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MINI-REVIEW

Metabolic Regulation Including Anaerobic Metabolism in *Paracoccus denitrificans*

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Abstract

Under anaerobic circumstances in the presence of nitrate *Paracoccus denitrificans* is able to denitrify. The properties of the reductases involved in nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase are described. For that purpose not only the properties of the enzymes of *P. denitrificans* are considered but also those from *Escherichia coli*, *Pseudomonas aeruginosa*, and *Pseudomonas stutzeri*. Nitrate reductase consists of three subunits: the α subunit contains the molybdenum cofactor, the β subunit contains the iron sulfur clusters, and the γ subunit is a special cytochrome *b*. Nitrate is reduced at the cytoplasmic side of the membrane and evidence for the presence of a nitrate–nitrite antiporter is presented. Electron flow is from ubiquinol via the specific cytochrome *b* to the nitrate reductase. Nitrite reductase (which is identical to cytochrome cd_1) and nitrous oxide reductase are periplasmic proteins. Nitric oxide reductase is a membrane-bound enzyme. The bc_1 complex is involved in electron flow to these reductases and the whole reaction takes place at the periplasmic side of the membrane. It is now firmly established that NO is an obligatory intermediate between nitrite and nitrous oxide. Nitrous oxide reductase is a multi-copper protein. A large number of genes is involved in the acquisition of molybdenum and copper, the formation of the molybdenum cofactor, and the insertion of the metals. It is estimated that at least 40 genes are involved in the process of denitrification. The control of the expression of these genes in *P. denitrificans* is totally unknown. As an example of such complex regulatory systems the function of the *fnr*, *narX*, and *narL* gene products in the expression of nitrate reductase in *E. coli* is described. The control of the effects of oxygen on the reduction of nitrate, nitrite, and nitrous oxide are discussed. Oxygen inhibits reduction of nitrate by prevention of nitrate uptake in the cell. In the case of nitrite and nitrous oxide a competition between reductases and oxidases for a limited supply of electrons from primary dehydrogenases seems to play an important role. Under some circumstances NO formed from nitrite may inhibit oxidases, resulting in a redistribution of

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electron flow from oxygen to nitrite. *P. denitrificans* contains three main oxidases: cytochrome aa_3, cytochrome *o*, and cytochrome *co*. Cytochrome *o* is proton translocating and receives its electrons from ubiquinol. Some properties of cytochrome *co*, which receives its electrons from cytochrome *c*, are reported. The control of the formation of these various oxidases is unknown, as well as the control of electron flow in the branched respiratory chain. Schemes for aerobic and anaerobic electron transport are given. Proton translocation and charge separation during electron transport from various electron donors and by various electron transfer pathways to oxygen and nitrogenous oxide are given. The extent of energy conservation during denitrification is about 70% of that during aerobic respiration. In sulfate-limited cultures (in which proton translocation in the NADH-ubiquinone segment of the respiratory chain is lost) the extent of energy conservation is about 60% of that under substrate-limited conditions. These conclusions are in accordance with measurements of molar growth yields.

Key Words: *P. denitrificans*; denitrification; nitrate reductase; nitrite reductase; nitric oxide reductase; nitrous oxide reductase; nitric oxide; copper transport; cytochrome oxidases; proton translocation; charge separation.

Introduction

Paracoccus denitrificans is a Gram-negative, coccoid hydrogen-oxidizing bacterium, formerly known as *Micrococcus denitrificans*, and was first isolated by Beyerinck and Minkman (1910). It is a very versatile bacterium capable of growth under various growth conditions. Heterotrophic growth occurs in the presence of a large variety of carbon and energy sources, both under aerobic and under anaerobic conditions with nitrate, nitrite, or nitrous oxide as a terminal electron acceptor. Furthermore, autotrophic growth is possible with hydrogen, thiosulfate, methanol, or methylamine as a sole energy source. The taxonomic and physiological characteristics of *P. denitrificans* have recently been reviewed (van Verseveld and Stouthamer, 1990). At this stage it seems appropriate to mention that *P. denitrificans* is closely related to *Rhodobacter capsulatus* and that it has been suggested that *P. denitrificans* arose by loss of photophosphorylation from a member of the purple photosynthetic bacteria, particularly of the genus *Rhodobacter* (Fox *et al.*, 1980).

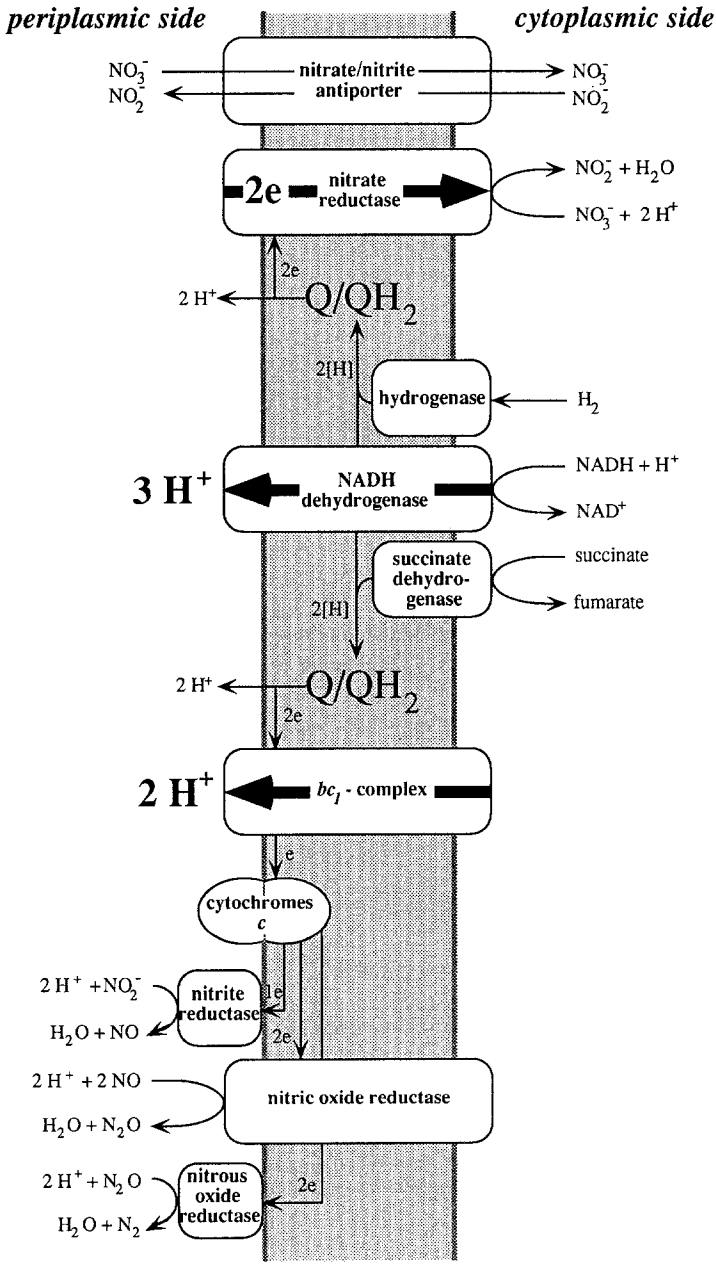
It has been emphasized that *P. denitrificans* harbors many mitochondrial features (John and Whatley, 1977; Albracht *et al.*, 1980). Based on this, *P. denitrificans* is thought to be a plausible ancestor of mitochondria of eukaryotic cells. For that reason it is a very popular organism for studying electron transfer and energy conservation (van Verseveld and Bosma, 1987). However, notwithstanding this marked mitochondrial similarity, also striking differences exist that should not be overlooked. One of these differences is the presence of multiple oxidases in the electron transport chain,

i.e., cytochromes aa_3, *o*, *co*, *d*, and *cd* (nitrite reductase with oxidase activity). The relative amount of each oxidase seems to vary widely with the growth conditions (Knobloch *et al.*, 1971; Lawford *et al.*, 1976; Willison and John, 1979; Porte and Vignais, 1980; Willison *et al.*, 1981; van Verseveld *et al.*, 1983). The mechanisms which regulate the formation of these various oxidases are not known. Under anaerobic conditions a number of nitrogenous oxides can act as electron acceptor. Electron transport to these nitrogenous oxides branches from the electron transport chain at various points (van Verseveld and Bosma, 1987; Ferguson, 1988; Stouthamer, 1988a,b). During denitrification a number of changes occur in the composition of the respiratory chain, e.g., denitrifying cells contain alternative oxidase(s), nitrate reductase, nitrite reductase, and nitrous oxide reductase but scarcely any cytochrome aa_3 (Vignais *et al.*, 1981). A parallel increase in the content of nitrate reductase and cytochrome *b*, suggesting the formation of a specific *b*-type cytochrome, has been reported (Calder and Lascelles, 1984). The cytochrome *c* composition is also variable (Bosma *et al.*, 1987a; Husain and Davidson, 1986).

These data indicate that the composition of the respiratory chain of *P. denitrificans* is very variable and very complex. This implies that the respiratory chain has a very flexible structure and this is of great importance for the capability of *P. denitrificans* to grow with a large number of different energy sources and under a large variety of environmental conditions. In this paper the biochemistry of anaerobic metabolism, the control of denitrification, the presence of various alternative oxidases, and a comparison of the bioenergetics of aerobic and anaerobic growth will be treated.

The Biochemistry and Bioenergetics of Denitrification in *P. denitrificans*

During denitrification nitrate is reduced to nitrogen. The capacity to denitrify is widely distributed among prokaryotes (Zumft, 1991). Denitrification is a very complex process and it may be estimated that at least 40 genes are involved to form the structural proteins and their prosthetic groups and cofactors, the components of specific transport mechanisms for uptake of metals (molybdenum and copper) and regulatory proteins. It is clear now that four reductases are involved in denitrification: nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase. These enzymes are formed only in the presence of nitrate under anaerobic circumstances. Under oxygen-limitation nitrous oxidase reductase activity and some nitrite reductase activity are formed (van Verseveld *et al.*, 1983). Various reviews emphasize the physiology and biochemistry (Hochstein and Tomlinson, 1988; Stouthamer, 1988a; Zumft *et al.*, 1988b) or the bioenergetics


periplasmic side
cytoplasmic side
NO3-
NO2-
nitrate/nitrite antiporter
NO3-
NO2-
2e
nitrate reductase
NO2- + H2O
NO3- + 2 H+
2e
2 H+
Q/QH2
2[H]
hydrogenase
H2
3 H+
NADH dehydrogenase
NADH + H+
NAD+
succinate dehydro-genase
succinate
fumarate
2[H]
2 H+
Q/QH2
2e
2 H+
bc1 - complex
e
cytochromes c
2 H+ +NO2-
H2O + NO
nitrite reductase
1e
2e
2 H+ + 2 NO
H2O + N2O
nitric oxide reductase
2 H+ + N2O
H2O + N2
nitrous oxide reductase
2e

of denitrification (Ferguson, 1988; Stouthamer, 1988b). Nitrate respiration in facultative microorganisms has been reviewed by Stewart (1988). The treatment here will be concentrated on *P. denitrificans*. However, when a certain reaction is known in more detail for other organisms (*Escherichia coli*, *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, or *Rhodobacter sp*), these aspects will be dealt with. We must conclude that in some aspects our knowledge of denitrification in *P. denitrificans* lags behind that in other organisms. This is due to the fact that a system for genetic analysis for *P. denitrificans* was unavailable for a long time (see Harms *et al.*, this issue).

A scheme for electron transfer to nitrogenous oxides for this organism is shown in Fig. 1. We will now treat the successive steps of denitrification separately.

The Reduction of Nitrate to Nitrite

This reaction is carried out by nitrate reductase, which is a membrane-bound enzyme. Therefore, the first step in the purification of nitrate reductase must be its solubilization from the cytoplasmic membrane. In *P. denitrificans* the nonionic detergent Nonidet P-40 was used for that purpose (Craske and Ferguson, 1986). The enzyme comprises three polypeptides, α, β, and γ, with estimated relative molecular masses of 127, 61, and 21 kDa. The enzyme is therefore similar to that of a large number of other organisms (Hochstein and Tomlinson, 1988; Stouthamer, 1988a). A preparation of the enzyme that lacks the γ-subunit can easily be obtained. This form of the enzyme is only active with reduced viologens as electron donor. The γ-subunit is a *b*-type cytochrome, which is a required component when duroquinol acts as the electron donor. This suggests that electron flow would be from ubiquinol via the specific cytochrome *b* to the nitrate reductase. Thus the bc_1 complex is not involved in electron flow to nitrate reductase, which confirms earlier schemes for electron transfer to nitrogenous oxides (Stouthamer *et al.*, 1982; Boogerd *et al.*, 1983a). This is in accordance with the observation that electron transport to nitrate is not inhibited by antimycin A or myxothiazol (Boogerd *et al.*,

Fig. 1. Diagram of the organization of the components of the electron transport chain of *Paracoccus denitrificans* involved in anaerobic respiration. Vectorial (bold type) proton translocation and scalar proton consumption and production (roman type) are indicated. Charge separation by electron transfer reaction is also given by bold arrows. The location of enzymes in the periplasmic space or at the cytoplasmic side of the membrane is given. For the sake of clearness electron transfer to Q is drawn in two directions. In the upper part Q is acting as electron donor to nitrate reductase and in the lower part as electron donor to the bc_1 complex. Q: ubiquinone-10. Although present, oxidases involved in electron transfer to oxygen (see Fig. 3) are not included in this diagram.

1983b; Parsonage *et al.*, 1986). By redox potentiometry two *b* type heme centers were identified in the γ-subunit with midpoint potentials of +95 and +210 mV (Ballard and Ferguson, 1988).

Nitrate reductase is a molybdoenzyme. The molybdenum is covalently linked to a pterin derivative, where it occurs as the molybdenum cofactor. For this compound, called molybdopterin, a molecular structure has been proposed by Johnson and Rajagopolan (1982). The enzyme contains three to four [4Fe–4S] and one [3Fe–3S] cluster (Johnson *et al.*, 1985). Electron paramagnetic resonance studies clearly show the participation of Fe–S centers and the molybdenum in the nitrate-reducing activity of the enzyme. It has been suggested that the iron–sulfur clusters and the molybdenum are associated with the α subunit, which is therefore thought to be the catalytic subunit (Chaudhry and MacGregor, 1983). So the β subunit was supposed to be devoid of metal and was suggested to be involved in mediating subunit interactions and membrane association. However, these suggestions were shown to be incorrect. Recently the nucleotide sequence of the *narGHJI* operon of *E. coli* was completed (Blasco *et al.*, 1989). The *narGHJ* genes code for the α, β, and γ subunits of nitrate reductase, respectively. The molecular weights of the α, β, and γ subunits were 138.7, 57.7, and 25.5 kDa, respectively. By comparison of the amino acid sequence of the α-subunit with that of other proteins, five segments were detected which are highly homologous with parts of the molybdoproteins biotinsulfoxide reductase, dimethylsufoxide reductase, and formate dehydrogenase of *E. coli*. A proposed role for these regions is the binding of the molybdenum cofactor to the catalytic subunit α. The amino acid sequence failed to reveal any structure capable of binding iron to the α-subunit. However, cysteine arrangements typical of iron–sulfur centres were found in the β-subunit. These observations suggest that during nitrate reduction electrons are provided by quinols to the γ-subunit (the *b*-type cytochrome) and subsequently transferred to the β-subunit. The β-subunit delivers them to the molybdenum cofactor, where nitrate reduction takes place. The results indicate that the α-subunit is the active site for nitrate reductions. The results also suggest that the nitrate reductase complex involves four polypeptides. The function of the *narI* gene product (molecular weight 26.5 kD) is not known.

In *P. denitrificans* nitrate is reduced at the cytoplasmic side of the membrane. In cell-free extracts nitrate reductase reduces both nitrate and chlorate readily (Forget, 1971). However, intact cells reduce chlorate at a very low rate, which is strongly increased by the addition of Triton-X-100 (John, 1977). Furthermore, purified preparations of nitrate reductase are very sensitive to inhibition by thiocyanate. In intact cells nitrate reduction is only inhibited by high concentrations of thiocyanate (Boogerd *et al.*, 1983a). Due to the existence of the protonmotive force, the concentrations of the

anion thiocyanate in whole cells will be much lower than in the outer bulk phase, which explains the difference in sensitivity of nitrate reduction to thiocyanate in whole cells and in cell-free preparations. The ability of the cell to discriminate between nitrate and chlorate (John, 1977) suggests the presence of a specific carrier system in the cytoplasmic membrane. Studies on the transport of nitrate were performed by measuring H_2 consumption or N_2O production after addition of nitrate to a cell suspension (Boogerd *et al.*, 1983a). It was found that a lag phase in H_2 consumption or N_2O production appeared whenever the membrane potential was dissipated by addition of uncoupling agents. However, these lag phases were not observed when nitrite was present at the moment of introduction of nitrate. When the reduction of nitrate by H_2 was studied in the presence of antimycin A, nitrate was only reduced to nitrite. Under these circumstances, in the presence of thiocyanate a lag in the H_2 production was observed upon the first addition of nitrate. No lag was observed when a second addition of nitrate was made. In that case the lag was prevented by the presence of nitrite formed after the first addition of nitrate. If antimycin A was omitted, which makes possible the further reduction of nitrite, a lag in the reduction of nitrate is also observed at the second, third, and fourth addition of nitrate. When the reduction of nitrous oxide is blocked by high concentration of thiocyanate, a long lag phase in N_2O production is observed after the addition of nitrate. When nitrite is added during the lag phase, nitrite and part of the nitrate are converted into N_2O. The reduction of nitrate comes at an immediate stop after the complete conversion of the nitrite added. Upon a second addition of nitrite, nitrite and part of the nitrate are again reduced to N_2O (Boogerd *et al.*, 1983a). These observations can be explained by the presence of two systems for the entry of nitrate in the cell: an active uptake process driven by the proton motive force and a nitrate–nitrite antiporter. The existence of the latter system has the advantage that after the reduction of nitrate has started its further uptake does not require energy (Fig. 1). The rate of swelling of spheroplasts has been studied to probe possible mechanisms of nitrate and nitrite transport across the cytoplasmic membrane of *P. denitrificans* (Parsonage *et al.*, 1985). In such experiments no evidence was found for the operation of nitrate–nitrite antiport or proton–nitrate symport. Recently the *narK* gene of *E. coli* was cloned and its sequence determined (Noji *et al.*, 1989). The *narK* gene product was characterized as a strongly hydrophobic transmembrane protein with 463 amino acid residues. A *narK* deletion mutant was unable to perform nitrate transport. The *narK* protein is thought to expel nitrite. This would mean that the *narK* gene product is the nitrate–nitrite antiporter.

In addition to the *narGHJI* and the *narK* gene the formation of active nitrate reductase was also abolished in *chlAM*, *chlB*, *chlD*, *chlEN*, and *chlG* mutants of *E. coli*. These mutants have been isolated as chlorate-resistant

mutants. These loci have been implicated in molybdate transport and the formation of the molybdenum cofactor (Stewart, 1988). Consequently, mutants deficient in these loci cannot form other molybdo proteins as well, e.g., formate dehydrogenase. Chlorate-resistant mutants have been isolated from *P. denitrificans* (Burke *et al.*, 1980). However, a genetic analysis has not been performed.

The Reduction of Nitrite

Nitrite reductases involved in denitrification have been purified from several bacteria and appear to be of two types: a multiheme enzyme and a copper-containing enzyme. The first type contains two types of prosthetic group: heme *c*, which is covalently linked to the protein, and a noncovalently bound heme *d* (Stouthamer, 1988a; Hochstein and Tomlinson, 1988). Therefore, this type of nitrite reductase, which is also found in *P. denitrificans*, is often referred to as cytochrome *cd*. The structure of *d*-type heme has recently been identified as a novel 1,3-porphyrindione (i.e., a dioxoisobacteriochlorin) (Chang *et al.*, 1986). The *d*-type heme is thus different from that in *d*-type cytochrome oxidase. The molecular weight of cytochrome *cd* is about 120 kDa, consisting of two identical subunits, each of which contains one *c*-type and one d_1-type heme (Stouthamer, 1988a; Hochstein and Tomlinson, 1988). Cytochrome *cd* has cytochrome oxidase activity. However, the main function is the reduction of nitrite. This is evident from the K_m values of the enzyme of *P. denitrificans* for O_2 and nitrite which were estimated as 80 and 6 μM, respectively (Timkovich *et al.*, 1982).

Cytochrome *cd* is a periplasmic enzyme. It has been shown that the protons consumed during reduction of nitrite (Meijer *et al.*, 1979; Boogerd *et al.*, 1981) are taken from the periplasmic side of the cytoplasmic membrane. The same applies to nitrous oxide reductase (Boogerd *et al.*, 1981). Furthermore, it appeared possible to prepare intact spheroplasts which were virtually depleted in nitrite reductase (Alefounder and Ferguson, 1980) and nitrous oxide reductase (Alefounder *et al.*, 1983). Recently, a highly improved purification procedure for cytochrome *cd* was published, which starts with periplasmic proteins (Carr *et al.*, 1989). During synthesis an apoform of cytochrome *cd* is translocated to the periplasm (Page and Ferguson, 1989). The insertion of the *c*-type and *d*-type heme then occurs in the periplasm. Recently the gene coding for cytochrome *cd* from *P. aeruginosa* has been cloned and its sequence determined (Silvestrini *et al.*, 1989). The amino terminus of the mature protein is located at lys-26, and the preceding 25-residue-long extension shows the features typical of signal peptides. The mature protein is made of 543 amino acids and has a molecular mass of

60204 Da. Analysis of the protein sequence in terms of hydrophobicity profile gives results consistent with the fact that the enzyme is fully water soluble and not membrane bound. These data confirm therefore the periplasmic location of cytochrome *cd*.

Electron transport to nitrite and N_2O involves cytochrome *c* (John and Whatley, 1977; Boogerd *et al.*, 1980). Recently it was established that *P. denitrificans* can form at least nine different *c*-type cytochromes (Bosma *et al.*, 1987a; Bosma, 1989). It is not yet known which of these *c*-type cytochromes are involved in electron transport to nitrogenous oxides. The end product of the reduction of nitrite by cytochrome *cd* is NO (Carr *et al.*, 1989). The biochemical pathway by which nitrite is converted into N_2O has long been a matter of controversy. Especially the role of NO as a possible, free, obligate intermediate between nitrite and N_2O has been a matter of much disagreement (Hochstein and Tomlinson, 1988). Earlier data on the formation and conversion of NO have been reviewed by Zumft and Kroneck (1990).

Recently it has been possible to trap NO, produced during denitrification, by extracellular hemoglobin (Goretski and Hollocher, 1988; Carr *et al.*, 1989; Kucera, 1989). Hemoglobin has a very high affinity for NO and the nitrosyl hemoglobin can be detected by spectrophotometeric methods. At high hemoglobin concentrations 1.3–1.8 nitrite was needed for the formation of one nitrosyl hemoglobin. Nitrogen not trapped appeared largely as N_2O in the presence of acetylene, which inhibits nitrous oxide reductase (Goretski and Hollocher, 1988). These results indicate that NO is a freely diffusible intermediate between nitrite and N_2O.

Till very recently N_2O was supposed to be the product of the reduction of nitrite by cytochrome *cd in vivo*. Experiments using the isotopes ^{15}N and ^{18}O have been performed to study the mechanism of N–N bond formation. The results indicate a sequential mechanism for nitrite reduction in denitrification, which means that N_2O originates via attack of nitrite on a coordinate nitrosyl (Weeg-Aerssens *et al.*, 1988; Shearer and Kohl, 1988). It is difficult to reconcile these findings with a pathway in which NO is an obligate intermediate between nitrite and N_2O. This is a point which needs clarification. The isolation of mutants which are deficient in nitric oxide reductase activity can be considered of great importance for resolving this point.

The Reduction of NO

Nitric oxide reductase has recently been purified from *P. stutzeri* (Heiss *et al.*, 1989). It is both in *P. stutzeri* (Heiss *et al.*, 1989) and in *P. denitrificans* (Carr *et al.*, 1989) a membrane-bound enzyme. The enzyme was purified from membrane fractions of *P. stutzeri* by solubilization with Triton X-100. The

enzyme consisted of two polypeptides with molecular masses of 38 and 17 kDa and contained heme-*b* and heme-*c* groups plus nonheme iron. The nitric oxide reductase transformed NO to N_2O. Thus nitric oxide reductase is the only enzyme known to be able to form the N–N bond in N_2O. Mutants of *P. stutzeri* defective in cytochrome *cd* (a *nir* mutant) were devoid of any nitrite-reducing activity and were still able to reduce NO (Zumft *et al.*, 1988a). In *Rhodobacter sphaeroides* f.s. *denitrificans* (Itoh *et al.*, 1989) and in *P. denitrificans* (Carr *et al.*, 1989) reduction of NO is inhibited by antimycin A and myxothiazol (Carr *et al.*, 1989), indicating that the bc_1 complex is involved in the transfer of electrons to the nitric oxide reductase. In accordance with this view it was observed that the rate of NO reduction was stimulated by uncouplers and that NO reduction was associated with ATP formation with a $P/2e^-$ ratio of 0.75 (Carr *et al.*, 1989). Since the protons consumed in the conversion of nitrite to N_2O or N_2 are taken from the periplasmic space (Meijer *et al.*, 1979; Boogerd *et al.*, 1981) NO must be reduced at the periplasmic side of the cytoplasmic membrane.

The Reduction of N_2O

Nitrous oxide reductase is a multi-copper protein (Zumft *et al.*, 1988b; Zumft and Kroneck, 1990). The best characterized system is that of *P. stutzeri*. The enzyme has a molecular mass of about 140 kDa and consists of two identical subunits (Coyle *et al.*, 1985). The enzyme contains eight copper atoms. After several papers which indicated the opposite, it has been agreed now that nitrous oxide reductase of *P. denitrificans* has similar properties as that of *P. stutzeri* (Snyder and Hollocher, 1987). Related enzymes have been found in several *Rhodobacter* sp. (McEwan *et al.*, 1985; Michalski *et al.*, 1986). It has been mentioned earlier that in *P. denitrificans* it is a periplasmic protein (Boogerd *et al.*, 1981; Alefounder *et al.*, 1983) and furthermore that the bc_1 complex is involved in electron transfer to the enzyme (Boogerd *et al.*, 1980; Alefounder and Ferguson, 1982). Recently the structural gene for nitrous oxide reductase of *P. stutzeri* (the *nosZ* gene) was cloned and its sequence determined (Viebrock and Zumft, 1988). An open reading frame coded for a protein of 638 amino acid residues (molecular mass 70822 Da) including a presumed signal sequence of 35 residues for protein export to the periplasmic space. The carboxy-terminal positioned residues cys 618, cys 622, and his 626 show a spacing which matches that of the proposed Cu_A site of the cytochrome aa_3 subunit II of *P. denitrificans* (Steinrücke *et al.*, 1987; Holm *et al.*, 1987). A multiplicity of spectroscopic data can be interpreted best by assuming a Cu_A site in nitrous oxide reductase (Scott *et al.*, 1989). However, in addition to a Cu_A site two

other types of Cu species are present in nitrous oxide reductase (Zumft and Kroneck, 1990).

Mutants deficient in nitrous oxide reductase activity of *P. stutzeri* have been obtained by random Tn5 mutagenesis (Zumft *et al.*, 1985). A *nos* coding region of ca. 8 kilobases was cloned (Viebrock and Zumft, 1988). It contained the structural gene (*nosZ*), a regulatory gene, and three genes (*nos D, F, Y*). Mutations in the latter genes lead to the formation of an apoenzyme. Probably these genes are involved in Cu transport as well as insertion of Cu into nitrous oxide reductase (Zumft and Kroneck, 1990). Another gene that is involved in Cu transport is the *nosA* gene. Mutants that are defective in the *nosA* gene product form nitrous oxide reductase that is inactive because it lacks copper (Mokkele *et al.*, 1987). The *nosA* gene product has been obtained in a homogenous form; it is a channel-forming protein when inserted into black lipid bilayers (Lee *et al.*, 1989). The molecular mass of the *nosA* gene product was about 65 kDa and it contained about 1 mole copper per protein molecule. It is located in the outer membrane. The synthesis of the *nosA* gene product is repressed by growth in a medium with a high copper concentration and by aerobiosis. The *nosA* gene product has been suggested to be a bifunctional protein, containing both a copper-binding and a pore-forming domain (Lee *et al.*, 1989). It is very interesting that the *nosA* gene product is the receptor of the bacteriophage ØP55 (Clark *et al.*, 1989). Mutant strains that do not attach ØP55 lack the *nosA* gene product and cannot grow with N_2O as hydrogen acceptor.

Control of Denitrification

There are two levels at which control of denitrification is exerted. The first is gene expression and the second is control of electron transport reactions. These two aspects of control will be discussed consecutively.

Control of Gene Expression

It is well known that the enzymes of denitrification are not formed under aerobic conditions. In general, it can be stated that the formation of a reductase for a certain substrate is prevented when a hydrogen acceptor with a higher energy-yielding potential is also present (Stouthamer, 1976). Thus in *E. coli* oxygen prevents the induction of nitrate reductase and nitrate prevents the induction of fumarate reductase (Stewart, 1988). In *P. denitrificans* the presence of oxygen prevents the formation of reductases for nitrogenous oxides (Lam and Nicholas, 1969; Sapshead and Wimpenny, 1972). At an earlier stage it was estimated that at least 40 genes are involved in the

denitrification process. It may be expected that these genes are coordinately controlled. There is nothing known about the molecular mechanism of this regulation. Gradually much more becomes known about the mechanism by which the formation of nitrate reductase is regulated in *E. coli* (Stewart, 1988). In this process the gene products of the *fnr*, *narX*, and *narL* genes are involved. The *fnr* gene is essential for the expression of a large number of genes for proteins, which have a function in anaerobic metabolism. The *fnr* gene has been cloned and its sequence has been determined (Shaw and Guest, 1982). The sequence of the *fnr* gene (Fnr) product shows regions of homology with the cyclic AMP receptor protein (CRP) (Shaw *et al.*, 1983). These considerations suggested that Fnr-mediated activation of gene expression occurs by a mechanism similar to that of CRP. Upstream of the transcription initation site of the *narGHJI* and the *frdABCD* (*frd* = fumarate reductase) operons a binding site for Fnr has been detected, which is located around −55 basepairs from the transcriptional initial site (Li and De Moss, 1988). The Fnr protein is dependent on Fe^{2+} ions for its activity and these metal ions bind to the cysteins in the N-terminal part of the protein (Trageser and Unden, 1989; Spiro *et al.*, 1989). The redox state of the metal in Fnr has been

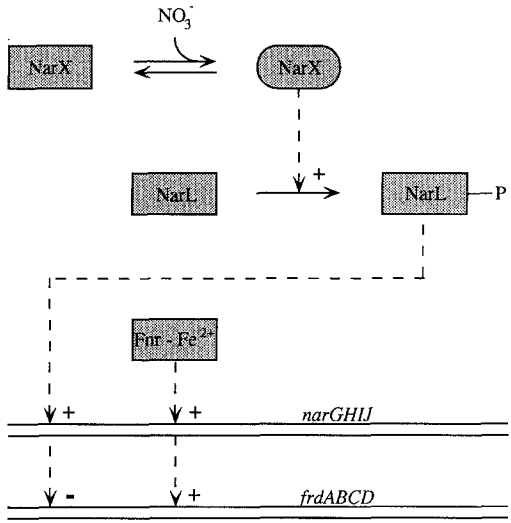
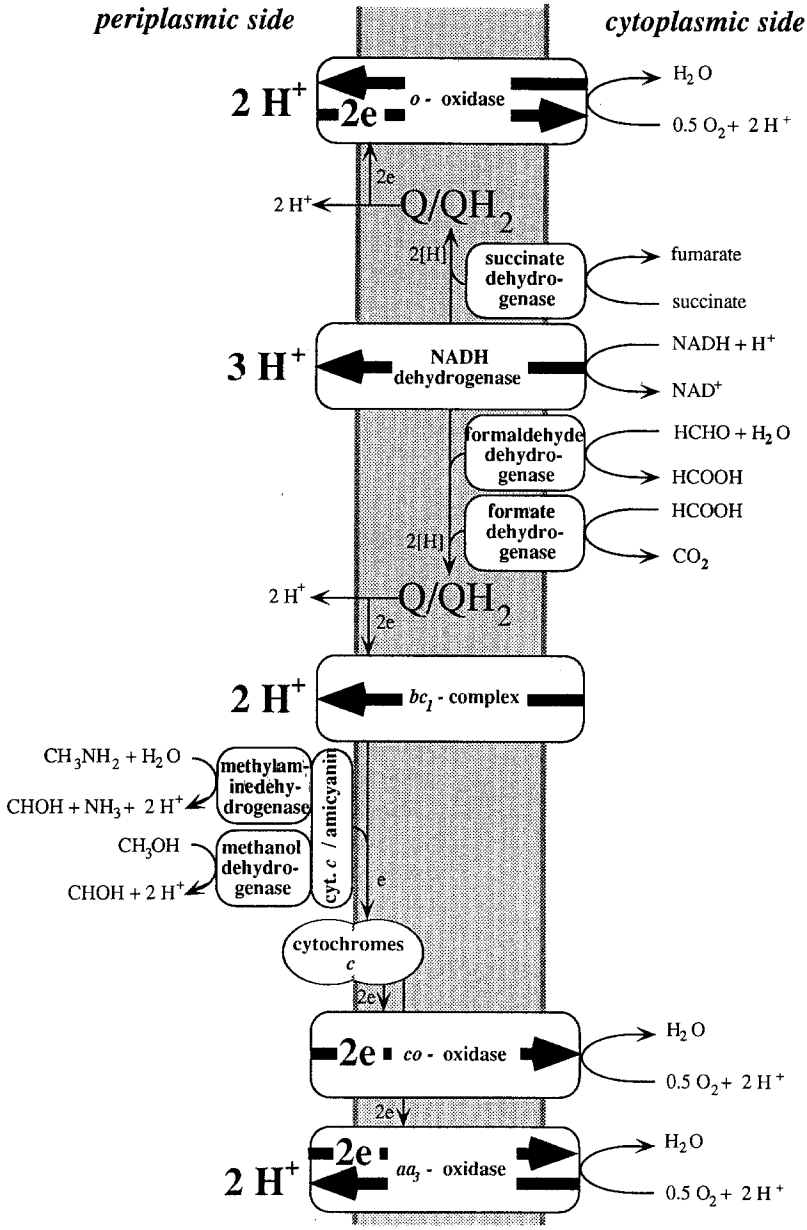


Fig. 2. Scheme for the regulation of the formation of nitrate reductase (*nar*) and fumarate-reductase (*frd*) in *Escherichia coli*. NarX is a nitrate-sensor protein, which undergoes a conformational change by binding nitrate. This conformational change activates the phosphorylation of NarL, which is a DNA binding protein. NarL-P activates transcription of the *narGHIJ* operon (indicated by +) and represses the transcription of the *frABCD* operon (indicated by −). The Fnr protein is a redox sensor. At a suitable redox potential the Fnr binds to the upstream region of the *nar* and *frd* operons and activates their transcription. The *nar* and *frd* operons are transcribed from left to right. Further explanation see text.

suggested to be very important for the activity of the protein. The *narX* and *narL* proteins have also been cloned and their sequence has been determined (Stewart and Parales, 1988; Kalman and Gunsalus, 1989). These genes form a two-component regulatory system (for a review on these systems, see Stock *et al.*, 1989). The *narX* gene product is the sensor protein. By binding of nitrate, a conformational change occurs, which activates phosphorylation of NarL. The NarL protein is the response regulator. After phosphorylation, the NarL-P protein binds to the upstream region of the *narGHIJ* operon. The binding occurs at a base sequence of about 27 base pairs located at about −200 basepairs from the transcriptional start site. Thus the NarL-P protein is a positive activator of gene expression of the *narGHIJ* operon. It is very interesting that the NarL-P protein is a repressor of the *frdABCD* operon and for the trimethylamine N-oxide reductase operon (Iuchi and Lin, 1987; Kalman and Gunsalus, 1989). In these cases activation by Fnr is overruled by repression by NarL-P. A scheme representing the regulation of the *nar* and *frd* operons by Fnr, NarL, and NarX is shown in Fig. 2. It is evident that the regulation system, which is even not yet completely known, is very complex.

Control of Electron Transport Reactions of Denitrification

The control of electron transport reactions of denitrification has been discussed by Ferguson (1988) and Stouthamer (1988a). It is an interesting question by what mechanism electron flow in branched respiratory chains is regulated. When nitrate-reducing cells of *P. denitrificans* are shifted to aerobic conditions, there is an immediate cessation of nitrate reduction (John, 1977). The artificial electron acceptor ferricyanide, which accepts electrons from cytochrome *c* (Boogerd *et al.*, 1981; van Verseveld *et al.*, 1981), mimics oxygen in blocking nitrate reduction (Alefounder *et al.*, 1981; Kucera *et al.*, 1981). Inhibition of electron flow to ferricyanide by antimycin A reversed this blockade. Similarly, interference with respiration by inhibiting terminal oxidases by hydroxylamine abolished the effect of oxygen on nitrate reduction. It has been shown that nitrous oxide and sometimes nitrite behave like oxygen by inhibiting nitrate reduction (Alefounder *et al.*, 1983; Kucera *et al.*, 1983a). Again nitrate reduction starts when electron flow to these nitrogenous oxides is prevented by antimycin A or acetylene (Alefounder *et al.*, 1983; Kucera *et al.*, 1983a). On the other hand, it has been demonstrated that in inverted membrane vesicles nitrate reduction occurred under aerobic conditions (Alefounder and Ferguson, 1980; Alefounder *et al.*, 1983; John, 1977; Kucera *et al.*, 1983a). It has been suggested therefore that the redox state of ubiquinone controls the movement of nitrate to its reductase (Alefounder *et al.*, 1983). A similar view has recently been reached for *P. aeroginosa*


periplasmic side
cytoplasmic side
2 H+
2e
o - oxidase
H2O
0.5 O2+ 2 H+
2e
2 H+
Q/QH2
2[H]
succinate dehydro-genase
fumarate
succinate
3 H+
NADH dehydrogenase
NADH + H+
NAD+
formaldehyde dehydro-genase
HCHO + H2O
HCOOH
formate dehydro-genase
HCOOH
CO2
2[H]
2 H+
Q/QH2
2e
2 H+
bc1 - complex
CH3NH2 + H2O
methylam-inedehy-drogenase
CHOH + NH3+ 2 H+
CH3OH
methanol dehydro-genase
CHOH + 2 H+
cyt. c / amicyanin
e
cytochromes c
2e
2e
co - oxidase
H2O
0.5 O2+ 2 H+
2e
2 H+
2e
aa3 - oxidase
H2O
0.5 O2+ 2 H+

(Hernandez and Rowe, 1987). Cells of *P. denitrificans* do not reduce nitrite (Kucera *et al.*, 1983b; Alefounder *et al.*, 1983) or N_2O (Alefounder and Ferguson, 1982; Kucera and Dadak, 1983) under aerobic conditions. In this case a competition between reductases and oxidases for a limited supply of electrons from primary dehydrogenases seems to play an important role (Alefounder *et al.*, 1981, 1983; Kucera *et al.*, 1981, 1983a,b). When nitrite is added to anaerobically grown cells that have been treated with an uncoupler or a low concentration of detergent, aerobic respiration is inhibited (Kucera and Dadak, 1983; Parsonage *et al.*, 1985). This results eventually in the complete redistribution of electron flow from oxygen to nitrite. This effect is due to the formation of NO (Kucera, 1989; Kucera *et al.*, 1986, 1987), which inhibits oxidase activity. It was shown that in the presence of an uncoupler NO production increased (Kucera, 1989). The reason for this is unknown. Recently it was shown that aerobic dentrification occurs in *Thiosphaera pantotropha* (Robertson and Kuenen, 1990). This may be due to a limited oxidase activity.

Alternative Oxidases

It has been mentioned before that *P. denitrificans* can form various oxidases. Of these the cytochromes aa_3 and *o* seem to be the most important. There is already a controversy for many years about the branchpoint in the electron transport pathways to cytochrome aa_3 and *o*. On the basis of inhibition studies with myxothiazol and antimycin A, it was concluded that branching occurs at ubiquinone and that the bc_1 complex is not involved in electron flow to cytochrome *o* (Kucera *et al.*, 1984; Parsonage *et al.*, 1986. In other laboratories the inhibition was less pronounced, however, and these have placed the branching point at cytochrome *b* (van Verseveld *et al.*, 1981; Boogerd *et al.*, 1981, 1983a) or at cytochrome *c* (van Verseveld and Bosma, 1987; van Verseveld and Stouthamer, 1991). Newer data give evidence for the presence of two alternative oxidases and this reconciles these contradictory findings. The alternative oxidases are cytochrome *o* and cytochrome *co* (Fig. 3).

Fig. 3. Diagram of the organization of the aerobic electron transport chain of *Paracoccus denitrificans*. Vectorial (bold type) proton translocation and scalar proton production (roman type) are indicated. Charge separation by electron transfer is also given by bold arrows. The location of enzymes in the periplasmic space or at the cytoplasmic side of the membrane is given. For the sake of clearness electron transfer to Q is drawn in two directions. In the upper part Q acts as electron donor to the alternative oxidase (cytochrome *o*); in the lower part Q transfers electrons to the bc_1 complex.

Cytochrome *o*

Recently mutants by gene replacement were obtained which lack cytochrome c_1 (Gerhus *et al.*, 1990; van Spanning *et al.*, unpublished results). These mutants are deficient in the whole bc_1 complex. Such mutants grow aerobically with the same maximum specific growth rate with mannitol and succinate (van Spanning *et al.*, unpublished results). The molar growth yield with those substrates is about 85% of that of the wild type. The results are consistent with electron transport to cytochrome *o* and the fact that the charge separation per electron pair in this pathway is two less than in the pathway to cytochrome aa_3 (Fig. 3). Recently it has been shown that the cytochrome *o* of *P. denitrificans* and of *E. coli* is proton translocating (Puustinen *et al.*, 1989). In these studies, mutant cells of *P. denitrificans* lacking cytochrome *c* or wild type cells in the presence of myxothiazol were used. In both cases the oxidation of succinate by spheroplasts was associated with the translocation of 1.5 to 1.9 H^+/e^- across the membrane. The same results were obtained with mutant cells lacking cytochrome *c* oxidase. Therefore under these conditions *P. denitrificans* gives the same results as *E. coli*, which does not contain a bc_1 complex (Anraku and Gennis, 1987). Based on this similarity we may conclude that *P. denitrificans* contains a cytochrome *o*, which receives its electrons directly from ubiquinol, and that it is proton translocating.

Cytochrome *co*

P. denitrificans is able to grow with methanol or methylamine as a sole source of carbon and energy. Each of these substrates is oxidized to formaldehyde by a specific methanol of methylamine dehydrogenase which functions in the periplasmic space (Alefounder and Ferguson, 1981; Husain and Davidson, 1985). In each dehydrogenase electrons are transferred via a pyrroloquinoline quinone prosthetic group (Duine *et al.*, 1980). During growth with methanol and methylamine a number of specific *c* type cytochromes are synthesized (Husain and Davidson, 1986; Bosma *et al.*, 1987a). From these data we may conclude that the bc_1 complex is not involved in electron transfer from methanol and methylamine dehydrogenase to oxygen.

In methanol-grown cells cytochrome aa_3 is the main oxidase (van Verseveld and Stouthamer, 1978a,b; van Verseveld *et al.*, 1979, 1981). For these reasons it is surprising that a mutant defective in cytochrome aa_3 (Willison *et al.*, 1981) is still able to grow with methanol (Harms *et al.*, 1985). Haltia *et al.* (1989) have isolated a mutant in which the gene for subunit III of cytochrome aa_3 is deleted. This mutant has a defect in the assembly of

cytochrome aa_3 and the cytochrome aa_3 oxidase activity is much lower than that in the wild type. Still this mutant can grow readily with methylamine as sole source of carbon and energy (J. Ras *et al.*, unpublished results). A mutant in which the gene for the periplasmic cytochrome c_{550} is deleted has been isolated by van Spanning *et al.* (1990). This mutation caused a shift in the electron flow from cytochrome aa_3 to an alternative oxidase. The mutation resulted in a 14% decrease in the growth yield during aerobic heterotrophic growth and in a 40% decrease in the maximum specific growth rate with methylamine. The findings mentioned above can be explained by assuming the presence of an alternative oxidase which receives its electron from a reduced *c*-type cytochrome. Strong evidence for the presence of such an alternative oxidase has been obtained and it has tentatively been identified as a cytochrome *co* (Bosma *et al.*, 1987b; Bosma, 1989). Ubiquinol oxidase activity was isolated from cells growth under different conditions. From aerobically growth cells a super complex containing the bc_1 complex and cytochrome aa_3 can be isolated (Berry and Trumpower, 1985; Bosma *et al.*, 1987b). However, from cells grown anaerobically with nitrate a membrane-protein fraction with cytochrome *c* oxidase activity could be obtained in which cytochrome aa_3 was spectroscopically (optical and EPR) not detectable. This indicates that the fraction contained an alternative oxidase. Potentiometric analysis indicated that the fractions contained, in addition to cytochrome bc_1 components, a cytochrome *b* with a midpoint potential around 130 mV and a *c*-type cytochrome with a midpoint potential around 345 mV. The *b* component was shown to bind CO. Finding additional cytochromes *c* in the active fractions suggests that this alternative oxidase is a *co*-type oxidase, which has properties similar to those in a number of other bacteria (Poole, 1988). In particular, a great similarity was found with the cytochrome *co* of another methylotrophic bacterium, *Methylophilus methylotrophus* (Carver and Jones, 1983; Froud and Anthony, 1984). This organism also contains cytochrome aa_3 and cytochrome *co*. From the molar growth yields of the various *P. denitrificans* mutants on methylamine we may conclude that electron transport to cytochrome *o* and cytochrome *co* is energetically equivalent, which implies that cytochrome *co* is not proton translocating.

Comparison of the Charge Separation for Aerobic and Anaerobic Respiration

This subject has been reviewed by Ferguson (1988) and Stouthamer (1988a,b). The stoichiometry of proton translocation over the cytoplasmic membrane upon addition of a limiting amount of oxidant ($\rightarrow H^+$/oxidant)

Table I. Proton Translocation ($\rightarrow H^+/2e^-$) and Charge Separation ($\infty q^+/2e$) during Electron Transport by Various Pathways to Oxygen and to Nitrogenous Oxides[a]

	$\rightarrow H^+/2e^-$			$\rightarrow q^+/2e$		
Electron donor	NADH	$FADH_2$ (H_2, succinate)	Methanol ($PQQH_2$)	NADH	$FADH_2$ (H_2, succinate)	Methanol ($PQQH_2$)
Electron acceptor						
Oxygen (via aa_3)	9	6	4	9	6	4
Oxygen (via *o* and *co*)	7	4	2	7	4	2
Nitrate	5	2	–	5	2	–
Nitrite	7	4	0	5	2	0
Nitric oxide	7	4	0	5	2	0
Nitrous oxide	7	4	0	5	2	0

[a]A dash means no transport.

has been measured for *P. denitrificans* grown under various conditions and for various electron acceptors. During electron transport of two electrons via the NADH-ubiquinone segment of the electron transport chain, two to three protons are translocated, the ubiquinol-cytochrome *c* segment translocates four protons, and the cytochrome *c*-oxygen segment transports two protons (van Verseveld *et al.*, 1981; Boogerd *et al.*, 1981, 1983a, 1984). However, it is not the protons, but the charge separation across the membrane, that is important in energy conservation, and thus the localization of the oxidases and reductases as cytoplasmic or periplasmic is of utmost importance (Ferguson, 1988; Stouthamer, 1988a,b). Figures 1 and 3 show the translocation of protons and the localization of the enzymes involved. The data for proton translocation and charge translocation for various pathways to oxygen and to nitrogenous oxides are shown in Table I.

Two important conclusions may be drawn from the data in Table I. First, transfer of a pair of electrons to nitrate, nitrite, nitric oxide, or nitrous oxide will result in the same charge separation, independent of the nitrogenous oxide used as electron acceptor. This is valid inspite of the fact that fewer proton-translocating sites are passed during transfer to nitrate than to the other nitrogenous oxides. This energetic equivalence of all five electrons required for reduction of nitrate to N_2 is explained by the difference in localization with respect to the cytoplasmic membrane of, on the one hand, nitrate reductase and, on the other hand, nitrite reductase and nitrous oxide reductase. Second, the charge separation is always higher during electron transfer to oxygen ($\rightarrow q^+/2e^-$ for NADH 9 or 7) than during electron transfer to nitrogenous oxides ($\rightarrow q^+/2e^-$ for NADH 5).

P. denitrificans has been grown in the chemostat with various carbon sources and hydrogen acceptors (Boogerd *et al.*, 1984). Proton translocation studies with these cells indicate that in heterotrophically grown cells electron transport is mainly to the alternative oxidase(s). During growth with methanol or with mannitol/methanol mixtures electron transport is mainly to cytochrome aa_3 (van Verseveld and Stouthamer, 1978a,b; van Verseveld *et al.*, 1979). During sulfate-limited growth, proton translocation in the NADH–ubiquinone segment of the respiratory chain is lost (Meijer *et al.*, 1977a,b). Under all these conditions molar growth yields have been measured. These have been analyzed by forming assimilation and dissimilation equations by Boogerd *et al.* (1984). Theoretical overall $P/2e^-$ ratios were calculated for growth under a variety of culture conditions on the basis of the schemes for electron transport and associated proton and charge translocation (Figs. 1 and 3, Table I). Experimental overall $P/2e^-$ ratios were calculated by using the specific rate of ATP synthesis determined in cultures grown under aerobic substrate-limited conditions for growth with the same substrate under other conditions. Under all conditions the experimental

$P/2e^-$ and the theoretical $P/2e^-$ ratios were very similar. In sulfate-limited cultures the extent of energy conservation was about 60% of that under substrate-limited conditions. The growth yields for growth under aerobic and denitrifying conditions indicate that the extent of energy conservation during denitrification was about 70% of that during aerobic respiration. Furthermore, the growth yields with nitrate and nitrite as hydrogen acceptor are the same. All these data are in accordance with the conclusions given above about electron transport pathways and charge translocation. Therefore the analysis of molar growth yields gives additional support for the schemes given (Figs. 1 and 3, Table I).

Conclusions

From this treatment we may conclude that already very much is known about the bioenergetics of *P. denitrificans*. Knowledge on some reactions of the denitrification pathway is still scarce. A characterization of the alternative oxidases is urgently needed. However, the molecular aspects of the regulation of the formation of electron transfer components and enzymes of the denitrification pathway are completely unknown. Future research to gather more information on this aspect will be of great importance.

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