Biochimica et Biophysica Acta, 638 (1981) 181–191
Elsevier/North-Holland Biomedical Press

BBA 48168

RESPIRATION-DRIVEN PROTON TRANSLOCATION WITH NITRITE AND NITROUS OXIDE IN *PARACOCCUS DENITRIFICANS*

F.C. BOOGERD, H.W. VAN VERSEVELD and A.H. STOUTHAMER

Department of Microbiology, Biological Laboratory, Vrije Universiteit, De Boelelaan 1087, 1007 MC Amsterdam (The Netherlands)

(Received May 25th, 1981)

Key words: Respiratory chain; Proton translocation; Denitrification; Nitrite reduction; Nitrous oxide reduction; (Paracoccus denitrificans)

(1) $\vec{H}^+$/electron acceptor ratios have been determined with the oxidant pulse method for cells of denitrifying *Paracoccus denitrificans* oxidizing endogenous substrates during reduction of O_2, NO_2^- or N_2O. Under optimal H^+-translocation conditions, the ratios $\vec{H}^+/O$, $\vec{H}^+/N_2O$, $\vec{H}^+/NO_2^-$ for reduction to N_2 and $\vec{H}^+/NO_2^-$ for reduction to N_2O were 6.0–6.3, 4.02, 5.79 and 3.37, respectively. (2) With ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylenediamine as exogenous substrate, addition of NO_2^- or N_2O to an anaerobic cell suspension resulted in rapid alkalinization of the outer bulk medium. H^+/N_2O, H^+/NO_2^- for reduction to N_2 and H^+/NO_2^- for reduction to N_2O were −0.84, −2.33 and −1.90, respectively. (3) The H^+/oxidant ratios, mentioned in item 2, were not altered in the presence of valinomycin/K^+ and the triphenylmethylphosphonium cation. (4) A simplified scheme of electron transport to O_2, NO_2^- and N_2O is presented which shows a periplasmic orientation of the nitrite reductase as well as the nitrous oxide reductase. Electrons destined for NO_2^-, N_2O or O_2 pass two H^+-translocating sites. The $\vec{H}^+$/electron acceptor ratios predicted by this scheme are in good agreement with the experimental values.

Introduction

About 15 genera of bacteria are able to denitrify, i.e., reduce ionic nitrogenous oxides to gaseous products [1]. As stated by Zumft and Cárdenas [2], one should specify substrates and products of a given reaction in order to prevent confusion about the studied conversion. *Paracoccus denitrificans* can reduce NO_3^- to N_2 in a dissimilative way and is thus able to grow anaerobically in the presence of NO_3^-. The reduction of NO_3^- to NO_2^- is accomplished by the respiratory nitrate reductase, a molybdenum-containing iron-sulphur protein [3]. The nitrate reductase is linked to the respiratory chain in the region of the *b*-type cytochromes [4]. The NO_3^- binding site of the nitrate reductase is most probably located at the inner aspect of the cytoplasmic membrane [5–7]. The subsequent reduction of NO_2^- involves a soluble two-haem (*c*- and *d*-type) nitrite reductase [8,9]. Electron transport to NO_2^- occurs via *c*-type cytochromes [10]. There are strong indications that the H^+-consuming side of the nitrite reductase is situated at the periplasmic face of the cytoplasmic membrane [6,11], although also a cytoplasmic orientation of the nitrite reductase has been claimed [5]. The product of NO_2^- reduction by cytochrome *cd* has not yet been determined, but H_2O has been shown to be the product of reduction of O_2 by

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $TPMP^+$, triphenylmethylphosphonium cation; $\vec{q}^+/2e^-$ ($\vec{H}^+/2e^-$), number of charges (protons) translocated across the cytoplasmic membrane during flow of $2e^-$ to an electron acceptor.

the nitrite reductase [12]. Cytochrome(s) *c* is involved in electron transport to N_2O [13]. Recently, an in vitro assay for nitrous oxide reductase has been described [14]. Although these authors spoke of an intracellular nitrous oxide reductase, they have not indicated the orientation with respect to the cytoplasmic membrane.

With this knowledge of the anaerobic respiratory chain in mind it becomes very interesting to obtain information about the efficiency of oxidative phosphorylation with O_2 and the different intermediates of denitrification. Because the coupling of oxidation and phosphorylation is mediated by an electrochemical H^+ gradient [15], it is a logical assumption that the orientation (cytoplasmic or periplasmic) of H^+ consumption with regard to the inner membrane during reduction of O_2 or nitrogenous oxides will have an influence on the generation of a $\Delta\bar{\mu}_{H^+}$.

In a previous paper [13] we suggested that N_2O could be an obligate intermediate in the conversion of NO_2^- to N_2, in contrast to earlier publications [11, 16] of this laboratory. One of the reasons to doubt the role of N_2O as an intermediate was the observation that N_2O did not result in acidification of the outer medium in an oxidant pulse type of experiment. In those experiments, valinomycin and SCN^- were used to eliminate the membrane potential in order to measure respiration-driven H^+ translocation. However, it appeared that both the nitrate reductase and the nitrous oxide reductase activities were rather sensitive to SCN^-. The inhibition of the nitrate reductases from several bacteria by SCN^- has been described earlier [17–19]. When SCN^- was omitted from the reaction medium, a pulse of N_2O resulted in fast acidification of the outer bulk phase, followed by H^+ back-flow. So a re-evaluation of the role of N_2O was necessary.

In this study we have used the oxidant pulse method for determination of $\vec{H}^+/2e^-$ ratios using O_2 and nitrogenous oxides as oxidants with *P. denitrificans*, grown under denitrifying conditions. $\vec{H}^+/2e^-$ stoicheiometries observed upon reduction of O_2 or nitrogenous oxides by endogenous substrates and exogenous ascorbate/TMPD revealed a consistent picture of the H^+-translocating properties of the anaerobic electron-transport chain with respect to O_2, N_2O and NO_2^-.

Materials and Methods

Microorganism and growth conditions

Paracoccus denitrificans NCIB 8944 was the experimental organism. Anaerobic continuous cultures were performed in a Bioflo C30 chemostat (New Brunswick Scientific Co., Inc., New Brunswick, NJ, U.S.A.). The culture volume was about 275 ml. The pH was automatically maintained at a value of 7.0 by addition of 0.1 M HCl. A continuous flow of N_2 (containing less than 4 ppm O_2) over the culture and through the medium maintained anaerobic conditions during the chemostat runs. Bacteria were grown in the liquid medium described by Chang and Morris [20] with succinate as carbon and energy source, NH_4Cl as nitrogen source and NO_3^- as electron acceptor.

Two kinds of anaerobic chemostat culture provided cells for the measurement of H^+ translocation. (I) Bacteria grown with 40 mM KNO_3 and 50 mM sodium succinate at 37°C and a specific growth rate (μ) of 0.23 h^{-1}. (II) Bacteria grown with 40 mM KNO_3, 50 mM sodium succinate and 0.3% yeast extract (Oxoid) at 35°C and μ of about 0.2 h^{-1}.

Measurement of H^+ translocation in intact cells

Treatment of bacterial cells. After at least six generation times, cells were harvested from continuous culture, subsequently washed twice with 150 mM KCl/1.5 mM glycylglycine buffer (pH 7.0) and then resuspended in this buffer at approx. 30 mg dry wt. cells/ml. This cell suspension was used immediately for experimentation.

Reaction cell and electrode system. The system was as described by Meijer et al. [21] with the following changes. The combined glass electrode was connected to a Philips pH-meter (type PW 9409, Philips, The Netherlands) and the recorder output of the pH-meter was taken to a Kipp recorder (BD-8 recorder, Kipp and Zonen, Delft, The Netherlands). The recorder sensitivity was 10 mV for a full-scale response of 20.0 cm. A full-scale recorder deflection at 10 mV corresponded to 0.10 pH units. The response time of the total system, i.e., time for injection, mixing, and electrode, pH meter and recorder responses, was about 1 s. The temperature of the reaction cell was thermostatically maintained at 25°C.

Actual measurement procedure. The reaction cell was filled with 3 ml O_2-free 150 mM KCl/1.5 mM glycylglycine buffer (pH 7.0) and 150 μl of the above-mentioned cell suspension. After addition of the desired mixture of permeant ions, electron-transport inhibitors and substrates, the solution was flushed during 3 min with pure N_2 containing less than 4 ppm O_2 (AGA gas B.V., Amsterdam, The Netherlands). After this period a continuous flow of N_2 was directed at the liquid surface to prevent diffusion of O_2 from the air into the reaction medium. The cells were allowed to equilibrate for 60 min before measurements were started. Experiments were performed at a pH of the outer medium between 6.5 and 7.0. O_2 was introduced into the anaerobic suspension by injecting air-saturated 150 mM KCl at 25°C (mostly 10 μl, containing 4.7 ngatom O [22]). NO_2^- or $Fe(CN)_6^{3-}$ was introduced by injecting O_2-free 150 mM KCl/1 mM KNO_2 or 150 mM KCl/1 mM $K_3Fe(CN)_6$ at 25°C (mostly 5 μl, containing 5 nmol NO_2^- or $Fe(CN)_6^{3-}$). N_2O was introduced by injecting water saturated at 25°C with a gas mixture of 5% N_2O and 95% N_2 (mostly 6 μl, containing 7.35 nmol N_2O). The percentage of N_2O was checked gas chromatographically with the aid of a 5% N_2O, 95% N_2 calibration mixture (AGA gas B.V., Amsterdam, The Netherlands). The deflection of the pH recorder was calibrated by injecting a known amount of anaerobic 150 mM KCl/1 mM HCl at 25°C. The NO_2^-, $Fe(CN)_6^{3-}$ and HCl solutions were freshly prepared each day from 10 mM KNO_2, $K_3Fe(CN)_6$ and HCl (Titrisol, Merck) standard solutions. Ascorbate and TMPD solutions were prepared and adjusted to pH 7.0 just before use. Valinomycin, antimycin A and rotenone were added as ethanolic solutions. KSCN and triphenylmethylphosphonium bromide were added as aqueous solutions. Control experiments made clear that addition of anaerobic 150 mM KCl did not result in any deflection of the recorder pen. The respiration-driven acidification or alkalinization of the outer medium was compared with HCl-driven acidification to calculate the quantity of H^+ translocated per electron acceptor reduced, i.e., the H^+/electron acceptor ratio. The H^+/electron acceptor ratios were corrected by extrapolating the tangent of the initial decay phase back to the moment of electron acceptor addition.

Results

$\vec{H}^+$/oxidant ratios with endogenous substrates

In the first instance, the effect of varying concentrations of valinomycin on $\vec{H}^+/O$ and $\vec{H}^+/N_2O$ was studied in order to determine the optimal conditions for H^+ translocation. The results of these experiments are summarized in Table I. It can be seen that the mean value of $\vec{H}^+/O$ is higher than that of $\vec{H}^+/N_2O$ for each given range of valinomycin concentrations and that both ratios have a maximum value at 11–13 μg valinomycin/ml. Especially the $\vec{H}^+/O$ ratio showed some variance from batch to batch and also within one batch, which is expressed in the rather large standard deviations. When the cells were preincubated with 12 μg valinomycin/ml and 80 mM SCN^-, the mean $\vec{H}^+/O$ value was 5.99 ± 0.41 (16) and no H^+ translocation with N_2O was observed. The $\vec{H}^+/O$ stoicheiometry of about 6 was not greatly influenced by using different combinations of valinomycin and SCN^- concentrations in the ranges 4–32 μg/ml and 9–173 mM, respectively, as indicated by the mean $\vec{H}^+/O$ value of 6.15 ± 0.62 (20) for all the tested combinations. So, in the presence of more than 9 mM SCN^-, an $\vec{H}^+/O$ value of about 6 is always measured regardless of the valinomycin concentration in the range 4–32 μg/ml. A value of 6 for the $\vec{H}^+/O$ ratio is apparently the value which can be determined under optimal H^+-translocation conditions with cells of an

TABLE I

EFFECT OF VALINOMYCIN CONCENTRATION ON $\vec{H}^+/O$ AND $\vec{H}^+/N_2O$

Cells from type I chemostat cultures were washed twice with 150 mM KCl/1.5 mM glycylglycine buffer (pH 7.0) and subsequently incubated in the same buffer for 90 min at 25°C under anaerobic conditions with different amounts of valinomycin. The $\vec{H}^+/O$ and $\vec{H}^+/N_2O$ ratios with endogenous substrates are presented as averages ± S.D. with the number of pulses in parentheses.

Valinomycin (μg/ml)	$\vec{H}^+/O$	$\vec{H}^+/N_2O$
4.4– 4.9	3.77 ± 0.64 (22)	2.06 ± 0.54 (15)
8.7–10.7	4.18 ± 1.24 (7)	2.87 ± 0.66 (7)
11.0–13.0	4.99 ± 0.90 (10)	3.73 ± 0.36 (8)
13.5–15.3	4.05 ± 0.60 (17)	3.39 ± 0.44 (9)

NO_3^--limited chemostat culture. This means that even the maximal $\vec{H}^+/O$ value of 4.99 measured in the presence of only valinomycin is not as high as possible. And as a logical consequence, the $\vec{H}^+/N_2O$ value is likewise an underestimation. Because increasing the valinomycin concentration up to 50 μg/ml (as suggested in Ref. 5) or lengthening the preincubation time (as suggested in Refs. 5 and 23) up to 6 h did not yield a higher $\vec{H}^+/O$ or $\vec{H}^+/N_2O$ value, we tried to establish optimal H^+-translocation conditions otherwise. In further experiments a concentration of 12 μg valinomycin/ml was routinely added to the reaction cell.

At first, the effect of a combination of valinomycin and low concentrations of SCN^- (below 9 mM) on H^+ translocation with O_2 and N_2O was studied. However, no satisfactory results were obtained, which is probably caused by an almost complete inhibition of the nitrous oxide reductase activity even at those low concentrations. In accordance with this view, dual-wavelength experiments with cytochrome *c* showed that 1 mM SCN^- caused 85% inhibition and 9 mM resulted in 100% inhibition of electron transport from endogenous substrates to N_2O.

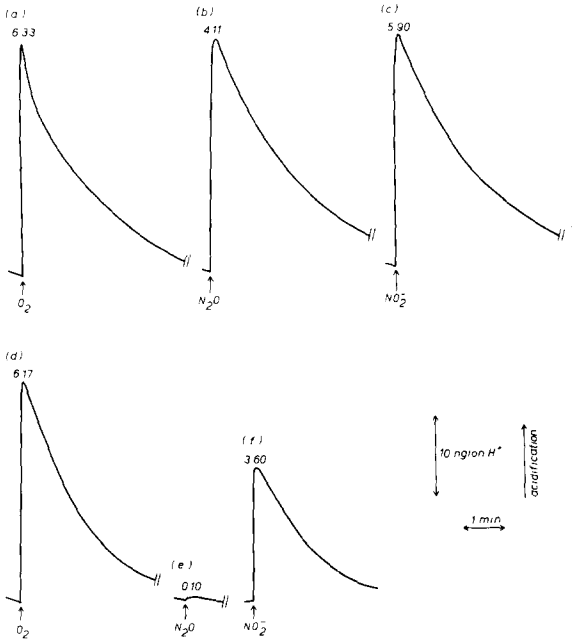

Fig. 1. Respiration-driven H^+ translocation in cells of *P. denitrificans* grown in an NO_3^--limited chemostat at $\mu = 0.2\ h^{-1}$. Cells from type II chemostat culture were used. In a–c, 12 μg valinomycin/ml and 5 mM $TPMP^+$ were present, while in d–f in addition 100 mM KSCN was present. The anaerobic cell suspension was pulsed with O_2 (a and d, 4.7 ngatom O), N_2O (b and e, 7.35 nmol N_2O) or NO_2^- (c and f, 5 nmol NO_2^-). The observed $\vec{H}^+$/oxidant ratio is shown for each oxidant pulse.

Then we started to search for a suitable substitute for SCN^-. It had to be a permeant ion with no inhibiting action on the nitrous oxide reductase activity. The lipophilic, synthetic cation $TPMP^+$ turned out to be a very elegant substitute for SCN^-. When cells of an NO_3^--limited continuous culture were preincubated with 12 μg valinomycin/ml and 5 mM $TPMP^+$, injection of O_2, NO_2^- or N_2O resulted in rapid acidification of the outer bulk phase. This is illustrated in Fig. 1. The fast acidification phase was following by a slow decay to the initial level with a half-time of about 60–80 s for each electron acceptor. Addition of 100 mM SCN^- had no effect on $\vec{H}^+/O$ (Fig. 1a and d), diminished $\vec{H}^+/N_2O$ to almost zero (Fig. 1b and e) and lowered $\vec{H}^+/NO_2^-$ (Fig. 1c and f). Table II summarizes the $\vec{H}^+/O$, $\vec{H}^+/N_2O$ and $\vec{H}^+/NO_2^-$ stoicheiometries measured this way. The $\vec{H}^+/O$ value of 6.32 is hardly different from those in the presence of valinomycin and SCN^- (5.99 and 6.15). Moreover, in the presence of 100 mM SCN^- together with valinomycin and $TPMP^+$, a mean $\vec{H}^+/O$ value of 6.21 is found. So a value of $\vec{H}^+/O$ just above 6 seems a realistic stoicheiometry for cells of NO_3^--limited chemostat cultures of *P. denitrificans*.

The $\vec{H}^+/N_2O$ value with valinomycin and $TPMP^+$ is 4.02, while in the same system plus 100 mM SCN^- a value of 0.11 is measured. The $\vec{H}^+/NO_2^-$ ratio measured with valinomycin and $TPMP^+$ is 5.79, which is lowered to 3.37 in the presence of 100 mM SCN^-. The observation that the $\vec{H}^+/NO_2^-$ value decreases and the $\vec{H}^+/N_2O$ value is virtually reduced to zero by addition of SCN^-, while the $\vec{H}^+/O$ ratio is not influenced by this addition, can be taken as evidence for reduction of NO_2^- to N_2O in the presence of SCN^-.

The overall reactions of the various electron acceptors are given in Eqns. 1–4.

$$O + 2e^- + 2H^+ \rightarrow H_2O \quad (1)$$

$$NO_2^- + 3e^- + 4H^+ \rightarrow 0.5N_2 + 2H_2O \quad (2)$$

$$NO_2^- + 2e^- + 3H^+ \rightarrow 0.5N_2O + 1.5H_2O \quad (3)$$

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad (4)$$

Bearing in mind a periplasmic consumption [6,11] of $3H^+$ per NO_2^- reduced to $0.5N_2O$ (Eqn. 3), $6.37H^+$ is therefore the correct stoicheiometry of H^+ translocation for $2e^-$ passing from NADH to NO_2^-. Because *c*-type cytochromes are involved in the reduction of N_2O [13] and NO_2^- [10,17], it is likely that electrons transported to N_2O and NO_2^- share a common route. So it is expected that the amount of H^+ translocated on an electron basis is equal for the reduction of N_2O to N_2 and NO_2^- to N_2O, provided that the segment of the respiratory chain between cytochrome *c* and N_2O or NO_2^- is not H^+ translocating. Evidence in favour of the latter assumption will be presented below. Comparison of an $\vec{H}^+/N_2O$ stoicheiometry of 4.02 with an $\vec{H}^+/2e^-$ value of 6.37 for the reduction of NO_2^- to $0.5N_2O$ strongly suggests that the two H^+ consumed per N_2O reduced to N_2 (Eqn. 4) are taken from the periplasmic side of the cytoplasmic membrane. Moreover, the $\vec{H}^+/NO_2^-$ value found in the absence of SCN^- (Eqn. 2) is 5.79. Corrected for the H^+ consumed from the periplasmic side of the cytoplasmic membrane and expressed on an electron basis, the $\vec{H}^+/2e^-$ value for the reaction $NO_2^- \rightarrow 0.5N_2$ is 6.53. A value for $\vec{H}^+/2e^-$ of this reaction can also be calculated from the measured $\vec{H}^+$/electron acceptor ratios for the reduction of NO_2^- to N_2O (Eqn. 3) and N_2O to N_2 (Eqn. 4). From Eqns. 2–4, the following equation can be derived for this calculation:

$$\vec{H}^+/2e^-(NO_2^- \rightarrow 0.5N_2) = [\vec{H}^+/NO_2^-(\rightarrow 0.5N_2O) + 0.5(\vec{H}^+/N_2O) + \text{number of } H^+ \text{ consumed}][1.5]^{-1} \quad (5)$$

This calculation yields an $\vec{H}^+/2e^-$ value of 6.25 for the reduction of NO_2^- to $0.5N_2$, if it is assumed that the $4H^+$ consumed in Eqn. 2 are taken from the periplasmic side of the cytoplasmic membrane. This value is in good agreement with the value of 6.53 calculated directly from the conversion of NO_2^- to $0.5N_2$. The consistency of the $\vec{H}^+/2e^-$ values for the reduction of NO_2^- and N_2O obtained in various ways can be regarded as evidence in favour of the assumptions made in these calculations.

H^+/oxidant ratios with ascorbate/TMPD

To obtain more evidence in favour of the above-mentioned proposals we also used ascorbate (+TMPD) as exogenous substrate. At pH 7.0 ascorbate is a 2 electron and 1 H^+ donor, which donates its electrons via TMPD to cytochrome *c* [24]. The overall reactions of ascorbate (A) with NO_2^-, N_2O and $Fe(CN)_6^{3-}$ are given in Eqns. 6–9.

$$1.5AH^- + NO_2^- + 2.5H^+ \rightarrow 1.5A + 0.5N_2 + 2H_2O \quad (6)$$

$$AH^- + NO_2^- + 2H^+ \rightarrow A + 0.5N_2O + 1.5H_2O \quad (7)$$

$$AH^- + NO_2^- + 2H^+ + A + 0.5N_2O + 1.5H_2O \quad (8)$$

TABLE II

STOICHEIOMETRIES OF H^+ TRANSLOCATION IN *P. DENITRIFICANS* WITH O_2, N_2O OR NO_2^- OXIDIZING ENDOGENOUS SUBSTRATES

Cells from type II chemostat cultures were used. Without KSCN the reaction cell contained 12 μg valinomycin/ml and 5 mM $TPMP^+$ and with KSCN 12 μg valinomycin/ml, 5 mM $TPMP^+$ and 100 mM KSCN. The $\vec{H}^+$/electron acceptor ratios are given as averages ± S.D. with the number of pulses in parentheses. The $\vec{H}^+/2e^-$ stoicheiometries corrected for H^+ consumption due to reduction of N_2O and NO_2^- (as discussed in the text) are also presented.

Without KSCN			With KSCN		
Electron acceptor reaction	$\vec{H}^+$/electron acceptor	$\vec{H}^+/2e^-$	Electron acceptor reaction	$\vec{H}^+$/electron acceptor	$\vec{H}^+/2e^-$
$O \rightarrow H_2O$	6.32 ± 0.37 (32)	6.32	$O \rightarrow H_2O$	6.21 ± 0.65 (18)	6.21
$N_2O \rightarrow N_2$	4.02 ± 0.35 (27)	6.02	–	0.11 ± 0.09 (10)	–
$NO_2^- \rightarrow 0.5N_2$	5.79 ± 0.33 (27)	6.53	$NO_2^- \rightarrow 0.5N_2O$	3.37 ± 0.57 (18)	6.37

$$AH^- + 2Fe(CN)_6^{3-} \rightarrow A + 2Fe(CN)_6^{4-} + H^+ \quad (9)$$

When these reactions are followed under conditions unfavourable for the measurement of H^+ translocation, only the scalar H^+ produced or consumed in the periplasmic space will be seen as pH changes in the outer bulk phase. According to Eqns. 6–9 the following theoretical H^+/electron acceptor stoicheiometries will be expected: H^+/NO_2^- ($\rightarrow 0.5N_2$) = –2.5, H^+/NO_2^- ($\rightarrow 0.5N_2O$) = –2.0, H^+/N_2O = –1.0 and $H^+/2Fe(CN)_6^{3-}$ = +1.0 (a negative sign denotes H^+ consumption, a positive sign H^+ production). A typical experiment is shown in Fig. 2. Injection of a small amount of N_2O resulted in rapid alkalinization of the medium (Fig. 2a). Since the cytoplasmic membrane of *P. denitrificans* is characterized by a low H^+ permeability [25], the rapidity of H^+ disappearance is another indication of the periplasmic consumption of H^+ upon reduction of N_2O to N_2. Addition of NO_2^- resulted also in fast alkalinization of the outer bulk phase (Fig. 2b), as has been reported earlier by Meijer et al. [11]. An $Fe(CN)_6^{3-}$ pulse yielded the expected acidification from the non-enzymatic reaction with ascorbate (see Eqn. 9) [24]. In the presence of 100 mM KSCN the pH change with N_2O as oxidant was virtually abolished (Fig. 2d), the extent of alkalinization with NO_2^- diminished (Fig. 2e) and the extent of acidification with $Fe(CN)_6^{3-}$ remained unaltered (Fig. 2f), as was expected. The observed H^+/oxidant ratios are given in Table III. The measured values are in good agreement with the theoretical stoicheiometries. When for each suspension the H^+/NO_2^- ratio in the absence of SCN^- is substracted from that of H^+/NO_2^- in the presence of SCN^- the difference is –0.45 ± 0.16 [12], which is close to the measured ratio of –0.42 ± 0.04 [18] for $0.5 \times H^+/N_2O$. So the H^+/nitrogenous oxides stoicheiometries of Table III are consistent. The same experiments with ascorbate/TMPD as exogenous substrate were also performed in the presence of 12 μg valinomycin/ml and 5 mM $TPMP^+$ in order to determine the extent of H^+ translocation in the segment of the respiratory chain between cytochrome *c* and NO_2^- or N_2O.

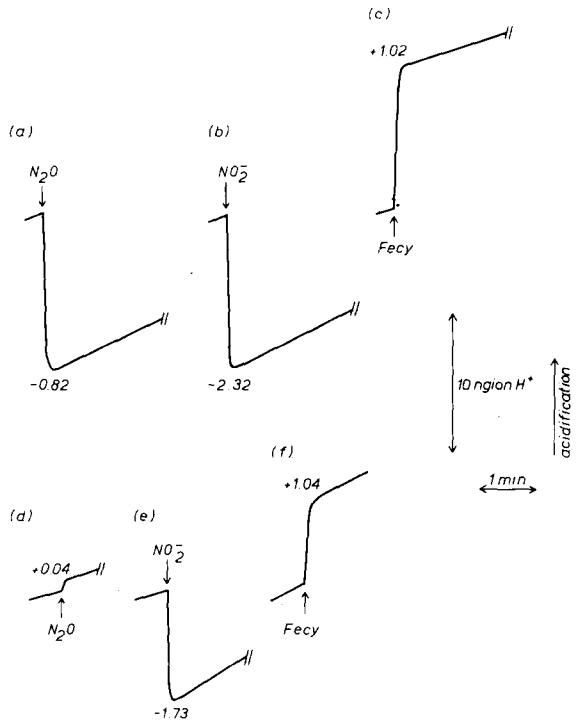

Fig. 2. pH changes induced by injection of N_2O, NO_2^- or $Fe(CN)_6^{3-}$ (Fecy) into an anaerobic suspension of *P. denitrificans* cells, grown in an NO_3^--limited chemostat at $\mu = 0.2$ h^{-1}, in the presence of ascorbate/TMPD as exogenous substrate. Cells from type II chemostat culture were used. The reaction cell contained 1.2 mM potassium ascorbate, 0.2 mM TMPD, 20 μM rotenone and 6.0 μg antimycin A/ml. In d–f, 100 mM KSCN was present. The amounts of oxidant added to the cell suspension were as follows: a and d, 14.7 nmol N_2O; b and e, 5 nmol NO_2^-; c, 20 nmol $Fe(CN)_6^{3-}$; and f, 10 nmol $Fe(CN)_6^{3-}$. The observed H^+/oxidant ratio is shown for each oxidant pulse.

If H^+ translocation does occur in these segments one expects that the translocated H^+ will alter the initial stoicheiometry of the pH change induced by addition of NO_2^- or N_2O. However, this was evidently not the case as can be seen from the average H^+/electron acceptor stoicheiometries presented in Table IV. It is clear that the same picture arises as in Table III. The values of H^+/N_2O of –0.81, H^+/NO_2^- ($\rightarrow 0.5N_2$) of –2.28 and $H^+/2Fe(CN)_6^{3-}$ of +0.98 are in good agreement with the corresponding ratios of –0.84, –2.33 and +0.99, respectively, from Table III. Addition of 100 mM SCN^- resulted again in elimination of H^+/N_2O, increased H^+/NO_2^- and the same $H^+/Fe(CN)_6^{3-}$. When in this case the H^+/NO_2^- values in the absence of SCN^- are subtracted from the H^+/NO_2^- ratios in the presence of SCN^- the difference is –0.51 ± 0.09 [12], which is almost equal to the theoretical difference and compatible with the measured value of –0.41 for $0.5 \times H^+/N_2O$.

TABLE III

H^+/OXIDANT STOICHEIOMETRIES FOR REDUCTION OF N_2O, NO_2^- OR $Fe(CN)_6^{3-}$ IN *P. DENITRIFICANS* CELLS BY ASCORBATE/TMPD

The reaction conditions were the same as those described in the legend of Fig. 2. The H^+/oxidant ratios are presented as averages ± S.D. with the number of pulses in parentheses. The stoicheiometries expected theoretically (Eqns. 6–9) are also included. (valinomycin and $TPMP^+$ absent.)

Without KSCN			With KSCN		
Electron acceptor reaction	Observed H^+/oxidant	Theoretical H^+/oxidant	Electron acceptor reaction	Observed H^+/oxidant	Theoretical H^+/oxidant
$N_2O \rightarrow N_2$	−0.84 ± 0.07 (18)	−1.0	–	+0.02 ± 0.03 (4)	0.0
$NO_2^- \rightarrow 0.5N_2$	−2.33 ± 0.14 (13)	−2.5	$NO_2^- \rightarrow 0.5N_2O$	−1.90 ± 0.24 (13)	−2.0
$2Fe(CN)_6^{3-} \rightarrow 2Fe(CN)_6^{4-}$	+0.99 ± 0.06 (4)	+1.0	$2Fe(CN)_6^{3-} \rightarrow 2Fe(CN)_6^{4-}$	+1.06 ± 0.07 (4)	+1.0

TABLE IV

H^+/OXIDANT STOICHEIOMETRIES FOR REDUCTION OF N_2O, NO_2^- AND $Fe(CN)_6^{3-}$ IN *P. DENITRIFICANS* BY ASCORBATE/TMPD IN THE PRESENCE OF VALINOMYCIN/K^+ AND $TPMP^+$

The conditions were the same as those described in Table III, except for the presence of 12 μg valinomycin/ml and 5 mM $TPMP^+$.

Without KSCN		With KSCN	
Electron acceptor reaction	Observed H^+/oxidant	Electron acceptor reaction	Observed H^+/oxidant
$N_2O \rightarrow N_2$	−0.81 ± 0.06 (19)	–	+0.01 ± 0.02 (6)
$NO_2^- \rightarrow 0.5N_2$	−2.28 ± 0.11 (15)	$NO_2^- \rightarrow 0.5N_2O$	−1.81 ± 0.11 (15)
$2Fe(CN)_6^{3-} \rightarrow 2Fe(CN)_6^{4-}$	+0.98 ± 0.05 (6)	$2Fe(CN)_6^{3-} \rightarrow 2Fe(CN)_6^{4-}$	+1.04 ± 0.10 (9)

Control experiments clearly demonstrated that no pH change was measured after addition of N_2O or NO_2^- to the reaction cell without *P. denitrificans* cells, while injection of $Fe(CN)_6^{3-}$ resulted in an H^+/$2Fe(CN)_6^{3-}$ value of +1.0, which is expected from a non-enzymatic reaction.

Discussion

Previous studies on H^+ translocation with N_2O as electron acceptor were hampered by the inhibiting effect of SCN^- on nitrous oxide reductase.

In contrast to the results of Kristjansson et al. [5], we were not able to find conditions under which reproducible and consistent $\vec{H}^+$/oxidant stoicheiometries were measured in the presence of only valinomycin/K^+ as the permeant ion with cells from an NO_3^--limited continuous cultures. However, these authors only mentioned the maximal $\vec{H}^+/O$ and $\vec{H}^+$/nitrogenous oxide values, measured with cells from anaerobic batch cultures of *P. denitrificans* and *Pseudomonas denitrificans*. Moreover, their presented maximal ratios are not consistent with each other. $\vec{H}^+/N_2O$, $\vec{H}^+/NO_2^-$ ($\rightarrow 0.5N_2O$) and $\vec{H}^+/NO_2^-$ ($\rightarrow 0.5N_2$) values of 4.3, 3.3 and 3.7, respectively, were measured. These maximal $\vec{H}^+/N_2O$ and $\vec{H}^+/NO_2^-$ ($\rightarrow 0.5N_2O$) values are in agreement with the mean values presented in this paper. However, from these two values an $\vec{H}^+/NO_2^-$ ($\rightarrow 0.5N_2$) value of 5.5 can be calculated (see Eqn. 5), which is significantly different from their measured value [5]. Therefore, we conclude that these authors did not measure under optimal H^+-translocating conditions.

With cells from NO_3^--limited chemostat cultures

$\vec{H}^+/O$ ratios between 6.0 and 6.3 are measured during oxidation of endogenous substrates in the presence of: (1) combinations of different concentrations of valinomycin and SCN^-, (2) valinomycin and $TPMP^+$ and (3) valinomycin, SCN^- and $TPMP^+$. Moreover, Van Verseveld et al. [26] have reported an $\vec{H}^+/O$ ratio of 6.21 in the presence of valinomycin and SCN^- for *P. denitrificans* cells grown under NO_3^- limitation with gluconate as carbon and energy source. So an $\vec{H}^+/O$ value of 6.0–6.3 for oxidation of endogenous substrates by cells from NO_3^--limited denitrifying chemostat cultures of *P. denitrificans* seems to be well established.

The combination of 12 μg valinomycin/ml and 5 mM $TPMP^+$ appeared to be suitable for the study of H^+ translocation with NO_2^- and N_2O. The results indicate that the H^+-consuming side of nitrite reductase and nitrous oxide reductase is the periplasmic side of the inner membrane. The evidence is as follows. (1) In the experiments with ascorbate/TMPD as exogenous substrate, the H^+/oxidant ratios were very close to the theoretical ones, when it is assumed that H^+ used in the reduction of NO_2^- to N_2O and N_2O to N_2 is taken from the periplasmic space (see Table III). The theoretical values for these ratios are completely different when the H^+-consuming side is the cytoplasmic side of the inner membrane. Furthermore, when cells from NO_3^--limited chemostat cultures, incubated with the electron-transport inhibitors rotenone and antimycin A, to inhibit the oxidation of endogenous substrates, were pulsed with N_2O or NO_2^- in the presence of ascorbate/TMPD, rapid alkalinization of the outer bulk medium could be observed (see Fig. 2). Since the cytoplasmic membrane of *P. denitrificans* has a low H^+ permeability [25], the rapidity of H^+ disappearance is additional evidence for the periplasmic orientation of the H^+-consuming side of these reductases.

The H^+/oxidant ratios were not influenced by the presence of valinomycin and $TPMP^+$, indicating that the possibility of an H^+ translocating character of the segment of the respiratory chain between cytochrome *c* and NO_2^- or N_2O can be excluded (see Table IV). (2) The electrons destined for NO_2^- and N_2O share the same route through the respiratory chain as far as the *c*-type cytochromes [10,13,17,27,28]. Since the segment of the respiratory chain between cytochrome *c* and NO_2^- or N_2O is not H^+ translocating, one would expect the same $\vec{H}^+/2e^-$ value for the reduction of NO_2^- to $0.5N_2O$ and N_2O to N_2. Moreover, electrons transported to O_2 also pass two sites of energy conservation. Therefore an $\vec{H}^+/2e^-$ value of about 6 is expected for the reduction of O_2, NO_2^- and N_2O. When the $\vec{H}^+$/electron acceptor ratios are corrected for the number of H^+ consumed in the reduction of NO_2^- and N_2O at the periplasmic side of the inner membrane these expected values are found. If this correction is made, consistent values between 6.02 and 6.53 are found for the $\vec{H}^+/2e^-$ ratios for the reduction of O_2, NO_2^- and N_2O (see Table II).

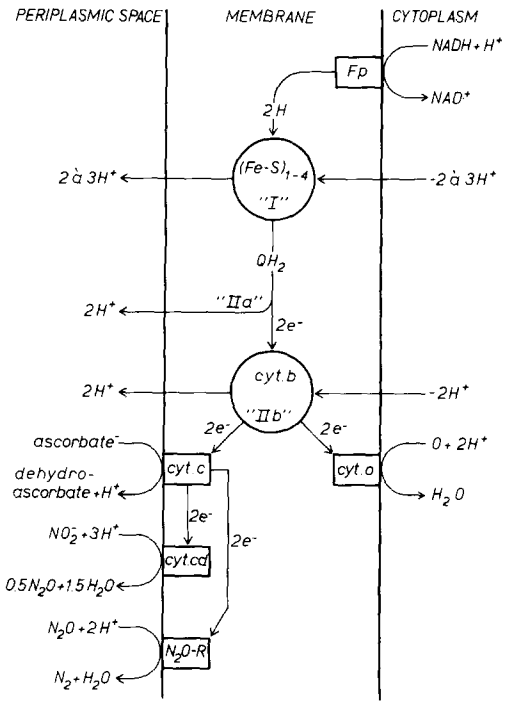

Fig. 3. Simplified scheme of H^+ translocation and electron transport to NO_2^-, N_2O and O_2 in denitrifying *P. denitrificans*. Fp, flavoprotein; Fe-S, iron-sulphur centre; QH_2. ubiquinol; cyt., cytochrome; N_2O-R, nitrous oxide reductase. I and IIa + IIb are the traditional sites of energy conservation.

The results of this study are summarized in a simplified scheme of electron transport to NO_2^-, N_2O and O_2 in dinitrifying *P. denitrificans* (Fig. 3). All electron donors and acceptors used as well as the places where H^+ translocation can occur are indicated. In Table V the evidence in favour of this scheme is summarized. The experimental $\vec{H}^+$/nitrogenous oxide ratios with endogenous substrates and the H^+/nitro-

TABLE V

SUMMARY OF THEORETICAL AND EXPERIMENTAL H^+/OXIDANT STOICHEIOMETRIES WITH NO_2^-, N_2O AND O_2 DURING OXIDATION OF ENDOGENOUS SUBSTRATES OR ASCORBATE/TMPD

The theoretical $\vec{H}^+$/oxidant ratios with endogenous substrates and the theoretical H^+/oxidant values with ascorbate/TMPD have been calculated on the basis of Fig. 3. (Column 1) It is assumed that H^+, consumed in reduction of the oxidants, is taken from the cytoplasm. (Column 2) It is assumed that H^+, consumed in reduction of the oxidants, is taken from the periplasm.

Endogenous substrates				Ascorbate/TMPD		
Electron acceptor reaction	Theoretical $\vec{H}^+$/oxidant		Experimental $\vec{H}^+$/oxidant [a]	Theoretical H^+/oxidant		Experimental H^+/oxidant [b]
	1	2		1	2	
$NO_2^- \rightarrow 0.5\ N_2$	9–10.5	5–6.5	5.79	+1.5	−2.5	−2.33
$NO_2^- \rightarrow 0.5\ N_2O$	6– 7	3–4	3.37	+1.0	−2.0	−1.90
$N_2O \rightarrow N_2$	6– 7	4–5	4.02	+1.0	−1.0	−0.84
$O \rightarrow H_2O$	6– 7	4–5	6.0–6.3			

[a] Mean $\vec{H}^+$/oxidant values from Table II.
[b] Mean H^+/oxidant values from Table III.

genous oxide values with ascorbate/TMPD are clearly not in agreement with cytoplasmic H^+ consumption upon reduction of NO_2^- to N_2. The experimental $\vec{H}^+/O$ ratio, however, is in accordance with cytoplasmic H^+ consumption upon reduction of O_2.

The framework of Fig. 3 is very similar to that presented by Van Verseveld et al. [24] for aerobically grown *P. denitrificans*. Besides the presence of the denitrifying enzymes, the most striking difference between this and the aerobic scheme is the absence of cytochrome aa_3. Although in a recent paper [29] cytochrome aa_3 was detected in vesicles of anaerobically grown *P. denitrificans* in batch culture, this cytochrome seems to be absent in cells of NO_3^--limited chemostat cultures [13].

Since the H^+/NO_2^- value in the presence of SCN^- with ascorbate/TMPD as substrate has a negative value of 1.8–1.9, and not 1.5, the product of NO_2^- reduction by cytochrome *cd* has been designated as N_2O and not as NO, despite the claim that the product of NO_2^- reduction by the purified *cd*-type nitrite reductase from *Thiobacillus denitrificans* [30] and *Pseudomonas perfectomarinus* [31] is NO. However, other authors have reported that purified nitrite reductases from *Pseudomonas aeruginosa* [32], *Alcaligenes faecalis* [33] and also *T. denitrificans* [34] convert NO_2^- to NO as well as N_2O. Furthermore, from *Ps. perfectomarinus* a cytoplasmic membrane fraction with tightly bound cytochrome *cd* was obtained, which reduced NO_2^- to N_2O in a stoicheiometric reaction without NO as free intermediate [35]. The authors suggested that cytochrome *cd* might catalyze the direct reduction of NO_2^- to N_2O. Since it has been shown that four electrons are transferred to O_2 by the nitrite reductase of *P. denitrificans*, resulting in the formation of H_2O [12], it is tempting to assume that in an analogous manner four electrons are transferred to two molecules of NO_2^-, resulting in the production of N_2O. Although the substructure and the haem content of the *Pseudomonas* nitrite reductase [36] are consistent with a four-electron reduction, at the moment, however, there is little experimental evidence supporting this view [2].

Concerning the role of N_2O in denitrification, there are now many experimental data consistent with the occurrence of N_2O as an obligate and free intermediate in *P. denitrificans* [5,13,14,37,38].

The segments of the respiratory chain where H^+ ejection does occur are tentatively assigned to the iron-sulphur centre 1–4 (site I), ubiquinol (site IIa) and cytochrome *b* (site IIb). Since in the aerobic respiratory chain of *P. denitrificans* electron transport through the ubiquinol-cytochrome *c* segment is coupled to ejection of $2H^+$ per transferred electron [24], we assume that the $\vec{H}^+/e^-$ stoicheiometry for this span is the same afted growth on NO_3^-. More-

over, there exists a general agreement as to the stoicheiometry of H^+ translocation for site II in mitochondria, i.e., $\vec{H}^+/e^- = 2$ [39]. Recently, a model for H^+ ejection in site II of the mitochondrial respiratory chain has been proposed [40] which is similar to the corresponding part of the scheme in Fig. 3 and of the aerobic scheme [24]. A fixed value of 4 for $\vec{H}^+/2e^-$ of site II means that the number of H^+ translocated per electron pair in site I has to be between 2 and 3.

Based on Fig. 3, the $\vec{q}^+/2e^-$ value (number of charges translocated across the cytoplasmic membrane during flow of $2e^-$ to an electron acceptor) for the different reductions can be calculated. The reduction of O_2 to H_2O results in a $\vec{q}^+/2e^-$ value of 6–7. The $\vec{q}^+/2e^-$ values for the reduction of NO_2^- to N_2O and for the reduction of N_2O to N_2 are both 4–5. Since the efficiency of oxidative phosphorylation is related to the $\vec{q}^+/2e^-$ ratio, we can conclude that the efficiency of oxidative phosphorylation during electron transport to NO_2^- and N_2O is the same when expressed on an electron basis. The efficiency of oxidative phosphorylation during electron transport to these hydrogen acceptors is 67–71% of that during electron transport to O_2.

These findings are very similar to the results of Koike and Hattori [41,42], derived from growth yield determinations with *Ps. denitrificans* under aerobic and denitrifying conditions. They conclude that oxidative phosphorylation occurs to a similar extent in the electron-transport chains associated with the reduction of NO_2^- to N_2O and N_2O to N_2 and that denitrification is about 40% less efficient than aerobic respiration [41,42]. It also explains the lower growth yield of *P. denitrificans* under denitrifying conditions compared with the aerobic growth found by Van Verseveld et al. [26]. They concluded that the lower growth yields are explained by the disfunctioning of either site I or site II phosphorylation, probably by the uncoupling effect of NO_2^- [16]. Now a more probable conclusion is that electron transport to NO_2^- and N_2O via sites I and II has an efficiency of about 70% of that during electron transport to O_2, which fits well with the $P/2e^-$ ratio calculated from growth experiments [26].

Acknowledgements

This investigation was supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

1 Payne, W.J. (1973) Bacteriol. Rev. 37, 409–452
2 Zumft, W.G. and Cárdenas, J. (1979) Naturwissenschaften, 66, 81–88
3 Forget, P. and DerVartanian, D.V. (1972) Biochim. Biophys. Acta 256, 600–606
4 Lam, Y. and Nicholas, D.J.D. (1969) Biochim. Biophys. Acta 172, 450–461
5 Kristjansson, J.K., Walter, B. and Hollocher, T.C. (1978) Biochemistry 17, 5014–5019
6 Alefounder, P.R. and Ferguson, S.J. (1980) Biochem. J. 192, 231–240
7 John, P. (1977) J. Gen. Microbiol. 98, 231–238
8 Scholes, P. and Smith, L. (1968) Biochim. Biophys. Acta 153, 363–375
9 Newton, N. (1969) Biochim. Biophys. Acta 185, 316–331
10 John, P. and Whatley, F.R. (1975) Nature 254, 495–498
11 Meijer, E.M., Van der Zwaan, J.W. and Stouthamer, A.H. (1979) FEMS Microbiol. Lett 5, 369–372
12 Timkovich, R. and Robinson, M.K. (1979) Biochem. Biophys. Res. Commun. 88, 649–655
13 Boogerd, F.C., Van Verseveld, H.W. and Stouthamer, A.H. (1980) FEBS Lett. 113, 279–284
14 Kristjansson, J.K. and Hollocher, T.C. (1980) J. Biol. Chem, 255, 704–707
15 Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research Ltd., Bodmin, U.K.
16 Meijer, E.M., Van der Zwaan, J.W. and Stouthamer, A.H. (1979) Eur. J. Biochem. 96, 69–76
17 Lam, Y. and Nicholas, D.J.D. (1969) Biochim. Biophys. Acta 172, 450–461
18 Van 't Riet, J., Van Ee, J.H., Wever, R., Van Gelder, B.F. and Planta, R.J. (1975) Biochim. Biophys. Acta 405, 306–317
19 Radcliffe, B.C. and Nicholas, D.J.D. (1970) Biochim. Biophys. Acta 205, 273–287
20 Chang, J.P. and Morris, J.G. (1962) J. Gen. Microbiol. 29, 301–310
21 Meijer, E.M., Van Verseveld, H.W., Van der Beek, E.G. and Stouthamer, A.H. (1977) Arch. Microbiol. 112, 25–34
22 Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147–1162
23 Scholes, P. and Mitchell, P. (1970) J. Bioenerg. 1, 309–323
24 Van Verseveld, H.W., Krab, K. and Stouthamer, A.H. (1981) Biochim. Biophys. Acta 635, 525–534
25 Scholes, P. and Mitchell, P. (1970) J. Bioenerg. 1, 61–72
26 Van Verseveld, H.W., Meijer, E.M. and Stouthamer, A.H. (1977) Arch. Microbiol. 112, 17–23

27 Matsubara, T. (1975) J. Biochem. (Tokyo) 77, 627–632
28 Walter, B., Sidransky, E., Kristjansson, J.K. and Hollocher, T.C. (1978) Biochemistry 17, 3039–3045
29 Kula, T., Stellwagen, E., Szentirmay, R. and Kuwana, T. (1981) Biochim. Biophys. Acta 634, 279–288
30 Legall, J., Payne, W.J., Morgan, T.V. and DerVartanian, D.V. (1979) Biochem. Biophys. Res. Commun. 87, 355–362
31 Zumft, W.G., Sherr, B.F., and Payne, W.J. (1979) Biochem. Biophys. Res. Commun. 88, 1230–1236
32 Wharton, D.C. and Weintraub, S.T. (1980) Biochem. Biophys. Res. Commun. 97, 236–242
33 Matsubara, T. and Iwasaki, H. (1972) J. Biochem. (Tokyo) 72, 57–64
34 Sawhney, V. and Nicholas, D.J.D. (1978) J. Gen. Microbiol. 106, 119–128
35 Zumft, W.G. and Vega, J.M. (1979) Biochim. Biophys. Acta 548, 484–499
36 Kuronen, T., Saraste, M. and Ellfolk, N. (1975) Biochim. Biophys. Acta 393, 48–54
37 Pichinoty, F., Mandel, M. and Garcia, J.L. (1977) Ann. Microbiol. (Paris) 128B, 243–251
38 McCarthy, J.E.G., Ferguson, S.J. and Kell, D.B. (1981) Biochem. J. 196, 311–321
39 Wikström, M. and Krab, K. (1980) Curr. Top. Bioenerg. 10, 51–101
40 Alexandre, A., Galiazzo, F. and Lehninger, A.L. (1980) J. Biol. Chem. 255, 10 721–10 730
41 Koike, J. and Hattori, A. (1975) J. Gen. Microbiol. 88, 1–10
42 Koike, J. and Hattori, A. (1975) J. Gen. Microbiol. 88, 11–19