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THE STOICHEIOMETRY OF RESPIRATION-DRIVEN PROTON TRANSLOCATION IN *PSEUDOMONAS* AM1 AND IN A MUTANT LACKING CYTOCHROME *c*

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This paper clarifies the role of cytochrome *c* in *Pseudomonas* AM1 by measuring the stoicheiometry of proton translocation driven by respiration of endogenous or added substrates in wild-type bacteria and in a mutant lacking cytochrome *c* (mutant PCT76). The maximum $\rightarrow H^+/O$ ratio (protons translocated out of the bacteria per atom of oxygen consumed during respiration) was about 4 and, except when respiration was markedly affected, this ratio was similar in mutant and wild-type bacteria. The $\rightarrow H^+/O$ ratios were unaltered when the usual oxidase (cytochrome a_3) was inhibited by 300μM-KCN and respiration involved the single cytochrome *b* functioning as an alternative oxidase. Ratios measured in cells respiring endogenous substrate and in cells loaded with malate or 3-hydroxybutyrate suggest that there are two proton-translocating segments operating during the oxidation of NADH. By contrast, during oxidation of formaldehyde or methylamine only one pair of protons is translocated. Proton translocation could not be measured with methanol as substrate, because its oxidation was inhibited (90–95%) by 5mM-KSCN. It is tentatively proposed that the electron-transport chain for NADH oxidation in *Pseudomonas* AM1 is arranged such that the NADH–ubiquinone oxidoreductase forms one proton-translocating segment and the second segment consists of ubiquinone and cytochromes *b* and a/a_3. The cytochrome *c* appears to be essential only for respiration and proton translocation from methanol (and possibly from methylamine); there is no conclusive evidence that cytochrome *c* ever mediates between cytochromes *b* and a/a_3 in *Pseudomonas* AM1.

Pseudomonas AM1 is a typical facultative methylotroph able to grow on a range of carbon and energy sources, including C_1 compounds but excluding methane (Quayle, 1972; Anthony, 1975*a*). A scheme for electron transport in this bacterium has been proposed (Widdowson & Anthony, 1975) in which methanol is oxidized by way of an unusual 'pteridoprotein' dehydrogenase (Anthony & Zatman, 1967), a soluble cytochrome *c* (Anthony, 1975*b*) and cytochrome a/a_3. It has been proposed that there is only one physiological oxidase (cytochrome a_3) (Widdowson & Anthony, 1975) and it was initially proposed that the oxidation of all other substrates (except perhaps methylamine) involves cytochromes *b*, *c* and a/a_3 (Anthony, 1970). However, results with a mutant lacking cytochrome *c* (mutant PCT76) suggested either that this cytochrome is involved only in the oxidation of methanol (and perhaps methylamine) or that there is an alternative route for electron transport between cytochromes *b* and a/a_3 in wild-type bacteria which becomes the sole route for electron transport in the cytochrome *c*-deficient mutant (Anthony, 1975*b*; Widdowson & Anthony, 1975).

It is well established that the nature and arrangement of electron-transport components in the bacterial membrane influences the stoicheiometry of respiration-driven proton translocation; bacteria with cytochromes *b*, *c* and a/a_3 usually have three proton-translocating segments, whereas bacteria with no cytochrome *c* usually have only two segments (Haddock & Jones, 1977; Jones, 1977). The present paper describes experiments aimed at elucidating the role of cytochrome *c* in *Pseudomonas* AM1 by measuring the stoicheiometry of respiration-driven proton translocation in wild-type bacteria and in the mutant lacking cytochrome *c* (mutant PCT76).

The stoicheiometry of proton translocation during oxidation of a given substrate is often taken as a measure of the P/O ratio for that substrate, the P/O ratio usually being half the measured $\rightarrow H^+/O$ ratio (see Mitchell, 1970; Jones, 1977). The $\rightarrow H^+/O$ ratios reported in the present paper may thus give some indication of the amount of ATP likely to be

available for growth as a result of oxidative phosphorylation in *Pseudomonas* AM1. Discussions of growth yields of methylotrophs usually assume a P/O ratio of 3 for oxidation of formate by way of NADH, and because these bacteria always have cytochrome *c* this has perhaps been a reasonable assumption (see van Dijken & Harder, 1975). However, the only proton-translocation measurements so far published for methylotrophs indicate a maximum P/O ratio of 1 for all substrates (Tonge *et al.*, 1977). This low value in an obligate methane-utilizer contrasts with the results in the present paper obtained with a facultative methanol-utilizer, in which a maximum P/O ratio of 2 for NADH oxidation is indicated.

Some of the conclusions given here have been presented to the Society for General Microbiology (O'Keeffe & Anthony, 1977; Anthony & O'Keeffe, 1977).

Materials and Methods

Chemicals

All chemicals (including carbonate dehydratase) were obtained from BDH Chemicals, Poole, Dorset, U.K., except for carbonyl cyanide *m*-chlorophenylhydrazone, oligomycin and valinomycin [all from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] and dicyclohexylcarbodiimide (from Aldrich Chemical Co., Gillingham, Dorset, U.K.).

Organisms and growth media

Pseudomonas AM1 (N.C.I.B. 9133) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K. Mutant PCT76 was isolated by Dr. Patricia Dunstan of this department and has been described previously (Anthony, 1975*b*); this mutant lacks cytochrome *c* and methylamine dehydrogenase and grows on all the same substrates as wild-type bacteria except for methanol, ethanol and methylamine. Stock cultures of wild-type *Pseudomonas* AM1 were maintained on methylamine/agar, and of the mutant on succinate/agar. The defined liquid growth medium was that described by Anthony & Zatman (1964), carbon sources being used at 0.2%, except for methanol and methylamine (0.4%).

Growth and harvesting of bacteria

Bacteria were grown as 1-litre batch cultures in 2-litre baffled flasks at 30°C on a rotary shaker. Unless otherwise stated cells were harvested at late-exponential phase, washed twice at 2°C in 140mM-KCl and resuspended in this medium to a cell density of 120mg dry wt./ml; they were stored at 0–4°C and used for determination of $\rightarrow H^+/O$ ratios within 10h of harvesting.

Measurement of cytochrome oxidation rates

These measurements were made exactly as described previously (Widdowson & Anthony, 1975), except where stated.

Measurement of $\rightarrow H^+/O$ ratios

The pulse-oxidant technique used was essentially that of Mitchell & Moyle (1967). The pH was measured with Russel CMAT/72 electrodes with a Pye–Unicam 290 pH-meter and recorded on a Servoscribe RE 541.2 potentiometric recorder with full-scale deflection corresponding to a ΔpH of 0.1. The 6ml reaction vessel contained, in a 4ml reaction mixture, 66mM-KSCN, 140mM-KCl, 18–25mg dry wt. of washed bacterial suspension and KCN (300μM or 4mM), or respiratory substrate when required. The concentration of substrates was 2.5–5.0mM, except for ascorbate (1.5mM) plus tetramethylenephenylenediamine (4mM). Solutions to be added were made anaerobic by passage of a stream of water-saturated O_2-free N_2. Any residual O_2 in the bacterial suspensions was removed by their own respiration and anaerobiosis was maintained by passing a stream of N_2 over the surface of the bacterial suspension, which was incubated at 30°C. Although the $\rightarrow H^+/O$ ratios were constant when measured in the pH range 5.8–7.5 all experiments described below were done at a starting pH of 6.6–6.8. After 45min to equilibrate cell suspensions, O_2 was added in 5–20μl volumes of air-saturated 140mM-KCl, at least four different volumes being used for each determination. It was assumed that 10μl of air-saturated KCl at 30°C contains 4.45ng-atoms of O. The resulting acidification of the external medium was standardized with 5–20μl volumes of anaerobic 2mM-HCl. The change in pH on addition of O_2 or HCl was complete within 2–4s and the ΔpH was calculated as described by Mitchell & Moyle (1965), extrapolation being made back to the time taken for half the initial pH rise to occur. Graphs relating the ΔpH and the amount of standard HCl or O_2 injected were plotted and used to calculate the $\rightarrow H^+/O$ ratios (ng-ions of H^+ translocated from the bacteria into the external medium per ng-atom of O consumed). As is usually the case with measurements of respiration-driven pH changes, our methods do not permit a distinction between proton extrusion and uptake of OH^- ions (West & Mitchell, 1972).

Measurement of bacterial respiration

These measurements were made with a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.) and, unless otherwise stated, the same reaction mixtures were used as for the proton-translocation measurements. When inhibitors were used these were preincubated with the cells for at least 10min before measurement of respiration rates.

Results

Proton translocation driven by endogenous respiration in Pseudomonas AM1

Fig. 1 shows typical pH traces obtained when various amounts of O_2 (dissolved in 140mM-KCl) were injected into anaerobic suspensions of *Pseudomonas* AM1. The decay of the ΔpH showed first-order kinetics with a $t_{\frac{1}{2}}$ (half-life) of usually about 60s. The uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (2nmol/mg dry wt.) abolished the response to subsequent additions of O_2. Injection of 5–20μl volumes of anaerobic 2M-NaCl caused no decrease in the pH of the medium, showing that, unlike *Escherichia coli* (West & Mitchell, 1974), *Pseudomonas* AM1 has no Na^+/H^+ antiporter system. Inclusion of carbonate dehydratase (40μg/ml) in the incubation had no effect on the measured $\rightarrow H^+/O$ ratio in wild-type or mutant bacteria in the absence or presence of added respiratory substrates.

Fig. 2 shows the effect on measured $\rightarrow H^+/O$ ratios of various thiocyanate concentrations in methanol- and succinate-grown cells. A concentration of 66mM-KSCN was chosen for all further experiments, and it was confirmed that this was a suitable concentration for use with all bacteria grown on all the substrates used in this work. The low $\rightarrow H^+/O$ ratio of about 0.5 was always found in the absence of KSCN in wild-type and mutant bacteria regardless of growth substrate. Neither valinomycin (5μg/ml) nor KNO_3 (70mM) was a suitable alternative to thiocyanate as a means of dissipating the membrane potential which is necessary for measurement of maximum proton translocation (Mitchell & Moyle, 1965). Bacteria were not sensitized to the action of valinomycin by prior washing with 2mM-EDTA. The adenosine triphosphatase inhibitors oligomycin and dicyclohexylcarbodi-imide had no effect on respiration or on the measured $\rightarrow H^+/O$ ratio.

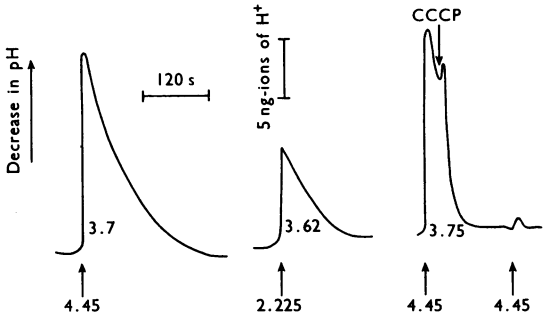


Fig. 1. *pH changes resulting from addition of O_2 to anaerobic suspensions of Pseudomonas AM1*
The pH-measuring apparatus and a detailed description of the methods are given in the Materials and Methods section. Cells were grown on methanol as the sole carbon and energy source: about 6mg dry wt./ml was incubated at 30°C in a 4ml reaction volume containing 140mM-KCl and 66mM-KSCN at pH6.6–6.8. The amount of O_2 (ng-atoms) added in each pulse of air-saturated KCl is indicated; addition of anaerobic KCl elicited no change in pH. The addition of uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 48nmol) is indicated by an arrow. The $\rightarrow H^+/O$ ratio is indicated for each oxygen pulse.

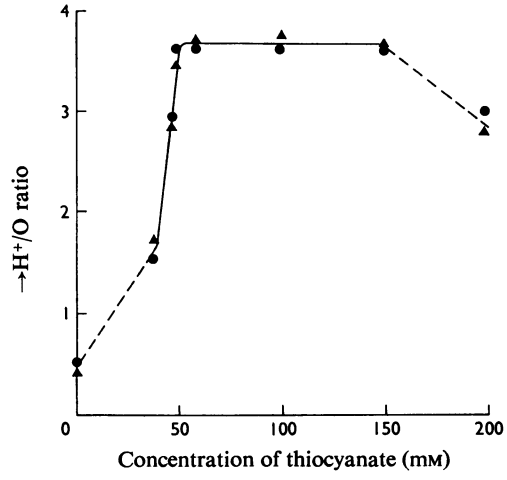


Fig. 2. *Variation of the $\rightarrow H^+/O$ ratio with thiocyanate concentration in Pseudomonas AM1*
Cells were grown on methanol (●), or on succinate (▲) as the sole carbon and energy source. $\rightarrow H^+/O$ ratios were determined as described in the Materials and Methods section in the presence of various concentrations of thiocyanate.

Effect of growth phase and growth substrate on the stoicheiometry of proton translocation coupled to endogenous respiration in Pseudomonas AM1 and in mutant PCT76 lacking cytochrome c

To test possible variations in $\rightarrow H^+/O$ ratio during growth, bacteria were harvested at ten different sampling times up to 24h into the stationary phase and the proton translocation coupled to endogenous respiration was measured. It was shown that the measured $\rightarrow H^+/O$ ratios were not influenced by the phase of growth for bacteria growing on methanol, methylamine, succinate or malate. This contrasts with results obtained in *Paracoccus denitrificans*, where the respiratory substrate varied during growth, thus leading to a wide variation in $\rightarrow H^+/O$ ratios (Lawford *et al.*, 1976).

The results in Table 1 show that the $\rightarrow H^+/O$ ratio

Table 1. *→H⁺/O ratios measured during respiration of endogenous substrate in Pseudomonas AM1 and mutant PCT76 lacking cytochrome c*

Cells were grown in batch culture and harvested at the end of the experimental phase of growth. The →H⁺/O ratio was measured as described in the Materials and Methods section; the values are presented as the means±S.E.M., with the numbers of determinations for separate batches of cells in parentheses. The range of endogenous respiration rates with wild-type bacteria was 5–15nmol of O_2 taken up/min per mg dry wt. and the range of values for mutant PCT76 was 8–15nmol/min per mg.

Carbon source for growth	→H⁺/O ratio: In *Pseudomonas* AM1	→H⁺/O ratio: In mutant PCT76
Methanol	3.45±0.23 (12)	–
Methylamine	3.80±0.11 (11)	–
Malate	3.36±0.27 (6)	3.8 ±0.15 (3)
Succinate	4.20±0.26 (11)	3.7 ±0.21 (6)
3-Hydroxybutyrate	3.69±0.30 (6)	3.42±0.07 (2)
Average →H⁺/O ratio	3.81±0.10 (46)	3.64±0.14 (11)
→H⁺/O ratio measured in the presence of 300μM-KCN	3.95±0.40 (6)	3.90±0.25 (4)

measured during endogenous respiration (average 3.81) was hardly affected by the growth substrate used and that the stoicheiometry was unaltered in the mutant lacking cytochrome *c* (average 3.64). This stoicheiometry indicates the presence of two translocating segments coupled to endogenous respiration in wild-type and mutant *Pseudomonas* AM1.

Effect of cyanide on the stoicheiometry of proton translocation coupled to endogenous respiration in wild-type and mutant bacteria

The results in Table 1 show that the →H⁺/O ratios in wild-type and mutant bacteria were unaffected by 300μM-KCN. This concentration was previously shown to have little effect on the endogenous respiration rate or on the oxidation of cytochrome *b* but to inhibit the oxidation of cytochromes *c* and a/a_3, and to inhibit respiration of all added substrates to about the endogenous value (Widdowson & Anthony, 1975). It was concluded that the usual oxidase in *Pseudomonas* AM1 is always cytochrome a_3 and that the slowly autoxidizable cytochrome *b* (mid-point potential 9mV) might act as an alternative oxidase in the presence of 300μM-KCN. That this is also the case during these studies on proton translocation was shown by repeating these experiments (and achieving the same results) with exactly the same cell suspensions and reaction mixtures (containing 66mM-KSCN and 140mM-KCl) as were used for the determination of →H⁺/O ratios. A higher concentration of cyanide (4mM) completely abolished respiration, proton translocation and oxidation of all cytochromes in wild-type and mutant *Pseudomonas* AM1.

Stoicheiometry of proton translocation coupled to respiration of exogenous substrates in Pseudomonas AM1 and in mutant PCT76 lacking cytochrome c

Although preferable for these experiments, it was impossible to diminish the endogenous respiration markedly by washing or by incubating harvested bacteria in growth medium with no carbon source for up to 24h. This is possibly because the endogenous substrate is NADH, which is constantly replenished by oxidation of 3-hydroxybutyrate, the hydrolysis product of poly-(3-hydroxybutyrate), which constitutes up to 10% of the dry weight of the bacteria. The concentration of storage product was insufficiently diminished by growth in carbon-limited continuous culture to decrease the endogenous respiration rate.

The →H⁺/O ratios for bacteria respiring with a variety of exogenous substrates are given in Table 2. The concentration of thiocyanate (66mM) used had very little effect on the rates of oxidation of endogenous substrate, formaldehyde or 3-hydroxybutyrate, but the oxidation of other substrates was inhibited to some extent (methylamine, 25% inhibition; formate, malate and succinate, 45%; ascorbate/tetramethylenephenylenediamine, 64%; methanol, 90–95%). Methanol oxidation was also 90% inhibited with 5mM-KSCN, and thus determination of →H⁺/O ratios for methanol oxidation was impossible. The presence of methanol had no effect on proton translocation driven by respiration of endogenous substrate. With the substrates listed in Table 2 the rate of decay of the ΔpH was about the same as that measured with endogenous substrate, but it was up to 10 times faster when cells were incubated with formate at a suitable concentration (40mM) for maximum respiration, and this prevented meaningful measurements of the →H⁺/O ratio for formate. The →H⁺/O ratios measured in mutant PCT76 were unaffected by methanol, methylamine or ascorbate/tetramethylenephenylenediamine because cytochrome *c* is essential for oxidation of these substrates.

In wild-type bacteria, incubation with methylamine, succinate, malate or 3-hydroxybutyrate all diminished the →H⁺/O ratio from about 4 (3.81) to about 3 (2.8–3.2), and incubation with formaldehyde or ascorbate/tetramethylenephenylenediamine diminished the ratio still further, to about 2.2. In mutant PCT76 incubation with succinate had the same affect, whereas incubation with malate or 3-hydroxybutyrate had less affect on the ratio than in wild-type bacteria.

Table 2. *→H⁺/O ratios and respiration rates measured with various added substrates in Pseudomonas AM1 and mutant PCT76*
Measurements of →H⁺/O ratios and respiration rates were as described in the Materials and Methods section. Values are presented as the means±S.E.M., with the numbers of determinations from separate batches of cells given in parentheses. Respiration rates with added substrate have been corrected for the endogenous rates measured in the same batch of cells. Respiration rates were measured in the same incubation mixture as was used for measurement of →H⁺/O ratios. Respiration rates are expressed as nmol of O_2 consumed/min per mg dry wt.

	Pseudomonas AM1		Mutant PCT76	
Respiratory substrate	→H⁺/O ratio	Respiration rate	→H⁺/O ratio	Respiration rate
Endogenous	3.81±0.10 (46)	5–15	3.64±0.14 (11)	8–15 (11)
Methylamine	3.12±0.15 (14)	12.5±4.75 (14)	–	–
Succinate	3.19±0.31 (10)	17.9±1.94 (10)	3.03±0.31 (6)	24±4.8 (6)
Malate	2.89±0.14 (10)	28.0±4.9 (10)	4.07±0.36 (3)	28±9.4 (3)
3-Hydroxybutyrate	3.09±0.31 (9)	5.1±2.8 (9)	3.58±0.35 (4)	11±2.1 (4)
Formaldehyde	2.21±0.33 (10)	17.8±6.1 (10)	2.90±0.27 (4)	8±0.4 (4)
Ascorbate/tetramethyl-enephenylenediamine	2.28±0.27 (5)	10.3±4.2 (5)	–	–

The diminished effect of formaldehyde on the →H⁺/O ratio in the mutant was probably due to the lowered respiration rate.

pH changes resulting from addition of anaerobic substrates to anaerobic suspensions of Pseudomonas AM1 and mutant PCT76

In an attempt to interpret the →H⁺/O ratios measured with exogenous substrates the possibility of substrate-linked influx or efflux of protons or OH^- ions during measurements of →H⁺/O ratios was investigated. In these experiments small amounts of anaerobic substrates (in a volume of 3–15 µl, final concentration in reaction mixture 2.5 mM) were injected into the same cell suspensions as were used for proton-translocation experiments and the pH was recorded. The pH of the substrate was adjusted before injection to that of the cell suspension and pH changes were standardized in the usual way with anaerobic 2 mM-HCl.

In wild-type *Pseudomonas* AM1 rapid pH changes occurred (15–50 ng-ions of H^+ in 4 s), but injection of substrate led to no pH changes in the mutant. In wild-type bacteria there was no change in pH on addition of methanol, but the pH increased on addition of succinate, malate and 3-hydroxybutyrate, and decreased with methylamine and formaldehyde. The pH changes were the same in the presence or absence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone or of thiocyanate. The simplest interpretations of these observations is that the pH changes result from rapid entry of substrate into the cells and that anions enter together with a proton, whereas methylamine enters in the uncharged form, leaving its proton in the surrounding medium. There is no straightforward explanation for the result with formaldehyde nor for the lack of pH changes in the mutant with no cytochrome *c*.

Although these preliminary observations cannot be used quantitatively and await further investigation with ^{14}C-labelled substrates, they do perhaps aid interpretation of the results in Table 2. If proton movements across the cell membrane are occurring with concomitant uptake of respiratory substrate during the O_2 pulse in experiments measuring →H⁺/O ratios, then the true values will be higher than those recorded in Table 2 for malate and 3-hydroxybutyrate (probably giving values approaching the endogenous value of about 4), and lower than those recorded for formaldehyde and methylamine (probably decreasing towards a value of 2). The higher →H⁺/O ratios measured with malate and 3-hydroxybutyrate in mutant PCT76, in which no substrate-linked proton movements were recorded, support this interpretation. That the →H⁺/O ratio measured with succinate is lower than that measured during respiration with endogenous substrate in wild-type bacteria, and lower than that measured with malate and 3-hydroxybutyrate in mutant PCT76, suggests that there are fewer proton-translocating segments associated with succinate respiration than with these other substrates; the observation that succinate may be transported with a proton, however, confuses the interpretation of these results.

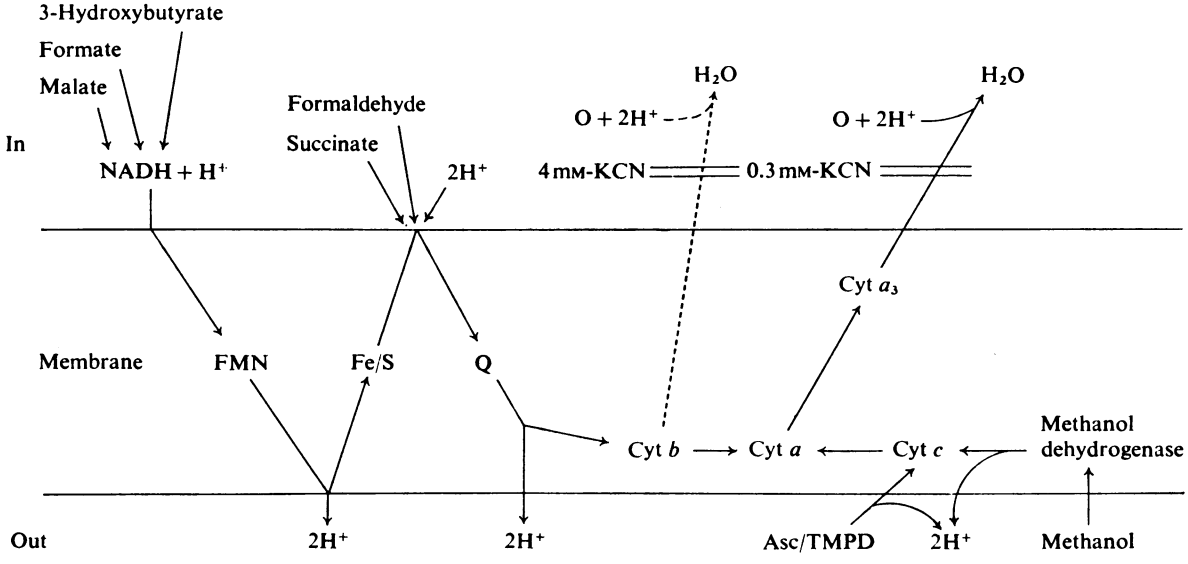
Discussion

Because *Pseudomonas* AM1 has cytochromes *a*, *b* and *c*, it might be expected that during respiration the same three proton-translocating segments (or loops) would operate as in other bacteria having this particular complement of cytochromes (see Haddock & Jones, 1977; Jones, 1977). If this were the case then segment 1 would be NADH–ubiquinone oxidoreductase, and segments 2 and 3 (or 2 plus 3 in a 'Q cycle') would involve two species of cytochrome *b*, cyto-

chrome c and cytochrome a/a_3. However, the observation that *Pseudomonas* AM1 has only one cytochrome b (Widdowson & Anthony, 1975) and that the maximum $\rightarrow H^+/O$ ratio measured during endogenous respiration in wild-type bacteria and also in the mutant lacking cytochrome c was about 4 suggests that the endogenous substrate is NADH and that there are only two proton-translocating segments involved in oxidation of NADH (Scheme 1). Cytochrome c is not involved in either of these segments; the first segment is NADH–ubiquinone oxidoreductase; the second segment involves cytochrome b and operates between ubiquinone and cytochrome a/a_3. These suggestions are supported by the observation that the $\rightarrow H^+/O$ ratio is unchanged (about 4) in wild-type and mutant bacteria when the usual oxidase, cytochrome a_3 (Widdowson & Anthony, 1975), is inhibited by 300μM-KCN and respiration is entirely by way of the cytochrome b functioning as an alternative oxidase.

A mixture of ascorbate and tetramethylenephenylenediamine was not oxidized by the mutant lacking cytochrome c, and this mixture did not reduce any of the cytochromes in the mutant, although cytochromes c and a/a_3 are reduced by this mixture in wild-type bacteria. These observations, together with the stoicheiometry of proton translocation measured during respiration with ascorbate/tetramethylenephenylenediamine in wild-type bacteria ($\rightarrow H^+/O$ ratio of about 2), suggest that cytochrome c is reduced on the outer side of the bacterial membrane and that oxygen is reduced by cytochrome a_3 on the inner side; these two cytochromes are thus able to constitute part of a proton-translocating segment in wild-type *Pseudomonas* AM1.

The proposed Scheme 1 is the most simple that will accommodate all the data so far available. It proposes, in effect, two electron-transport chains with cytochrome a/a_3 as a common component. The chains have been drawn in this way to emphasize that cytochrome c is only essential for electron transport and proton translocation when methanol or ascorbate/tetramethylenephenylenediamine (and possibly methylamine) is the respiratory substrate. Neither our previous results (Anthony, 1975*b*; Widdowson & Anthony, 1975) nor those described in the present



Scheme 1. *Proposed scheme for respiration-driven proton translocation in Pseudomonas AM1*

The arrows indicate flow of electrons or protons; they do not necessarily imply a direct reaction between components. The Scheme proposes that reduction of cytochromes b and c occurs on the outer side of the membrane and that reduction of molecular oxygen always occurs on the inner side. The oxidation of methanol, formaldehyde or succinate does not necessarily occur on the side of the membrane indicated here. The broken line indicates the flow of electrons to oxygen from cytochrome b that occurs when 0.3mM-KCN inhibits the usual cytochrome oxidase (cytochrome a_3). Abbreviations: FMN, flavoprotein of NADH dehydrogenase; Fe/S, iron–sulphur protein(s) of the NADH–ubiquinone oxidoreductase complex; Q, ubiquinone/ubiquinol; Cyt, cytochrome; Asc/TMPD, ascorbate/tetramethylenephenylenediamine.

paper are sufficient to determine whether or not cytochrome *c* might ever form a (non-essential) link between cytochromes *b* and a/a_3 in the wild-type bacteria. The results presented above suggest that no advantage in respiration rate nor proton translocation is conferred by such a link. The main observation previously taken to indicate that cytochrome *c* might be involved in oxidation of all substrates in wild-type bacteria was that its steady state of reduction increased with increasing flux to oxygen through the electron-transport chain regardless of the respiratory substrate used (Anthony, 1975*b*). However, this observation is also consistent with Scheme 1, where cytochrome *c* does not mediate between cytochromes *b* and a/a_3, provided that the extent of reduction of cytochrome *c* always tends to reflect that of cytochrome *a*.

Although the inhibition of methanol oxidation by KSCN prevented measurement of proton translocation with this substrate, the involvement of cytochromes *c* and a/a_3 in methanol oxidation has been established (Anthony, 1975*b*; Widdowson & Anthony, 1975), and thus it may be concluded that a $\rightarrow H^+/O$ ratio of at least 2 is possible during the oxidation of methanol to formaldehyde by way of the separate methanol dehydrogenase–cytochrome *c*–cytochrome a/a_3 proton-translocating segment.

Because the mutant lacking cytochrome *c* is also deficient in methylamine dehydrogenase it is not possible to state whether the cytochrome *c*–cytochrome a/a_3 segment or the ubiquinol–cytochrome a/a_3 segment is responsible for the proton translocation associated with methylamine oxidation.

If the proton-translocating segments are arranged as described in Scheme 1, then the $\rightarrow H^+/O$ ratio for those exogenous substrates oxidized by way of NAD^+-linked dehydrogenases (formate, 3-hydroxybutyrate and malate) should be 4, whereas that for substrates not involving NAD^+ (succinate, formaldehyde, methanol and methylamine) should be only 2. Except when respiration is affected by loss of cytochrome *c* (as with methylamine and methanol) these $\rightarrow H^+/O$ ratios should be unaltered in mutant PCT76. The results of measurements of $\rightarrow H^+/O$ ratios in the presence of exogenous substrates are consistent with these predictions. However, because it proved impossible to abolish the endogenous respiration before doing these experiments, and because of the proton movements associated with the presumed transport of exogenous substrates (in wild-type bacteria), the results with exogenous substrates are not unequivocal.

The results presented here suggest that if the P/O ratio is half the measured $\rightarrow H^+/O$ ratio, then during growth of *Pseudomonas* AM1 on methanol the P/O ratios will be 2 for formate oxidation, 1 for formaldehyde oxidation and probably 1 for methanol oxidation. A total value of 4 for complete oxidation of methanol to CO_2 is consistent with the P/O values measured with membrane preparations of a similar bacterium (Netrusov *et al.*, 1977).

These values for P/O ratios are also in accordance with the values predicted by Goldberg *et al.* (1976) from growth yields of bacteria such as *Pseudomonas* AM1 growing on methanol by way of the serine pathway. However it should be noted that, because the NAD(P)H supply, as well as the ATP supply, is a major factor limiting the growth yields of these bacteria, such predictions of P/O ratios from measurements of growth yields are of limited value. Indeed, it has been shown that, because of the NAD(P)H-limitation in these bacteria, if the total P/O ratio for oxidation of formaldehyde to CO_2 is 3 as indicated in the present paper then measured growth yields will be hardly affected by increasing the P/O ratio from 1 to 2 or 3 for the first step in methanol oxidation (Anthony, 1978).

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