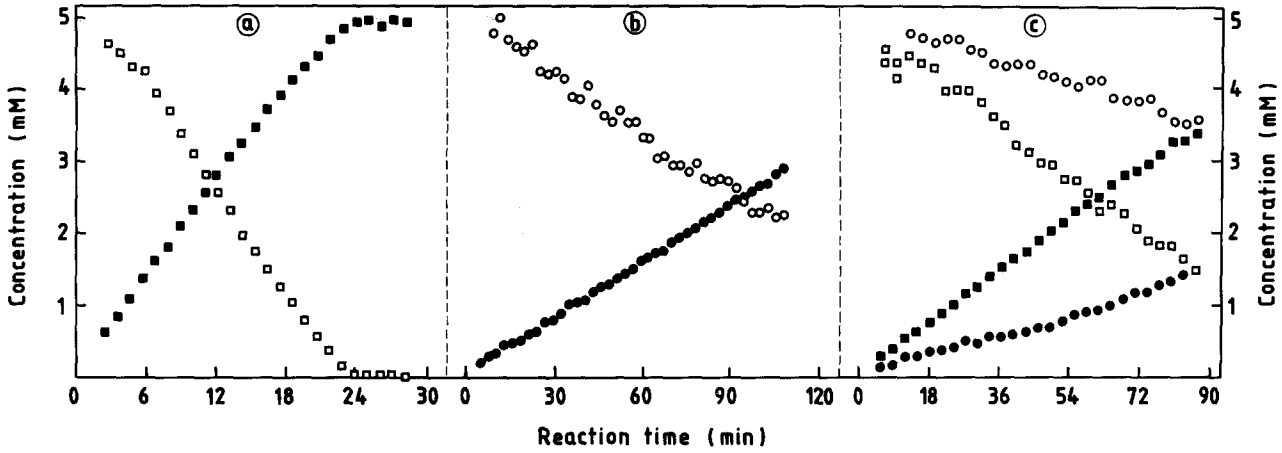

Fig. 1a–c. Time courses of TMAO and DMSO reduction together with TMA and DMS production by cells of *Rb. capsulatus* (260 μM bacteriochlorophyll): (**a**) Reduction of 5 mM TMAO alone (□——□, TMAO; ■——■, TMA); (**b**) Reduction of 5 mM DMSO alone (○——○, DMSO; ●——●, DMS); (**c**) Reduction of a mixture of 5 mM DMSO and 5 mM TMAO (□——□, TMAO; ○——○, DMSO; ■——■, TMA; ●——●, DMS). Note that the time scale is different in each of the three plots

and washing of cells was carried out according to McEwan et al. (1983). The bacteria were finally resuspended in RCV medium in which the malate concentration had been reduced to 1 mM to minimise interference of ^{1}H NMR signals from malate with ^{1}H signals to be monitored. The dense suspension of cells was stored on ice and used for experiments within 14 h. Bacteriochlorophyll concentration was measured as described previously (Clayton 1963).

Preparation of NMR samples

Rates of TMAO and/or DMSO reduction were measured as follows: 10–100 μl of the resuspended cell culture was added to a 5 mm o.d. NMR tube. Sufficient RCV medium containing 1 mM malate was then added to the suspension such that after addition of the appropriate amount of TMAO and/or DMSO (taken as zero reaction time) the final volume was 500 μl. TMAO and DMSO were added from concentrated stock solutions in the RCV medium. Myxothiazol and rotenone were added from stock solutions in deuterated methanol and FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) was added as a solution in ethanol. For study of the effects of illumination an NMR tube containing the cell suspension together with TMAO was placed in a water bath (30 °C) at a distance of 4 cm from a 250 W actinic light source that was focussed and passed through a Wratten 88A filter onto the sample.

NMR methods

^{1}H NMR spectra were acquired at 300 MHz using a Bruker WM300 spectrometer operated at 30 °C in the pulsed Fourier transform mode. A short period (30 s) was allowed for thermal equilibration of the sample within the probe before commencing acquisition of spectra.

Spectra were the result of 32–128 transients averaged into 8192 data locations using a sweep width of 4 kHz. Transients were obtained using a simple $\pi/2$ pulse ($\sim$ 5 μs) preceded by 500 ms of selective irradiation at the resonance frequency of water; the transient repetition time was 1.524 s.

DSS (0.25% in ^2H_2O), which was present in a small coaxial capillary within the NMR tube, was used as the chemical shift reference at 0.000 ppm. The ^2H_2O in the capillary was used for field/frequency locking. Sample tubes were spun at 20 Hz to average out magnetic field inhomogeneities. At this speed there was no significant tendency of cells to sediment in the sample tube.

Free induction decays were multiplied by a decaying exponential with a line-broadening factor of 0.5 Hz before Fourier transformation. Concentrations of products and reactants in time courses of TMAO and DMSO reduction were obtained from the relevant NMR peak intensities using the extinction coefficient method described previously (Vandenberg et al. 1986). For kinetic analysis of spectral data, the reaction-time was taken as the mid-point of each spectral accumulation.

Results

DMSO, DMS, TMAO and TMA are ideally suited for analysis by NMR because in each molecule there are either six or nine equivalent, non-coupled protons. Thus a singlet spectrum is observed in each case. The four singlets from these compounds are resolved from one another (TMAO, 3.261 ppm; TMA, 2.891 ppm; DMSO, 2.724 ppm; DMS, 2.106 ppm).

The time course of reduction of TMAO and appearance of TMA in a suspension of *Rb. capsulatus* in growth medium was readily followed by NMR (Fig. 1a). Similar results were obtained for conversion of DMSO to DMS (Fig. 1b), although in this case the DMSO signal was partially obscured by a resonance from malate, the carbon source for growth. The apparent delay at the beginning of some time courses is attributed to the period required for complete temperature equilibration of the samples. Comparison of Fig. 1a and 1b shows that DMSO was reduced at a rate of 0.09 μmol (μmol bacteriochlorophyll)$^{-1}$ (min)$^{-1}$. This is 12% of the rate for TMAO reduction which was 0.76 μmol (μmol bacteriochlorophyll)$^{-1}$ (min)$^{-1}$. When the two substrates were present together, the rates of reduction of each could be monitored simultaneously, both from the disappearance of TMAO and DMSO and the appearance of TMA and DMS (Fig. 1c). It is evident that the rate of

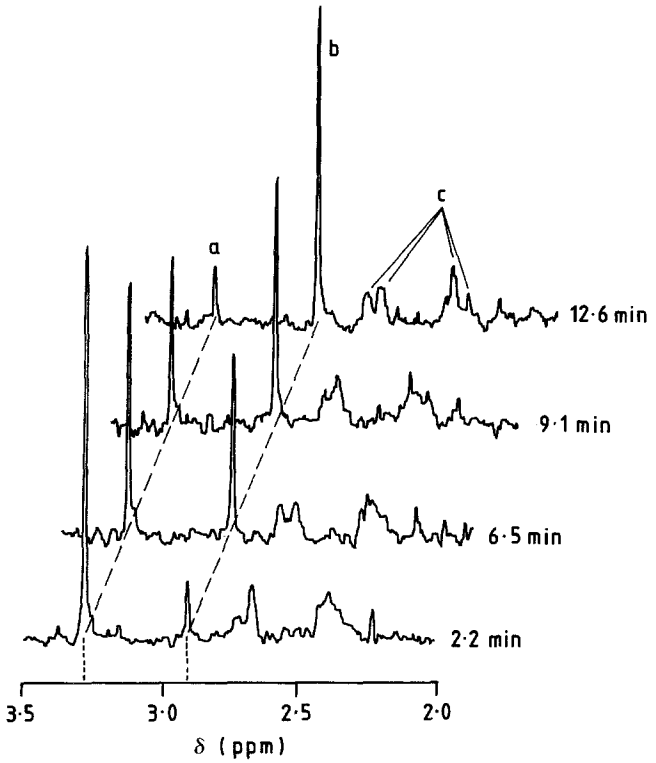

Fig. 2. ^{1}H NMR spectra of a suspension of *Rb. capsulatus* (50 μM bacteriochlorophyll) to which TMAO (final concentration 0.5 mM) was added at zero time. Spectral assignments: *a* TMAO methyl protons; *b* TMA methyl protons; *c* malate c-2 and c-3 protons. Each spectrum represents the average of 32 transients acquired over approximately 50 s; other experimental conditions as in Methods section

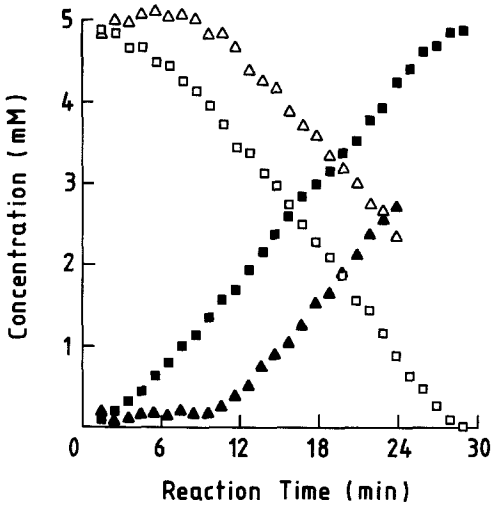

Fig. 3. Inhibition by oxygen of the reduction of TMAO to TMA catalysed by cells of *Rb. capsulatus* (60 μM bacteriochlorophyll). □——□ and ■——■ represent TMAO and TMA concentrations in a control experiment. △——△ and ▲——▲ represent TMAO and TMA concentrations in an experiment in which 0.5 mM H_2O_2 (from which O_2 was generated by endogenous catalase) was added simultaneously with the TMAO

reduction of TMAO [now 0.16 μmol (μmol bacteriochlorophyll)$^{-1}$ (min)$^{-1}$] was strongly inhibited in the presence of DMSO. The reduction rate of the DMSO [0.06 μmol (μmol bacteriochlorophyll)$^{-1}$ (min)$^{-1}$] was less inhibited by the presence of TMAO so that the total rate of reduction of both substrates was intermediate between that for TMAO or DMSO alone.

The experiments shown in Fig. 1 were done with initial concentrations of 5 mM for both TMAO and DMSO which permitted acquisition of data with short accumulation times. By decreasing the quantity of the cells used in the assay, it was also possible to follow the reduction of TMAO or DMSO when the initial concentrations were lower. For example, when the initial concentration of TMAO was 0.5 mM the sequential spectra shown in Fig. 2 were obtained. When the data were plotted in the form shown in Fig. 1, a linear rate of TMAO reduction was evident down to the lowest concentration of TMAO that could be observed. Linearity of the reduction rate was also observed in similar experiments using initial concentrations of either 0.2 mM TMAO or 0.5 mM DMSO. 0.2 mM TMAO represents an approximate lower limit to the initial concentration of substrate that can be studied by the NMR continuous assay procedure. However, considerably lower concentrations can be detected in extracts made from cell suspensions. 0.01 mM TMAO was detected after accumulation of transients for 3 h.

Electron transport to either nitrate (McEwan et al. 1984) or nitrous oxide (McEwan et al. 1985) is blocked in *Rb. capsulatus* by the presence of oxygen. A similar inhibition was observed for electron flow to TMAO (Fig. 3). Assuming complete reduction of the oxygen generated by catalase activity during the period when reduction of TMAO was inhibited (Fig. 3) a rate of oxygen reduction of 0.9 μmol O_2 (μmol bacteriochlorophyll)$^{-1}$ (min)$^{-1}$ can be calculated. This is typical for cells of this strain of *Rb. capsulatus* (Richardson et al. 1986). Inhibition of electron transport to either nitrous oxide (McEwan et al. 1985a) or nitrate (McEwan et al. 1984) by oxygen is independent of the proton electrochemical gradient across the cytoplasmic membrane. The same was found to be the case for electron transport to TMAO; in experiments similar to those shown in Fig. 3 the presence of 40 μM FCCP did not alter the inhibition caused by oxygen or the rate of TMAO reduction catalysed under anaerobic conditions by the cells at a bacteriochlorophyll concentration of 300 μM.

In contrast to the inhibition by oxygen, the inhibitory effect of illumination upon electron transport to nitrous oxide (McEwan et al. 1985a) or nitrate (McEwan et al. 1984) is relieved when the proton electrochemical gradient is dissipated by protonophores. Electron transport to TMAO was shown to follow a similar pattern.

The lack of suitable apparatus for illuminating the suspensions of cells inside the NMR spectrometer meant that the rate of TMAO reduction by cells in the light was estimated in the following way. A sample of cells contained in an NMR sample tube was illuminated (see Methods) for 10 min and then transferred to the dark conditions inside the spectrometer. The subsequently recorded NMR signals (Fig. 4a) gave both the initial concentrations of TMAO and TMA immediately after the transfer, together with the ensuing rate of conversion of TMAO to TMA. Comparison of the data obtained in such an experiment with that from an experiment in which the cells were placed in the spectrometer without the period of illumination shows that the rate of TMAO reduction was inhibited by 70% during the period of illumination (Fig. 4a). A similar experimental procedure was used to show that in the presence of FCCP the inhibition by light was relieved (Fig. 4b).

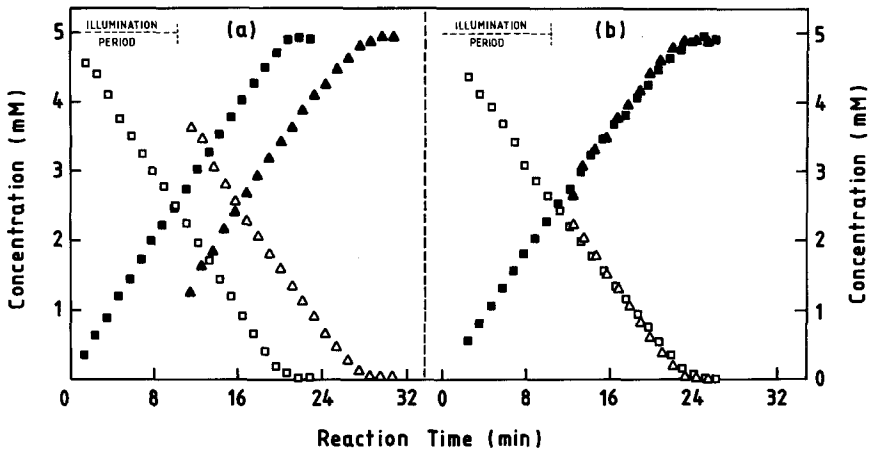

Fig. 4a, b. Inhibition by illumination of the reduction of TMAO by cells of *Rb. capsulatus* (300 μM bacteriochlorophyll). **(a)** □——□ and ■——■ represent TMAO and TMA concentrations under dark conditions (inside the spectrometer). △——△ and ▲——▲ represent TMAO and TMA concentrations in an experiment in which the cells were illuminated as indicated for 10 min from zero time before being placed in the NMR spectrometer. **(b)** □——□ and ■——■ represent TMAO and TMA concentrations under dark conditions in the presence of 40 μM FCCP; △——△ and ▲——▲ represent TMAO and TMA concentrations in an experiment in which the cells were illuminated in the presence of 40 μM FCCP for 10 min from zero time before being placed in the NMR spectrometer

By use of an indirect method for assay of electron transport to the DMSO/TMAO reductase of *Rb. capsulatus*, evidence has been obtained that whereas rotenone is an inhibitor, myxothiazol is without effect (McEwan et al. 1983; Richardson et al. 1986). This evidence was supported by NMR experiments with which it was shown that rotenone (40 μM preincubated for 5 min with cells, 60 μM bacteriochlorophyll) caused almost complete inhibition of TMAO reduction under conditions that were otherwise similar to those used in Fig. 1a. Myxothiazol (10 μM preincubated for 5 min with cells, 60 μM bacteriochlorophyll) caused less than 10% inhibition, again under otherwise similar conditions to those shown in Fig. 1a.

Discussion

The results presented establish that NMR provides the best available assay for measuring the reduction by bacteria of TMAO or DMSO to TMA or DMS as well as the simultaneous reduction of both oxidants. The essential requirement of the NMR spectrometer is the capacity for suppression of the water resonance. The lower detection limit for TMAO in samples is approximately 0.01 mM which is significantly below that for other methods. Interference from other molecules will occur only rarely, as the methyl protons of TMAO have a characteristic chemical shift owing to their proximity to the uncommon N-oxide group. Similar considerations apply to DMSO, although in this case the chemical shift positions can overlap with those of common biological chemicals (e.g., malate). It is also suggested that NMR can be adopted as the method of analysis wherever TMAO, TMA, DMSO or DMS need to be determined in aqueous solution. Such information is needed not just in studies of bacterial metabolism but also in the food industry where TMA is a spoilage agent and in brewing where reduction of DMSO by yeasts can be significant. The approach outlined here might also be expected to find application in the study of organisms and their enzymes that oxidise either trimethylamine (Anthony 1982) or dimethylsulphide (de Bont et al. 1981) as well as related compounds including acetone (Taylor et al. 1980).

The linearity of the rates of reduction of both DMSO and TMAO even when the initial concentrations of these species were reduced to 0.5 mM or less (e.g., Fig. 2) implies that the apparent K_m values for these two substrates are less than approximately 0.1 mM. This is in agreement with the sharp end point to the duration of the carotenoid shift that was observed in experiments using DMSO as electron acceptor (Richardson et al. 1986). Use of non-physiological electron donors has suggested K_m values for DMSO and TMAO of 0.17 mM and 0.89 mM for reductases from *E. coli* (Bilous and Weiner 1985) and *Salmonella typhimurium* (Kwan and Barrett 1983). These values are higher than those indicated by the NMR method in which physiological reductants are used. The possibility of obtaining with non-physiological reductants K_m values that are misleadingly high has recently been illustrated by work with the respiratory nitrate reductases from both *E. coli* (Morpeth and Boxer 1985) and *Paracoccus denitrificans* (Craske and Ferguson 1986).

In previous work several lines of evidence indicated that a single enzyme in the periplasm of *Rb. capsulatus* is responsible for the reduction of TMAO and DMSO (McEwan et al. 1985b, 1987). Owing to the low apparent K_m values for TMAO and DMSO reduction, these substrates were saturating at the 5 mM concentrations used for the experiments of Fig. 1a, b. The rates of reduction calculated from those experiments thus yield the apparent relative k_{cat} (V_{max}) values, and that for TMAO is approximately eight-fold higher than the value for DMSO. The term "apparent" is used because the enzyme activity was measured under conditions in which the enzyme acted as the terminal component of a sequence of reactions. The data in Fig. 1c show that when the cells were presented with both TMAO and DMSO, the reduction of TMAO was markedly inhibited compared with the change in the rate of DMSO reduction. This result can be understood in terms of two substrates competing for a single enzyme. In general, the ratio of rates in such circumstances will be given (Fersht 1985):

$$v_{TMAO}/v_{DMSO} = (k_{cat}/K_m)_{TMAO}\,[TMAO]/(k_{cat}/K_m)_{DMSO}\,[DMSO],$$

where v_{TMAO} and v_{DMSO} are the rates of reduction of TMAO and DMSO when both substrates are present together and where the square brackets denote concentrations. As the initial concentrations of TMAO and DMSO were equal in the experiment shown in Fig. 1c, and k_{cat} (apparent) is greater for TMAO than for DMSO by a factor of eight (Fig. 1a and b), then the ratio of rates ($v_{TMAO}/v_{DMSO} = 2.5$) observed with both substrates present can be accounted for if the apparent K_m for DMSO is approximately three-fold lower than K_m apparent for TMAO. The results shown in Fig. 1c are thus explicable in terms of a single enzyme that can use both substrates, rather than two distinct enzymes for the two substrates. If the latter case were to apply here, one should expect inhibition of DMSO reduction by TMAO to be more marked as a result of a mechanism based upon competition for available electrons.

The observation that the inhibition of reduction of TMAO by illumination was prevented by addition of a protonophore to collapse the proton electrochemical gradient, implies that the inhibitory effect upon reduction of TMAO was exerted through this gradient exerting a thermodynamic backpressure upon respiration. Such a mechanism has been proposed previously for the inhibitory effects of light upon reduction of nitrate and nitrous oxide (McEwan et al. 1984, 1985; Ferguson et al. 1987). On the other hand, the inhibition of TMAO reduction by oxygen was not reversed by a protonophore, again resembling earlier observations on the oxygen-inhibition of nitrate reduction (McEwan et al. 1984). The exact mechanism through which oxygen inhibits these reactions is not known.

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