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PROTON PUMP COUPLED TO CYTOCHROME *c* OXIDASE IN *PARACOCCUS DENITRIFICANS*

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Summary

The proton translocating properties of cytochrome *c* oxidase in whole cells of *Paracoccus denitrificans* have been studied with the oxidant pulse method.

$\rightarrow H^+/2e^-$ quotients have been measured with endogenous substrates, added methanol and added ascorbate (+TMPD) as reductants, and oxygen and ferricyanide as oxidants. It was found that both the observed $\rightarrow H^+/O$ with ascorbate (+TMPD) as reductant, and the differences in proton ejection between oxygen- and ferricyanide pulses, with endogenous substrates or added methanol as a substrate, indicate that the *P. denitrificans* cytochrome *c* oxidase translocates protons with a stoichiometry of $2H^+/2e^-$. The results presented in this and previous papers are in good agreement with recent findings concerning the mitochondrial cytochrome *c* oxidase, and suggest unequal charge separation by different coupling segments of the respiratory chain of *P. denitrificans*.

Introduction

Paracoccus denitrificans is a bacterium that shows many mitochondrial features. When the organism is grown aerobically, the plasma membrane shows a remarkable similarity with the inner mitochondrial membrane, including the composition of the electron transport chain [1,2]. Using EPR spectroscopy it has been shown that all paramagnetic groups of the mitochondrial respiratory chain are also found in the plasma membrane of *P. denitrificans*. Their properties suggest that they are embedded in very similar protein environments in the

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

two systems [3]. Recently it was shown that cytochrome *c* oxidase isolated from *P. denitrificans* contains only two polypeptides [4] compared with at least six in the mitochondrial enzyme [5].

In the last three years experimental evidence from intact mitochondria, submitochondrial particles and reconstituted vesicles accumulated suggesting that mitochondrial cytochrome *c* oxidase functions as a proton pump [6—17]. It has been shown that the proton pump functions as an intrinsic property of the enzyme rather than as a separate molecular entity [7,8,15].

At this time no conclusive evidence exists about proton pumping cytochrome oxidases in bacteria, although the observed $\rightarrow H^+/O$ ratio of 3.5 with methanol as externally added substrate to *P. denitrificans* cells indicates the functioning of cytochrome *c* oxidase as a proton pump [18]. This contrasts with the finding (Ludwig, B., personal communication) that with cytochrome *c* oxidase, isolated from *P. denitrificans* and reconstituted into phospholipid vesicles, proton pumping activity was low or absent. Also *P. denitrificans* seems a very suitable organism to investigate the proton translocating properties of bacterial cytochrome *c* oxidase, because after growth on methanol a methanol dehydrogenase (with a quinone-like prosthetic group) exists that donates its electrons to cytochrome *c* [19]. This supplies us with a physiological electron donor, donating its electrons to cytochrome *c*.

So far the results of proton translocation with methanol as electron donor in *P. denitrificans* do not exclude the occurrence of proton translocation mediated by the quinone prosthetic group of methanol dehydrogenase. In this paper we demonstrate the proton pumping function of cytochrome *c* oxidase in whole cells of *P. denitrificans* by (i) comparing $\rightarrow H^+/2e^-$ values obtained with oxygen and ferricyanide as oxidants, using cells incubated with added methanol or without exogenous substrate and (ii) determination of the $\rightarrow H^+/2e^-$ value with cells incubated with ascorbate (+TMPD) and pulsed with oxygen. Both methods yield a $\rightarrow H^+/2e^-$ ratio close to 2 for the span cytochrome *c* to oxygen.

Materials and Methods

Paracoccus denitrificans NCIB 8944 was grown aerobically at 35°C and pH 7.5 in the liquid medium described by Chang and Morris [20] with methanol (100 mM) as carbon and energy source and NH_4Cl as nitrogen source. The nutrient was supplemented with Difco yeast extract (0.01%) and $NaHCO_3$ (0.05%) [21]. Cells used for proton translocation experiments were grown in a methanol-limited (100 mM) aerobic chemostat culture as described before [22, 23]. Measurement of proton translocation was carried out in a double-walled glass chamber, termostated at 25°C and kept anaerobic with a continuous stream of nitrogen over the liquid. pH changes were measured with a glass microelectrode (type 7 GR 241, Electrofact NV, Amersfoort, The Netherlands) and calibrated with an anaerobic standard solution of HCl (Titrisol, Merck). The measuring chamber was filled with 3.1 ml oxygen-free 100 mM KSCN/50 mM KCl/1.5 mM glycylglycine buffer at pH 7.0, and 150 μl bacterial suspension (containing 25 mg dry weight bacteria per ml) (see also Ref. 18).

Ferricyanide, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate solutions were prepared freshly each day. A mutant of *P. denitrifi-*

cans lacking cytochrome *c* was isolated according to the methods described by Willison and John [24].

Results

Cells of *Paracoccus denitrificans* used for the measurement of respiration driven proton translocation were grown in methanol-limited continuous culture at 35°C with a specific growth rate (μ) of 0.11 h^{-1}. Methanol-grown cells were used for measuring the stoicheiometry of respiration driven proton translocation for two reasons: (i) the prosthetic group of methanoldehydrogenase is a physiological electron donor to cytochrome *c* and that methanol is really a substrate for electron transport has been shown by the observation that inhibition by antimycin A and rotenone of the velocity of both electron transport and proton extrusion is abolished by the addition of methanol (results not shown, but see Ref. 18). (ii) the relative concentration of complex IV of the respiratory chain is significantly higher with methanol as a growth substrate than with heterotrophic growth substrates [3,18].

When according to Moyle and Mitchell [25], cytochrome *c* oxidase does not act as a proton pump, it is expected that pulses of oxygen or ferricyanide (which in mitochondria accepts electrons from cytochrome *c*) will yield the same stoicheiometry of proton translocation [26]. Before drawing conclusions from experiments with *P. denitrificans* it is important to establish whether ferricyanide accepts electrons from cytochrome *c* also in the bacterial respiratory chain. To show the obligate presence of cytochrome *c* for the reduction of ferricyanide, we used a cytochrome *c* deficient mutant of *P. denitrificans*. When a cell suspension of the mutant was pulsed with ferricyanide, proton translocation was very slow or absent (not shown), which indicates that a fast rate of proton translocation (Fig. 1) is due to the presence of cytochrome *c*. Fig. 1 shows a sequential series of oxidant pulses with endogenous substrate (a, b) or methanol (c—h) as reductants. Most of the endogenous substrate will be NADH + H^+. Only part of the endogenous respiration will be caused by oxidation of the quinone-like cofactor of methanol dehydrogenase, because the absence of methanol will prevent the re-reduction of this cofactor. In the absence of added methanol, pulsing with either oxygen (a) or ferricyanide (b) leads to rapid acidification followed by a slow decay to a final level. After an oxygen pulse this level is identical to the initial level, however after a ferricyanide pulse this level amounts to a net production of one H^+ per ferricyanide added. The extent of the initial acidification is greater in an oxygen pulse than in a ferricyanide pulse. In the presence of the uncoupler CCCP the decay to the final level is greatly accelerated (not shown), indicating that the acidification is due to proton translocation. After addition of methanol, the extents of the acidification phases are diminished (c, d), because oxidation of methanol, via cytochromes *c* and aa_3 will become predominant. When also rotenone and antimycin have been added (e, f), an oxygen pulse still leads to acidification in excess of 3.9 $H^+/2e^-$ above the final level (e), while a ferricyanide pulse does no longer lead to acidification above the final level of $2H^+/2e^-$ (f). In the presence of CCCP (g, h) again final levels are reached faster.

$$RH_2 + 2Fe(CN)_6^{3-} \rightarrow R + 2Fe(CN)_6^{4-} + 2H^+ \quad (1)$$

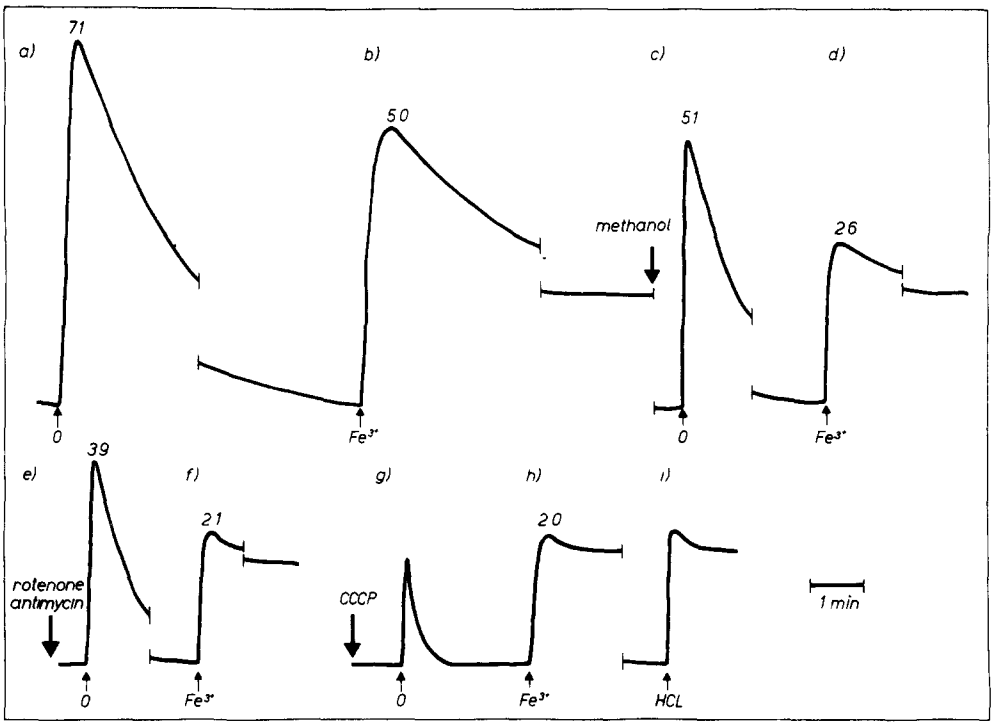

Fig. 1. Respiration driven proton translocation in cells of *P. denitrificans* grown in a methanol-limited chemostat at $\mu = 0.11\ h^{-1}$. The anaerobic cell suspension was pulsed with oxygen (O) by injecting 20 µl of air-saturated 150 mM KCl (9.4 ng atoms of oxygen) (a, c, e and g) or with ferricyanide (Fe^{3+}) by injecting 20 µl of an anaerobic solution of 1 mM $K_3Fe(CN)_6$ (b, d, f and h) with a microsyringe. Traces were calibrated by injecting 20 µl of an anaerobic solution of 10^{-3} N HCl (i). Additions of 80 mM methanol (c), 30 µM rotenone, 6.7 µg/ml antimycin A (e) and 6 µM CCCP (g) (all final concentrations) are indicated by a thick arrow. The observed $\rightarrow H^+/2e^-$ ratio is shown for each oxidant pulse.

Eqn. 1, in which RH_2 and R stand for reduced an oxidized hydrogen donor, respectively, predicts, after backflow, a net production of $2H^+/2e^-$ when ferricyanide is reduced by endogenous substrate or methanol. We found a net production of 1.94 ± 0.04 $H^+/2e^-$ (four experiments) with endogenous substrate and of 1.78 ± 0.16 $H^+/2e^-$ (four experiments) with methanol. In Table I a comparison is made between the extent of acidification with the two oxidants. From the final column it can be seen, that the difference amounts to nearly $2H^+$ ejected per $2e^-$. Since we know that ferricyanide accepts electrons from cytochrome *c*, the found difference in stoicheiometry between the two oxidants for endogenous substrate and methanol must be due to proton translocation driven by electron transport between cytochrome *c* and oxygen.

To reinforce the finding of proton translocation coupled to electron transport from cytochrome *c* to oxygen we used ascorbate (+TMPD) as exogenous electron donor.

Electrons from ascorbate are donated to cytochrome *c* via TMPD. Absence of oxidation of ascorbate/TMPD by the cytochrome *c*-negative mutant of *P. denitrificans* (our own observation, and see also Ref. 24) showed that the ob-

TABLE I

OBSERVED $\rightarrow H^+/2e^-$ RATIOS FOR WHOLE CELLS OF *PARACOCCUS DENITRIFICANS* GROWN AEROBICALLY IN THE CHEMOSTAT UNDER METHANOL-LIMITING CONDITIONS AT A μ VALUE OF 0.11 h^{-1}

Values are presented as averages ± S.D. with the number of experiments in parentheses. Oxygen or $Fe(CN)_6^{3-}$ pulses of respectively 9.4 ngat or 20 nmol jave been given to an anaerobic cell suspension in 1.5 mM glycylglycine buffer (pH 7), containing 100 mM KSCN and 50 mM KCl.

Substrate	$\rightarrow H^+/O$	$\rightarrow H^+/2Fe(CN)_6^{3-}$	$\rightarrow H^+/2e^-$ cyt. *c* oxidase
Endogenous	7.12 ± 0.24 (4)	5.26 ± 0.34 (4)	1.86 ± 0.18 (4)
160 mM methanol *	4.04 ± 0.29 (6)	2.11 ± 0.11 (6)	1.94 ± 0.22 (6)
0.4 mM ascorbate ** + 0.1 mM TMPD	2.81 ± 0.14 (5)	1.04 ± 0.05 (5) ***	1.77 ± 0.17 (5)
	Mean value of all experiments		1.86 ± 0.14 (15)

* and ** endogenous respiration inhibited by 30 μM rotenone and 6.7 μg/ml antimycin A solubilized in respectively methanol (*) and dimethylformamide (**). *** Non-enymatic.

served stoicheiometry of proton translocation is due to electron transport from cytochrome *c* to oxygen and a scalar production of one H^+ per ascorbate oxidized. The overall reactions of ascorbate oxidation are given in Eqns. 2 and 3.

$$AH^- + H^+ + \tfrac{1}{2}O_2 \rightarrow A + H_2O \quad (2)$$

$$AH^- + 2Fe(CN)_6^{3-} \rightarrow A + 2Fe(CN)_6^{3-} + H^+ \quad (3)$$

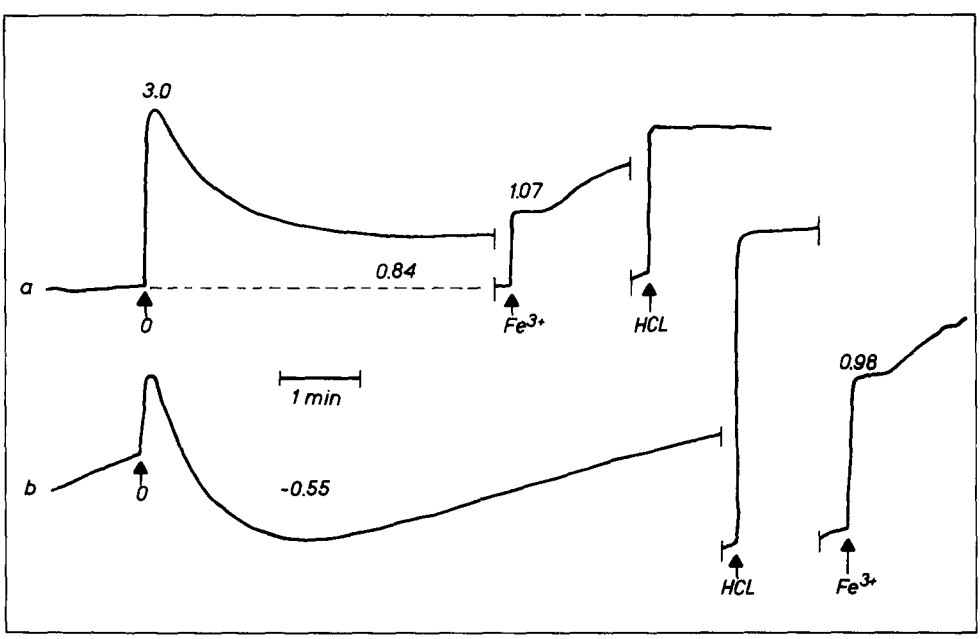

Fig. 2. Respiration-driven proton translocation in cells of *P. denitrificans* grown in a methanol-limited chemostat at $\mu = 0.11\ h^{-1}$. In (a), ascorbate (0.4 mM final concentration, pH 7) and TMPD (0.1 mM, final concentration, pH 7) and in (b) ascorbate, TMPD and CCCP (6 μM, final concentration) were added to the anaerobic cell suspension. Arrows indicate the moment of injection of 20 μl air-satured 150 mM KCl (9.4 ngatoms of oxygen) (O), 20 μl anaerobic solution of 1 mM $K_3Fe(CN)_6$ (Fe^{3+}) or 20 μl of anaerobic solution of 10^{-3} M HCl (HCl). The observed $\rightarrow H^+/2e^-$ ratio is shown for each oxidant pulse.

When cytochrome *c* oxidase of *P. denitrificans* does not act as a proton pump it is expected that an oxygen pulse results in the net consumption of 1 proton per $\frac{1}{2}O_2$ according to Eqn. 2.

Trace a in Fig. 2 shows, that an oxygen pulse results in an initial production of about $3H^+/2e^-$ in the extracellular medium. This value is expected, because at pH 7 ascorbate is a $1H^+,2e^-$ donor and it is assumed that electron transfer through cytochrome *c* oxidase leads to translocation of $2H^+/2e^-$. However, trace a does not decay to the level (uptake of $1H^+/2e^-$) predicted by Eqn. 2. Instead a net production of 0—0.84 $H^+/2e^-$ remains (depending on the batch of cells used).

Also the (non-enzymatic) oxidation of ascorbate by ferricyanide (trace a, final part) is after initial production of the $1H^+/2e^-$ expected, followed by a further acidification.

In the presence of uncoupler (Fig. 2 trace b) some net alkalinisation (0.4—0.85 H^+/O) is seen after the accelerated decay of the initial acidification, but also here acidic drifts (on a minute timescale) are observed.

These observations are explained by the property of dehydroascorbic acid (the oxidation product of ascorbic acid) to undergo an irreversible change, which is independent of the presence of oxidizing agents. The product of this change, 2,3-diketo-l-gulonic acid, and eventually following reactions yield products, that are stronger acids than dehydroascorbic acid [27]. The half-life of dehydroascorbic acid at pH 7.0 is only a few minutes, which explains the difficulty to observe the expected proton consumption, even after addition of CCCP. The second acidification phase is too slow to interfere with the initial fast acidification. (cf. the slow acidification after addition of ferricyanide in Fig. 2.) In *P. denitrificans* addition of CCCP never leads to the complete disappearance of proton extrusion like is observed in mitochondria.

Table I summarizes the acidification stoicheiometry of the ascorbate oxidation experiments. Translocation of $2H^+/2e^-$ by cytochrome *c* oxidase is clearly indicated.

Discussion

The oxidation of methanol and ascorbate/TMPD by whole cells of *Paracoccus denitrificans* was associated with an initial phase of H^+ release into the outside bulk phase. With both substrates the acidification phase is sensitive to the uncoupling agent CCCP, which suggests that H^+ ejection is a consequence of proton transport across the membrane.

The following observations support the notion that electron transport between cytochrome *c* and oxygen results in proton translocation across the membrane and for that purpose cytochrome *c* oxidase is the most likely candidate as a proton pump.

(i) Use of endogenous substrates as reductant resulted in a $\rightarrow H^+/2e^-$ quotient with ferricyanide as oxidant, that was 1.86 $H^+/2e^-$ lower than the $\rightarrow H^+/O$ quotient. It has experimentally been ascertained that ferricyanide accepts electrons from cytochrome *c* and as a consequence the difference between $\rightarrow H^+/O$ and $\rightarrow H^+/2$ ferricyanide must be due to protons transported across the membrane coupled to electron transport between cytochrome *c* and oxygen.

(ii) When methanol was used as exogenous substrate and endogenous respiration was inhibited by rotenone and antimycin A, we have shown with ferricyanide pulses that two protons are released from the membrane due to oxidation of methanol to formaldehyde and electron transport to cytochrome *c* via the quinone-like prosthetic group of methanol dehydrogenase. This release of $2H^+$ at the periplasmic side of the bacterial membrane due to electron transport from methanol to cytochrome *c* could mean that either the methanol dehydrogenase is located at the periplasmic side of the membrane or it is not and 2 hydrogen atoms are transported across the membrane by the quinone-like prosthetic group of methanol dehydrogenase. The difference between $\rightarrow H^+/O$ and $\rightarrow H^+/2$ ferricyanide quotients is 1.94 $H^+/2e^-$ and must again be due to proton translocation associated with electron transport between cytochrome *c* and oxygen.

(iii) With ascorbate as exogenous substrate, an oxygen pulse resulted in acidification of the outside bulk phase with a stoicheiometry of about $3H^+/2e^-$. One proton is released due to the oxidation of ascorbate to dehydroascorbate. Consequently about $2H^+$ are translocated across the membrane.

These observations are in contrast to findings with isolated and reconstituted bacterial cytochrome *c* oxidase. Ludwig and coworkers have found, that under those conditions little or no redox linked proton translocation is observed (Ludwig, B., personal communication) in contrast to similar experiments with the mammalian enzyme. An explanation of this discrepancy may be offered by the different subunit structure of the isolated bacterial enzyme, compared to the mammalian one.

In the mammalian enzyme, subunit III has been implicated in proton translocation by two findings: (i) Cytochrome oxidase lacking subunit III does not translocate protons when incorporated in phospholipid vesicles [28]. (ii) Proton translocation catalysed by cytochrome *c* oxidase is DCCD sensitive [15, 17], and DCCD binds most strongly to subunit III [15,17]. The fact that a protein with the properties of subunit III is lacking in isolated *P. denitrificans* cytochrome *c* oxidase suggests that the loss of proton translocation capacity during purification is the result of the loss of a subunit III-like protein. Experiments are in progress to test this possibility.

From the results of experiments presented in this paper and earlier results presented by others [29,30] and our group [18,31] we can make some assumptions about the proton and charge stoicheiometry in different segments of the respiratory chain of *P. denitrificans*. Fig. 3 shows a simplified scheme of the respiratory chain in the membrane. All used electron-donors and -acceptors as well as the places where proton translocation can occur are indicated.

We expect that at the traditional coupling sites 1 and 2, respectively, 3 and 2 protons are translocated across the membrane, driven by electron transport. Consequently, according to Fig. 3, the NADH/oxygen couple yields an expected $\rightarrow H^+/2e^-$ stoicheiometry of 9. Experimental values vary from 7 to 10. Hence, the expected charge separation, $\rightarrow q^+/2e$, is also 9 and could experimentally have a value of 7—10 and it is expected that the proton consumption in the inside bulk phase, $\leftarrow H^+/2e$, is 10. For all combinations of electron-donors and -acceptors the same calculation yields expected values of proton translocation and charge separation. These are presented in Table II together with the

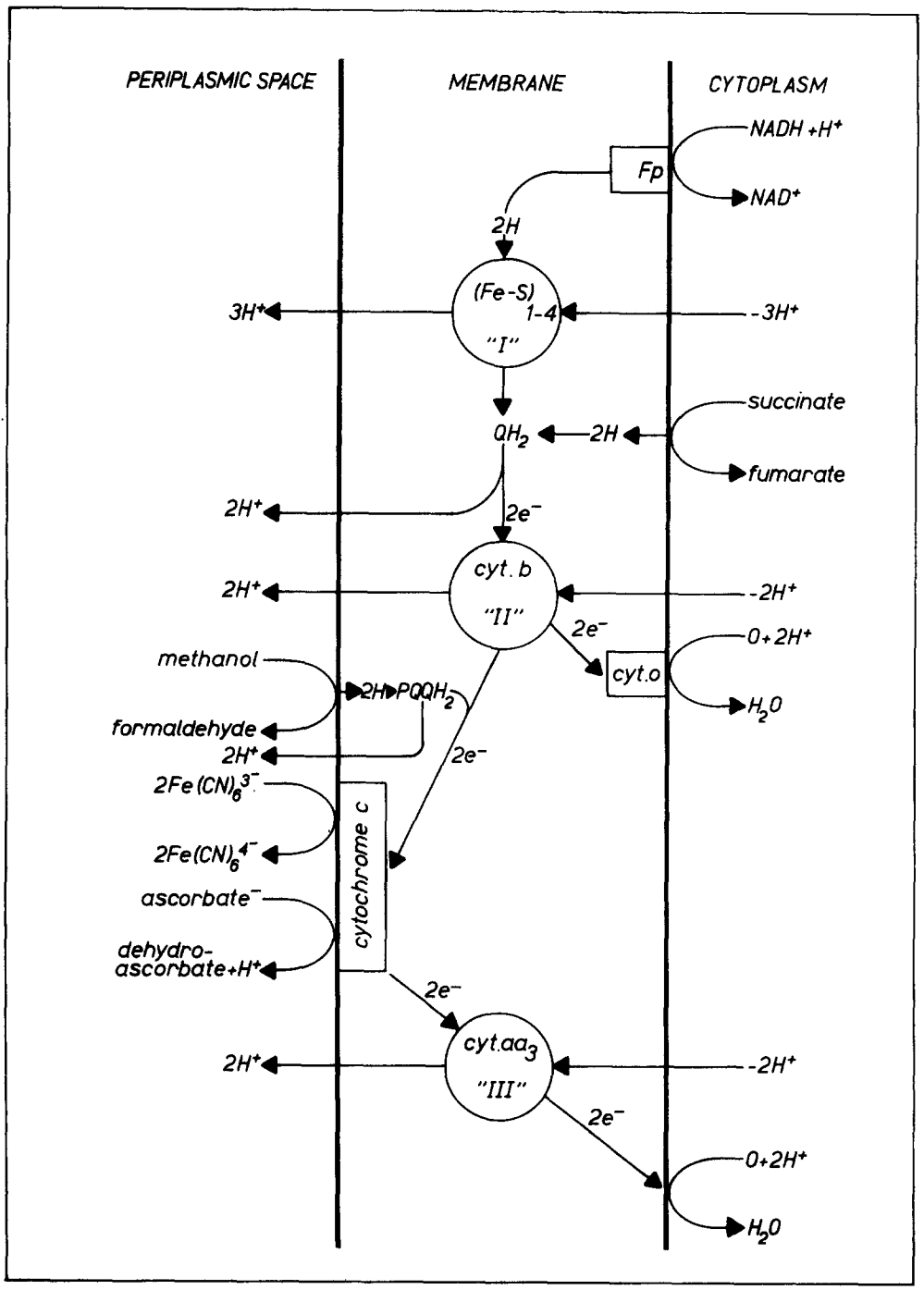

Fig. 3. Scheme showing stoicheiometry of proton translocation in aerobically grown *P. denitrificans*. Abbreviations: Fp, flavoprotein; Fe-S, iron-sulfur center; Q, ubiquinone; cyt., cytochrome; PQQ, pyrroloquinoline quinone, the quinone-like prosthetic group of methanol dehydrogenase [38]. I, II and III are the traditional coupling sites.

TABLE II

PROPOSED STOICHIOMETRIES OF PROTON TRANSLOCATION ($\rightarrow H^+/2e^-$), CHARGE TRANSLOCATION ($\rightarrow q^+/2e^-$), AND PROTON CONSUMPTION IN THE INSIDE BULK PHASE ($\leftarrow H^+/2e^-$)

Electron donor	Electron acceptor	Expected $\rightarrow H^+/2e^-$	$\rightarrow q^+/2e^-$	$\leftarrow H^+/2e^-$	Measured $\rightarrow H^+/2e^-$	Reference
NADH	Q *	3	3	4	3.5	31
NADH	O_2	9	9	10	7 —10	18, 29, 30
Succinate	cyt. *c* **	4	2	2	—	
Succinate	O_2	6	6	6	5 —6	18, 29
Methanol	cyt. *c* **	2	0	0	2	This paper
Methanol	O_2	4	4	4	4	18 and this paper
Ascorbate	O_2	3	4	4	3	This paper

* Ubiquinol.
** Ferricyanide.

measured $\rightarrow H^+/2e$ values. The calculation concerning the NADH/oxygen couple clearly demonstrates that it is not necessary to postulate proton translocation due to electron transport via site 0, the transhydrogenase site [32], to explain $\rightarrow H^+/2e^-$ ratios higher than 6. It will be clear from Table II that for the traditional sites 2 and 3 the measured values of $\rightarrow H^+/2e^-$ matches the expected values. In our opinion it is necessary to know not only the stoicheiometry of proton translocation but also the stoicheiometry of charge separation. At this moment experiments are in progress to determine the experimental values of charge separation.

As can be seen from Fig. 3 and Table II the charge separation is not equal in the traditional site segments of the respiratory chain as has been proposed before for mitochondria [13,33]. The calculated $\rightarrow q^+/2e^-$ ratio for the site 3 segment (cytochrome *c*/oxygen), the site 2 segment (ubiquinol/cytochrome *c*) and the site 1 segment (NADH/ubiquinol) are, respectively, 4, 2 and 1—3. If we assume that low $\rightarrow H^+/2e^-$ ratios with endogenous substrates are the result of interference of other electron donors than NADH, a $\rightarrow H^+/2e^-$ ratio of 9 is most likely, which indicates a $\rightarrow H^+/2e^-$ and $\rightarrow q^+/2e^-$ of 3 in the site 1 segment. Heterotrophically grown cells of *P. denitrificans* possess two functional sites of energy conservation and electrons from NADH to oxygen are most probably transported via cytochrome *o* (see Fig. 3) [18,31,34]. Sulphate-limited growth yields cells that have lost the capability of site 1 phosphorylation [31,35]. It has been shown that this loss of site 1 phosphorylation leads to a $\rightarrow H^+/O$ ratio on endogenous substrates which is decreased with 3—4 $H^+/2e^-$, while the $\rightarrow H^+/O$ ratio with succinate as exogenous substrate is not influenced. Consequently a $\rightarrow H^+/2e^-$ and $\rightarrow q^+/2e^-$ ratio of 3 for the site 1 segment of the respiratory chain seems very reasonable. In that case the relative charge translocation for site 1: site 2: site 3 is 3 : 2 : 4, which is in good agreement with both thermodynamic predictions [13] and experimental findings in mitochondria [33].

Recently it was concluded that yield studies [31,34,36] are not in accordance with an unequal charge separation in the different site segments of the respiratory chain [37]. However then we did neglect that when electron transport is carried out via cytochrome *o*, the charge separation in the site 2 segment

of the respiratory chain is not 2 charges/$2e^-$ but 4 charges/$2e^-$ (see Fig. 3). In the latter case also the yield studies are in good agreement with the charge separation as presented in Fig. 3.

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