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The location of dissimilatory nitrite reductase and the control of dissimilatory nitrate reductase by oxygen in *Paracoccus denitrificans*

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1. A method is described for preparing spheroplasts from *Paracoccus denitrificans* that are substantially depleted of dissimilatory nitrite reductase (cytochrome *cd*) activity. Treatment of cells with lysozyme + EDTA together with a mild osmotic shock, followed by centrifugation, yielded a pellet of spheroplasts and a supernatant that contained *d*-type cytochrome. The spheroplasts were judged to have retained an intact plasma membrane on the basis that less than 1% of the activity of a cytoplasmic marker protein, malate dehydrogenase, was released from the spheroplasts. In addition to a low activity towards added nitrite, the suspension of spheroplasts accumulated the nitrite that was produced by respiratory chain-linked reduction of nitrate. It is concluded that nitrite reduction occurs at the periplasmic side of the plasma membrane irrespective of whether nitrite is generated by nitrate reduction or is added exogenously. 2. Further evidence for the integrity of the spheroplasts was that nitrate reduction was inhibited by O_2, and that chlorate was reduced at a markedly lower rate than nitrate. These data are taken as evidence for an intact plasma membrane because it was shown that cells acquire the capability to reduce nitrate under aerobic conditions after addition of low amounts of Triton X-100 which, with the same titre, also overcame the permeability barrier to chlorate reduction by intact cells. The close relationship between the appearance of chlorate reduction and the loss of the inhibitory effect of O_2 on nitrate reduction also suggests that the latter feature of nitrate respiration is due to a control on the accessibility of nitrate to its reductase rather than on the flow of electrons to nitrate reductase.

An understanding of the generation of a protonmotive force by electron transfer chains requires a knowledge of the orientation of the various components of the chain with respect to their membrane so that sites of proton translocation, production and consumption can be identified. This information is of additional importance when the terminal electron acceptor is charged, as for instance during dissimilatory reduction of nitrate or nitrite by denitrifying bacteria. Movement of these ions across the plasma membrane must be understood in relation to the membrane potential component of the protonmotive force which would tend to exclude negatively charged ions from the cell.

In the case of the bacterium *Paracoccus denitrificans* there is evidence (John, 1977; P. John, personal communication; Kristjansson *et al.*, 1978), albeit of a relatively indirect kind, that nitrate is reduced at the inner side of the plasma membrane, and thus nitrate must gain entry to the cell against an adverse membrane potential (negative inside). The location of dissimilatory nitrite reductase activity in *P. denitrificans* has been assigned to the periplasmic side of the plasma membrane by Meijer *et al.* (1979) who studied the stoichiometry of proton consumption associated with the flow of electrons from ascorbate to nitrite. On the other hand, Kristjansson *et al.* (1978) were led by their studies on proton movements after addition to cells of pulses of nitrate or nitrite to conclude that nitrite is reduced inside the cell. A periplasmic site for nitrite reduction would imply that nitrite produced inside the cell by the reduction of nitrate must be transported out of the cell, and that overall reduction of nitrate to N_2 need not involve any net transfer of charge across the membrane as a result of the movement of inorganic anions, which act as electron acceptors. An internal location for reduction of both nitrite and nitrate means that the transfer of one negative charge into the cell must be compensated for during

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the overall conversion of nitrate to an uncharged gaseous product.

As nitrite reductase, cytochrome *cd*, from *P. denitrificans* is known to be a water-soluble protein that is absent from membrane vesicles (Newton, 1969; John & Whatley, 1970), a more direct approach to determining the location of this reductase would be to examine whether the enzyme is released during preparation of spheroplasts (Beacham, 1979). The present paper is concerned with experiments of this design which were carried out with *P. denitrificans* to resolve the conflicting experimental evidence on the location of this enzyme. This experimental approach has already been used by Wood (1978), who determined that the respiratory nitrite reductase of *Pseudomonas aeruginosa* has a periplasmic location, although work with ferritin-labelled antibodies led Saraste & Kuronen (1978) to suggest that the enzyme is found on the inner surface of the plasma membrane of that organism. Unfortunately the results of Wood (1978) cannot automatically be extrapolated to other organisms, as identical locations cannot be guaranteed for similar respiratory enzymes in different organisms as exemplified by the evidence suggesting that nitrate reductase is found only on the cytoplasmic side of the *Bacillus licheniformis* membrane but is a transmembrane protein in *Klebsiella aerogenes* (Wientjes *et al.*, 1979).

A characteristic feature of denitrifying bacteria is that the reduction of nitrate is very strongly inhibited by O_2 (John, 1977 and refs. therein). The mechanism of this inhibitory effect of O_2 is not known, but we show in the present paper that the control on nitrate reduction is retained by spheroplasts and may be associated with an inhibitory effect of O_2 on the accessibility of nitrate to its reductase on the cytoplasmic side of the plasma membrane.

Materials and methods

Growth of cells and preparation of spheroplasts

P. denitrificans N.C.I.B. 8944 was grown in a 2-litre batch culture with succinate as substrate and nitrate as the terminal electron acceptor as described by Burnell *et al.* (1975). The cells were harvested at the late logarithmic stage of growth (A_{550} 1.6–1.7 in a 1cm pathlength cuvette in a Unicam SP.500 spectrophotometer) by centrifugation for 20min at 4°C and 5000*g* (4100rev./min in the 6 × 1 litre rotor of an MSE Coolspin centrifuge), and washed once with 800ml of cold (4°C) 150mM-NaCl/10mM-Tris/HCl, pH7.3, and then suspended in approx. 10ml of 0.5M-sucrose/10mM-Tris/HCl, pH7.3. Half of this suspension was diluted to approx. 400ml with the same medium and used for the preparation of type-1 spheroplasts. In this procedure, following the method of John & Whatley (1970), 100mg of hen egg-white lysozyme dissolved in approx. 2ml of water was added and the suspension of cells was incubated at 30°C. Periodically spheroplast formation was ascertained (checked) by adding a 0.1ml sample to 2.4ml of water and monitoring the decrease in A_{550} due to swelling. When the absorbance dropped from approx. 0.15 to 0.05 in 10s or less the whole suspension was centrifuged at 4°C and 37000*g* (18000rev./min in the 8 × 50ml rotor of an MSE High Speed 18 centrifuge) for 10min and the spheroplasts were resuspended in approx. 5ml of 150mM-KCl/200mM-sucrose/20mM-Tris/acetate (pH7.3)/10mM-magnesium acetate.

The second half of the original cell suspension was used for the preparation of type-2 spheroplasts by using a method based on that of Witholt *et al.* (1976). The cell suspension was added to 200ml of 200mM-Tris/HCl (pH7.3)/0.5M-sucrose/0.5mM-EDTA (disodium salt) and 50mg of lysozyme dissolved in a little water was added. A mild osmotic shock was administered by the addition of 200ml of water and the suspension was incubated at 30°C for 40min. Spheroplasts were then centrifuged and resuspended in the same way as for type-1 spheroplasts. The type-1 spheroplasts exhibited no stickiness and had a similar coffee-brown colour to the whole cells whereas in comparison the type-2 spheroplasts had a pink tinge and the suspension was a little more viscous.

Concentration of the supernatant from the spheroplast preparation

The supernatants from the centrifugation of the spheroplasts were dialysed for 18h against 2 × 4 litres of 20mM-potassium phosphate buffer, pH7.3, at 4°C. A portion (100ml) of each type of supernatant was concentrated with an Amicon ultrafiltration apparatus fitted with a P30 membrane which retains species of mol.wt. above 30000.

Preparation of spheroplast lysates

A portion (2ml) of each type of spheroplast suspension was added to 18ml of water at room temperature. A trace of DNAase was added and, after approx. 25min, the suspension was centrifuged at 40000*g* for 40min. The supernatant (the lysate) was retained and the pellet was resuspended in approx. 5ml of the spheroplast resuspension medium.

Nitrite reductase assay

This was done by using a colorimetric assay for nitrite (Coleman *et al.*, 1978) in which 50μl aliquots of a reaction mixture (specified in the legends to the Tables and Figures) were periodically added to 3.7ml of 1% sulphanilimide in 1M-HCl. *N*-(1-Naphthyl)ethylenediamine dihydrochloride (0.3ml

of 0.02%) was added and after 20min at room temperature (20°C) the A_{540} was determined. The same procedure was also followed with a reaction mixture that lacked nitrite and the value for A_{540} obtained in this case was taken as a blank value. A calibration curve showed that the assay was linear for up to 1mM-nitrite in the aliquot.

Nitrate reductase assay

Changes in the concentration of nitrate were followed by using an Orion nitrate electrode (model 93-07) together with an Orion double-junction reference electrode (model 92-02). The output from the electrodes was measured with an Orion 701A pH/mV meter and displayed on a strip chart recorder. The same procedure was used for determining the concentration of chlorate, as the nitrate electrode is sensitive to chlorate (John, 1977). Measurements were done at room temperature.

Malate dehydrogenase assay

The reaction mixture contained the sample to be assayed in 100mM-potassium phosphate, pH 7.5, 0.2mM-oxaloacetic acid and 0.27mM-NADH; the decrease in A_{340} was measured (Markwell & Lascelles, 1978). An assay without oxaloacetic acid was also carried out so as to allow a correction for NADH oxidation via other reactions. In practice this correction was only significant in assays containing lysed membranes, which are expected to have NADH dehydrogenase activity.

Other determinations

d-Type cytochrome was measured as its pyridine–haemochrome (Newton, 1969). The Folin–Ciocalteau method of Lowry *et al.* (1951) was used for the determination of protein, with bovine serum albumin as standard.

Reagents

Horse heart cytochrome *c* was obtained from Boehringer (Lewes, East Sussex BN7 1LG, U.K.) and egg white lysozyme (grade 1) was purchased from the Sigma Chemical Co. (Poole, Dorset BH17 7NH, U.K.). All other reagents were of the highest grades commercially available.

Results

Nitrite reductase activity in spheroplasts

Type-1 spheroplasts retained appreciable nitrite reductase activity with either of two substrates tested (Fig. 1), but this does not rule out a periplasmic location for the enzyme because lysozyme treatment of exponential-phase cells of *P. denitrificans*, although causing osmotic instability (John & Whatley, 1970), does not result in any change of the

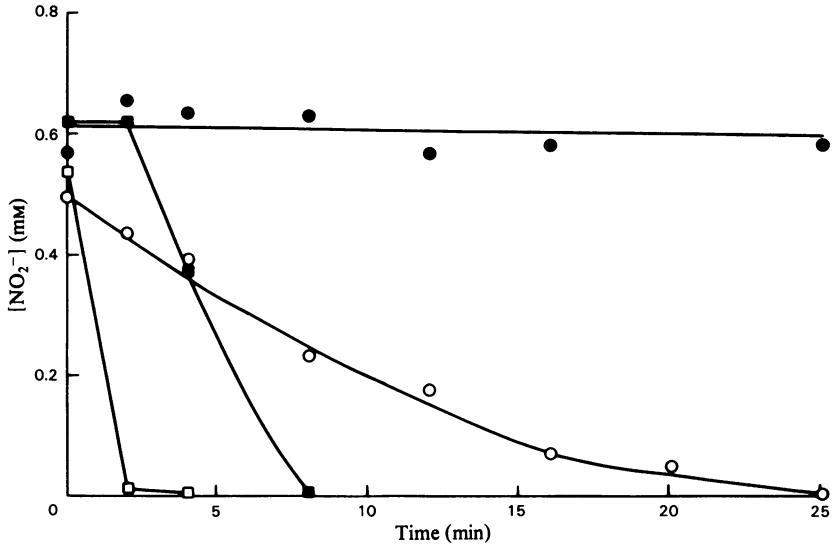

Fig. 1. *Comparison of nitrite reductase activities of type-1 and type-2 spheroplasts*

The reaction mixture contained 200mM-sucrose, 20mM-Tris/acetate, pH 7.3, 150mM-KCl, 10mM-magnesium acetate, 100μM-horse heart cytochrome *c* and 0.66mM-$NaNO_2$ in a total volume of 1ml. Additional components were: ●, 1mg of protein from type-2 spheroplasts + 10mM-sodium succinate; ■, 1.2mg of protein from type-1 spheroplasts + 10mM-sodium succinate; ○, 1.0mg of protein from type-2 spheroplasts + 10mM-sodium D-isoascorbate and 100μM-phenazine methosulphate; □, 1.2mg of protein from type-1 spheroplasts + 10mM-sodium D-isoascorbate and 100μM-phenazine methosulphate.

gross cell-wall structure (Scholes & Smith, 1968). On the basis that substantial loss of periplasmic proteins can be expected only when the cell wall is relatively extensively disrupted, an alternative preparation of spheroplasts was sought.

A very effective method for deriving spheroplasts from cells of Gram-negative bacteria has been introduced by Witholt *et al.* (1976). Essentially by this method, but with the modification of a 40min incubation with a greater amount of lysozyme than specified by Witholt *et al.* (1976), type-2 spheroplasts were prepared in which the nitrite reductase activity was severely depleted relative to type-1 spheroplasts (Fig. 1). A longer incubation with lysozyme led to further loss of nitrite reductase activity but the resulting spheroplast suspensions rapidly became too viscous for experimental use. Thus a 40min incubation was selected as the best compromise between maximizing loss of nitrite reductase activity and minimising the spontaneous lysis of the spheroplasts.

The conclusion that the type-2 spheroplasts are depleted in nitrite reductase activity would be invalid if the spheroplasts had lost one or more other components (e.g. cytochrome *c*) of the respiratory chain that are required to mediate electron flow between the substrates and nitrite reductase. It is known, however, that reduced phenazine methosulphate can directly reduce nitrite reductases of the cytochrome *cd* type (Robinson *et al.*, 1979; Zumft *et al.*, 1979), and so the use of ascorbate + phenazine methosulphate as substrate ought to provide a reliable assay for the nitrite reductase. As phenazine methosulphate can reduce horse heart cytochrome *c*, which can in turn act as an effective reductant for nitrite reductase of *P. denitrificans* (Robinson *et al.*, 1979), any failure of phenazine methosulphate to react directly with nitrite reductase should have been reflected by a stimulation of the rate of the nitrite reduction after adding horse heart cytochrome *c* to replace any absent endogenous cytochrome *c*. Although no such stimulation was observed, horse heart cytochrome *c* was routinely added to the assays of all spheroplasts. When succinate was substrate some stimulation by cytochrome *c*, approx. 20%, was observed with type-2 spheroplasts, although whether this was due to the added cytochrome *c* replacing endogenous cytochrome *c* is not known.

Evidence that type-2 spheroplasts had not lost other respiratory chain components in significant amounts was that the two types of spheroplast preparation catalysed very similar rates of electron flow to oxygen irrespective of whether succinate or ascorbate + *NNN'N'*-tetramethyl-*p*-phenylenediamine was the substrate. The result with the latter substrate is of particular interest, as it is believed that *NNN'N'*-tetramethyl-*p*-phenylenediamine is only an effective donor of electrons to *c*-type cytochromes (Willison & John, 1979; Sorgato & Ferguson, 1979). Hence the finding of similar respiration rates for both types of spheroplasts with ascorbate + *NNN'N'*-tetramethyl-*p*-phenylenediamine suggests that their cytochrome *c* content does not markedly differ, and this is consistent with the recent suggestion that cytochrome *c* of *P. denitrificans* is relatively firmly attached to the plasma membrane (Erecinska *et al.*, 1979).

If type-2 spheroplasts are considerably depleted of nitrite reductase then the supernatant collected after centrifugation of these spheroplasts would be expected to be relatively enriched in this activity. Table 1 shows that such a supernatant from type-2 spheroplasts had a much higher cytochrome *d* content, measured as a pyridine–haemochrome, than the corresponding supernatant from the type-1 spheroplast preparation. An inconsistency arose when the nitrite reductase activity of the two

Table 1. *Location of nitrite reductase in spheroplasts and extracts of P. denitrificans*

The type-1 and type-2 spheroplasts were prepared from a single batch of cells. The quantities given are those associated with the entire preparations of spheroplasts. Preparations contained 102 mg of protein (type-1) or 72.4 mg of protein (type-2). The assays of extracts were done under anaerobic conditions. A unit of activity is the reduction of 1 μmol of nitrite/min. Abbreviations: n.t., not tested; n.d., not detectable.

Spheroplast type ...	1		2	
	Nitrite reductase (units)	Pyridine haem (nmol)	Nitrite reductase (units)	Pyridine haem (nmol)
Spheroplasts	20.6	n.t.	1.9	n.t.
Supernatant from spheroplast preparation	1.3	3.75	1.7	27.3
Released upon lysing spheroplasts	9.4	16.4	n.d.	n.d.
Associated with pellet after lysis of spheroplasts	2.1	n.t.	n.d.	n.t.

supernatants was compared (Table 1), as, although a higher activity was always detected in the type-2 supernatant, the relative activities of the two types of supernatant were much closer than might have been expected from the relative *d*-type cytochrome contents. This result could be interpreted to mean that not all the *d*-type haem detected as a pyridine–haemochrome was derived from nitrite reductase. However, as there is no evidence for any other *d*-type cytochrome in anaerobically grown cells of *P. denitrificans* (John & Whatley, 1977), it seems probable that the low enzyme activity found in the supernatant from type-2 spheroplasts is a consequence of inactivation during the procedure for concentrating the supernatant. The concentrated supernatant from the type-2 spheroplast preparation was opalescent, whereas that from type-1 spheroplasts was clear, and this may be related to a loss of activity in the supernatant from type-2 spheroplasts.

When type-1 spheroplasts were lysed, readily detectable amounts of nitrite reductase were released, as shown both by the *d*-type pyridine–haemochrome determination and by enzymic assay (Table 1), whereas similar treatment of type-2 spheroplasts did not release nitrite reductase that was detectable by either procedure. This result confirms that the type-2 spheroplasts are greatly depleted of the cytochrome *cd*-type nitrite reductase. Nitrite reductase is absent from membrane vesicles (John & Whatley, 1970) and so is expected to be released irrespective of its cellular location upon exposing lysozyme-treated cells to osmotic shock.

Some nitrite reductase activity was found in the pellets after lysis of type-1 spheroplasts (Table 1) and this probably represents non-specifically attached cytochrome *cd*, or activity associated with unlysed cells. The total activity detected after lysing type-1 spheroplasts was less than that measured in the original intact spheroplasts, but this may mean that the enzyme loses activity after release from the spheroplasts under our conditions.

As nitrite is evidently produced by nitrate reductase inside the cell (John, 1977), the question arose as to whether nitrite reductase might have a dual location with some of the enzyme being located on the inner side of the plasma membrane. Although this possibility was unlikely in view of the absence of detectable nitrite reductase activity released upon lysing the type-2 spheroplasts (Table 1), the experiments shown in Fig. 2 were done as a further check on this point. In an assay of nitrate reduction with succinate as substrate type-2 spheroplasts almost quantitatively produced nitrite whereas a similar number of type-1 spheroplasts produced very much less nitrite (Fig. 2). The nitrate reductase activities of the two types of spheroplasts were almost identical (Fig. 3*a*), and so it is concluded that the loss of nitrite reductase activity from type-2

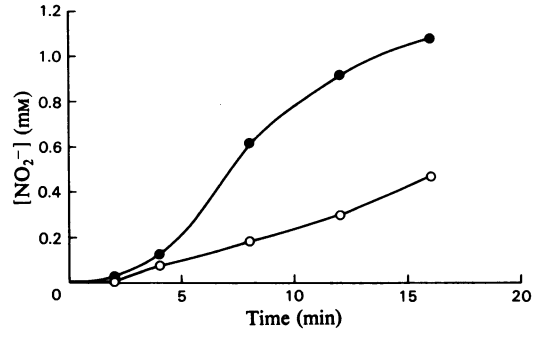

Fig. 2. *Conversion of nitrate into nitrite by the two types of spheroplast*

Type-1 (○, 1.1 mg of protein) or type-2 (●, 0.85 mg of protein) spheroplasts were added to a reaction mixture that contained, in a final volume of 1 ml: 200 mM-sucrose, 20 mM-Tris/acetate, pH 7.3, 10 mM-magnesium acetate, 150 mM-KCl, 100 μM-horse heart cytochrome *c* and 1.2 mM-KNO_3. At the times indicated 0.05 ml aliquots of the reaction mixture were analysed for nitrite as described in the Materials and methods section.

spheroplasts is correlated not only with the loss of activity towards added nitrite but also towards nitrite produced by the reduction of nitrate. It has also been reported that less than 10% of adeed nitrate is recovered as nitrite from respiring anaerobic cells (John, 1977), and thus the accumulation of nitrite in the suspension of type-2 spheroplasts is taken as evidence that there is no functional nitrite reductase on the cytoplasmic side of the plasma membrane.

Evidence for intactness of spheroplasts

Malate dehydrogenase was used as a marker for the leakage of cytoplasmic proteins from spheroplasts (Markwell & Lascelles, 1978). Very little of this enzyme activity was released during preparation of either type of spheroplast, but virtually all of the total detectable activity was released after osmotic shock of the spheroplasts (Table 2). The smaller amount of malate dehydrogenase activity released from type-1 spheroplasts might reflect incomplete lysis of this preparation in which the cell wall has been weakened to a lesser extent. Only a very small fraction of the total activity was retained in the pellet after lysis, and presumably this arose from enzyme that was trapped in, or adsorbed to, the pellet. The data (Table 2) thus show that, unless the dehydrogenase is severely inactivated in the supernatant fraction, the plasma membranes of the spheroplasts have not been rendered permeable to cytoplasmic

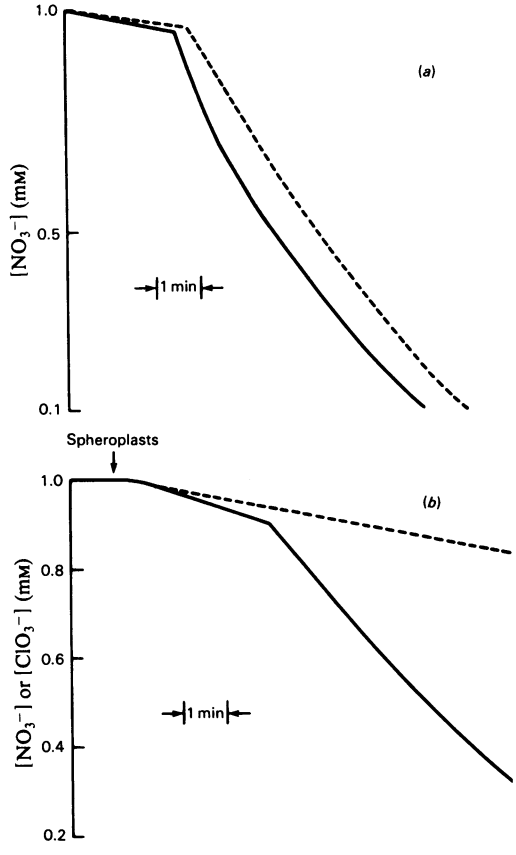

Fig. 3. *Assay of nitrate and chlorate reduction by spheroplasts of P. denitrificans*

(*a*) Type-1 (----) or type-2 (——) spheroplasts (0.5ml; 12.5 and 10mg of protein respectively) were added to 20ml of 450mM-sucrose/25mM-KCl/20mM-Tris/acetate (pH 7.3)/10mM-magnesium acetate/10mM-succinate/1mM-KNO_3. The point at which the nitrate reduction sharply increased corresponded closely to the time at which anaerobiosis was reached as judged by a parallel experiment in which the rate of O_2 reduction was measured with an oxygen electrode. The two types of spheroplast were prepared from a single batch culture of cells as described in the Materials and methods section. (*b*) Approx. 1mg of protein from type-2 spheroplasts from a single preparation was added to 30ml of 250mM-sucrose/20mM-Tris/HCl (pH 8.0)/10mM-sodium succinate and either 1mM-KNO_3 (——) or 1mM-$NaClO_3$ (----) which had been added as increments of 0.1mM to calibrate the approximately logarithmic electrode response. In both cases (*a*) and (*b*) the data have been replotted to show the change in nitrate or chlorate concentration on a linear scale.

proteins. It is particularly noteworthy that the extra depletion of nitrite reductase from type-2 spheroplasts is not paralleled by a higher leakage of malate dehydrogenase. Isocitrate dehydrogenase activity was found to be unsuitable as a marker for leakage of cytoplasmic components (Wood, 1978), as the concentrated supernatant fraction precipitated upon addition to the requisite assay.

Spheroplast integrity was studied further by testing whether, as in intact cells, nitrate was reduced only under anaerobic conditions and chlorate reduction was much slower than that of nitrate. Both types of spheroplast reduced nitrate at a much faster rate after anaerobiosis (Fig. 3*a*), although, unlike intact cells, nitrate reduction was not completely inhibited by O_2. This inhibitory effect of O_2 can be taken as evidence of a relatively intact plasma membrane for the reasons given below. Reduction of chlorate by type-2 spheroplasts was slow and, unlike nitrate reduction, was not accelerated at anaerobiosis (Fig. 3*b*). This is taken as evidence for intact spheroplasts, because cells reduce chlorate only when the integrity of the plasma membrane has been disrupted by addition of a detergent such as Triton X-100 (John, 1977). The restricted capacity of spheroplasts from *P. denitrificans* for reducing chlorate is in agreement with the recent observations made with spheroplasts from *Escherichia coli* (Kristjansson & Hollocher, 1979). The nitrate reductase in cells of *E. coli* also exhibits a latency towards chlorate (Kristjansson & Hollocher, 1979; P. John, personal communication).

Control of nitrate respiration by O_2 in cells

The molecular basis through which the inhibitory effect of O_2 is exerted on dissimilatory nitrate reduction by cells of *P. denitrificans* and other bacteria is not known (Stouthamer, 1976; John, 1977). An important finding was that inside-out phosphorylating membrane vesicles from *P. denitrificans* were able to reduce nitrate and O_2 simultaneously (John, 1977), and thus the question arises as to at which stage during the conversion of cells to vesicles the inhibitory effect of O_2 on nitrate reduction is lost. It is unlikely that this control is exerted by a periplasmic factor, because, with the type-2 spheroplasts, which are more depleted of periplasmic components, the inhibitory effect of O_2 is found to be comparable with that observed with type-1 spheroplasts (Fig. 3*a*).

The finding that the nitrate reductase in phosphorylating vesicles of *P. denitrificans* could reduce chlorate at similar rates to nitrate, but that cells were unable to reduce chlorate unless a detergent (Triton X-100) was added (John, 1977), raised the possibility that there is a permeability barrier or transport system in cells which permits the planar nitrate ion, but not the pyramidal chlorate ion, to reach the active site of nitrate reductase (John, 1977). Although it has been implied that the failure

Table 2. *Retention of malate dehydrogenase by spheroplasts*

The type-1 and type-2 spheroplasts were from the same preparation as used for the nitrite reductase determinations shown in Table 1. The activities of malate dehydrogenase are those associated with the entire preparations of spheroplasts. A unit of activity is the oxidation of 1 μmol of NADH by oxaloacetate/min.

	Malate dehydrogenase activity (units)	
Spheroplast type ...	1	2
Activity in supernatant from spheroplast preparation	3.3	3.8
Activity associated with pellet after lysis of spheroplasts	6.8	4.4
Activity released upon lysing spheroplasts	490	790
Activity found in supernatant (%)	0.7	0.5

of cells of *P. denitrificans* to reduce nitrate under aerobic conditions is due to the preferential flow of electrons down the aerobic respiratory chain rather than to nitrate reductase (John, 1977; Haddock & Jones, 1977), an alternative possibility is that the presence of O_2 prevents access of nitrate to its reductase.

If O_2 acts in this latter manner then any treatment that will allow the permeability barrier to be bypassed or destroyed will permit nitrate to be reduced in the presence of O_2, and thus an identical titre of Triton X-100 might permit both chlorate reduction and nitrate reduction under aerobic conditions. Fig. 4 shows that this prediction was confirmed by experiment. The appearance of nitrate reduction in Fig. 4 was not due to anaerobiosis, because from a parallel experiment with an oxygen electrode it was estimated that anaerobiosis would be reached after approx. 7min. Furthermore, the addition of H_2O_2, which reoxygenated the solution as a result of endogenous catalase activity (which was not inactivated by up to 0.02% Triton X-100), had only a slight inhibitory effect on the rate of nitrate reduction, which was similar to its effect on the rate of chlorate reduction. In other experiments it was shown that addition of 0.02% Triton X-100 immediately after introduction of 1mM-H_2O_2 resulted, after a short lag of approx. 20s (cf. Fig. 4), in nitrate reduction under conditions of high dissolved O_2 concentration. No interference with the nitrate electrode by Triton was observed.

The concentration of Triton X-100 required to permit either chlorate reduction or nitrate reduction in the presence of O_2 had a very slight stimulating effect (approx. 10%) on the aerobic respiration of the cells. Thus the appearance of nitrate reductase activity (Fig. 4) was not due to an inhibition by Triton of electron flow to O_2. When 1mM-KNO_3 was added to cells after treatment with 0.02% Triton X-100 the rate of O_2 reduction was inhibited by approx. 50%, consistent with a partition of electron flow between O_2 and nitrate as observed with inside-out vesicles (John, 1977). Addition of KNO_3 to untreated cells had no effect on the rate of O_2 reduction.

Although it cannot be absolutely excluded that Triton X-100 has a coincidental parallel effect on both (i) any control of the distribution of electrons between the aerobic respiratory chain and nitrate reductase and (ii) the permeability of the cytoplasmic membrane, these experiments (Fig. 4) point to the inhibition of nitrate reduction by O_2 being exerted as a control on the accessibility of nitrate to its reductase. If this interpretation is correct it follows that the two types of spheroplast used in the present work must have retained an intact cytoplasmic membrane, otherwise the observed failure to reduce nitrate in the presence of O_2 (Fig. 3) cannot be accounted for.

The failure of O_2 to inhibit completely nitrate reduction in spheroplasts (Fig. 3*a*), and the observation of some chlorate reduction by type-2 spheroplasts (Fig. 3*b*) suggests that, relative to cells, the spheroplasts have become slightly permeable to small ions or molecules. The type-2 spheroplasts, or at least a fraction of them, were probably the more permeable as the aerobic respiration rate was stimulated 2-fold by addition of NADH, whereas there was no stimulation in type-1 spheroplasts where NADH was presumably unable to reach its dehydrogenase on the cytoplasmic side of the membrane.

Discussion

A conclusion to be drawn from the present work

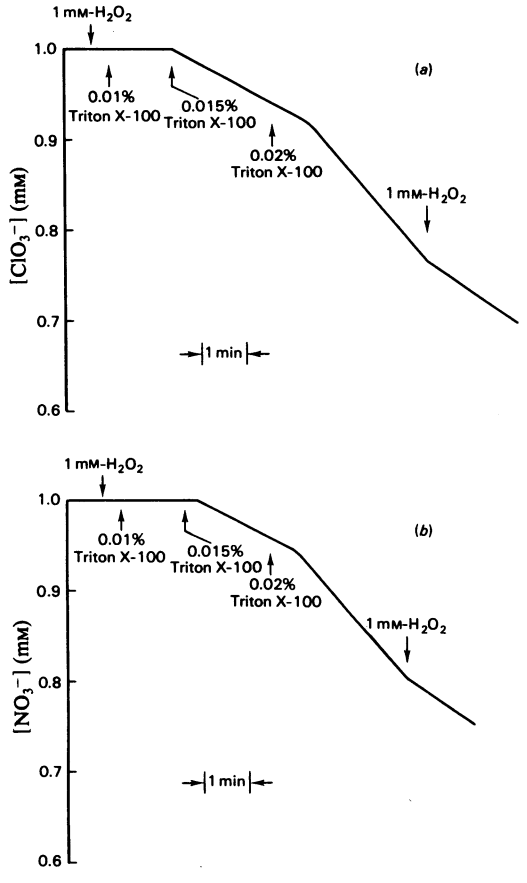

Fig. 4. *Effect of Triton X-100 on the reduction by cells of (a) chlorate and (b) nitrate in the presence of O_2*

Cells from an early stationary phase culture were harvested and washed as described in the Materials and methods section and finally were resuspended in 250mM-sucrose/20mM-Tris/HCl, pH 8.0. Reduction of chlorate or nitrate was measured in 30ml of the same medium to which 10mM-succinate, cells (approx. 110mg dry wt.) and either 1mM-KNO_3 or 1mM-$NaClO_3$ were added. Triton X-100 and H_2O_2 were added to give the final concentrations shown. The electrode was calibrated and the data replotted as described in the legend to Fig. 3.

is that dissimilatory nitrite reductase, cytochrome *cd*, is found in *P. denitrificans* either in the periplasmic space or loosely attached to the periplasmic side of the plasma membrane. This result agrees with the findings of Meijer *et al.* (1979), but disagrees with the proposal by Kristjansson *et al.* (1978) of a cytoplasmic location for this enzyme. Meijer *et al.* (1979) measured the stoichiometry of proton consumption associated with nitrite reduction by cells of *P. denitrificans* with the substrate ascorbate + *NNN'N'*-tetramethyl-*p*-phenylenediamine. This was assumed to donate electrons to the respiratory chain at the level of cytochrome *c*, which is often taken to be the physiological donor of reducing equivalents to nitrite reductase (see also below). The stoichiometry and insensitivity to the presence of permeant ions of this proton consumption corresponded very closely to that predicted by a model in which nitrite is reduced at the periplasmic surface of the cell, although the very sharp end point to the reaction presumably implies a lower K_m for nitrite than has been suggested previously for cytochrome *cd* (Newton, 1969; Saraste & Kuronen, 1978).

Kristjansson *et al.* (1978) measured the pH changes that followed the addition of small amounts of either nitrate or nitrite to intact cells that had available endogenous substrates. These pH changes were in general rather complex, and thus appear to have misled these workers into assigning a cytoplasmic location to nitrite reductase. Some of the evidence (Kristjansson *et al.*, 1978) for a cytoplasmic location was based on experiments in which nitrate was reduced via nitrite to nitrogenous gases, and thus a cytoplasmic enzyme might have reduced the nitrite formed from the reduction of nitrate, but no evidence for such an enzyme was found in the present work (Fig. 2). The movement and consumption of protons connected with the reduction of nitrate may be more easily studied in preparations of spheroplasts such as those described in the present work, in which complications due to the subsequent reduction of nitrite would be virtually absent.

A periplasmic site for nitrite reductase is consistent with cytochrome *c* being its immediate physiological electron donor, since it is expected that bacterial cytochrome *c* is located on the outer surface of the plasma membrane, as demonstrated in some cases (Garrard, 1972; Prince *et al.*, 1975). A possible role for azurin as the donor to nitrite reductase in *P. denitrificans* is now thought less likely because of a recent report in which it was shown that azurin is not, at least *in vitro*, a very effective donor of reducing equivalents to cytochrome *cd* (Martinkus *et al.*, 1980).

A location for the active site of nitrate reductase on the cytoplasmic side of the plasma membrane was suggested by the key observations of John (1977) on the latency in cells of nitrate reductase towards chlorate, and on the contrasting ability of the same enzyme to reduce chlorate at rates comparable with those of nitrate in phosphorylating inside-out vesicles. Further evidence for this location of the active site has come from studies of the pH dependence of azide inhibition of nitrate reduction in

both cells and inside-out vesicles of *P. denitrificans* (P. John, unpublished result). Experiments of a similar design originally led Garland *et al.* (1975) to conclude that in *E. coli* the active site of nitrate reductase was located at the outer face of the plasma membrane, but more recent experiments (Jones *et al.*, 1978) together with the work of Kristjansson & Hollocher (1979) indicate a location on the inner surface, as in *P. denitrificans*.

A single location for nitrite reductase in the periplasmic space or on the periplasmic side of the plasma membrane, together with an internal site of nitrate reduction, raises the tantalizing question as to how nitrite is exported from, and nitrate imported into, the cell. A nitrate–nitrite antiporter is an attractive possibility, but at present there is no experimental evidence for such a system (Garland *et al.*, 1975), which might also be postulated to work in the opposite direction in the nitrite-oxidizing bacterium *Nitrobacter winogradsky* (Cobley, 1976).

Taken together with the observation that inside-out vesicles can reduce O_2 and nitrate simultaneously (John, 1977), our observation that nitrate reduction by cells is possible in the presence of O_2 after addition of a low concentration of detergent (Fig. 4), suggests that the control by O_2 of nitrate reduction is likely to be exerted as a restriction on the accessibility of nitrate to its reductase, rather than as a control on the relative flow of electrons to O_2 or nitrate. This still begs the question of how the control is exerted, although the present work shows that periplasmic components are unlikely to be involved.

There has been some discussion of whether the dissimilatory nitrate reductase could also play a role in the assimilatory reduction of nitrate to nitrite (e.g. Sias & Ingraham, 1979). Our interpretation of the effect of O_2 on dissimilatory nitrate reduction suggests that such a role could only be played under aerobic growth conditions if the control by O_2 on the accessibility of nitrate to the dissimilatory reductase is lost. In any event, during nitrate assimilation and aerobic growth, nitrate must presumably enter the cell, in which case the dissimilatory nitrate reductase, if present, would, in our model for its control, be then able to reduce nitrate even in the presence of O_2.

Some variability has been found in the extent to which O_2 inhibits dissimilatory nitrate reduction by bacteria. For instance, John (1977) noted that when formate, but not succinate, was the donor of reducing equivalents some reduction of nitrate by *E. coli* under aerobic conditions was observed. Earlier Stouthamer (1976) reviewed the evidence on the influence of O_2 on the activity of nitrate reductase, and again found some variation as to the effectiveness of O_2 at inhibiting nitrate reduction. Thus in growing cultures of *Bacillus licheniformis* nitrate reduction did not cease upon introduction of O_2, whereas with wild-type *Ps. aeruginosa* nitrite production from nitrate was rapidly arrested. An interesting finding was that a mutant of *Ps. aeruginosa* which showed poor aerobic growth did not respond to aerobiosis in the same way and nitrate reduction continued for some time. Stouthamer (1976) concluded that the inhibition by O_2 of the activity of nitrate reductase *in vivo* was dependent on electron transport to O_2. However, it could equally be that the partial or complete absence of the proposed O_2 control mechanism on the movement of nitrate to its reductase was the basis for the observed instances of dissimilatory nitrate reduction under aerobic conditions.

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