Discussion Letter

Is a proton-pumping cytochrome oxidase essential for energy conservation in *Nitrobacter*?

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There is now considerable support for the proposal that mitochondrial cytochrome oxidase (cytochrome aa_3) has a proton-pumping activity [1–3]. This property of cytochrome oxidase is thermodynamically acceptable because the drop in redox potential across the cytochrome oxidase reaction is sufficient to drive the movement across the membrane of at least two positive charges, of which at least one must be a translocated proton, against accepted values of the mitochondrial proton electrochemical gradient [1–3]. However, there is disagreement [4] with the view that cytochrome oxidase is a proton pump and, as originally proposed by Mitchell [5], it would certainly be possible for the mitochondrial respiratory chain to generate a proton electrochemical gradient without any proton-pumping activity by cytochrome oxidase. The same arguments could be made for many bacteria, but the purpose of the present paper is to discuss for the species *Nitrobacter* how the operation of a proton-pumping cytochrome oxidase could be the only mechanism for generating a proton electrochemical gradient.

In *Nitrobacter* only one reaction provides energy for growth. This is the oxidation of nitrite by oxygen, a reaction that according to chemiosmotic principles [5] must be linked to the generation of a proton electrochemical gradient across the cell membrane. This gradient in turn drives ATP synthesis and also reversed electron transfer from nitrite to NAD(P) in order to provide reducing power for growth [6–8]. Electrons flow from nitrite to oxygen via a short respiratory chain that begins with a nitrite oxidase enzyme which is linked via a *c*-type cytochrome to a cytochrome oxidase generally thought to be of the aa_3-type [9–11]. Electron spin resonance studies in combination with redox titrations have shown that the nitrite oxidase has some strong similarities, including the presence of molybdenum, with the respiratory nitrate reductase enzyme of a denitrifying bacterium [12]. Indeed in cell-free membrane vesicle preparations from *Nitrobacter* under anaerobic conditions the nitrite oxidase can act as a nitrate reductase when a suitable reductant such as NADH is present [6–8].

The coupling of electron flow from nitrite to oxygen to the generation of a proton electrochemical gradient has to be understood against the following background. First, the midpoint potentials of the nitrate/nitrite couple (+420 mV) and of some of the components of the nitrite oxidase [12] are considerably higher than that of the *c*-type cytochrome (+274 mV) [9]. Second, in membrane vesicles with the opposite orientation to the intact cell the rate of oxidation of nitrite by oxygen is reduced by reagents that are expected to collapse the membrane potential, but not by reagents that collapse the pH gradient [13]. Inhibition of oxygen reduction cannot be attributed to a secondary effect of these reagents as direct electron-transport inhibitors, because the initiation of ATP synthesis, which is expected to decrease the membrane potential, is also accompanied by an inhibition of the rate of electron flow to oxygen [13]. The latter observation is paralleled by the finding that in elec-

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tron transport particles the extent of steady state reduction of cytochrome *c* by nitrite is considerably decreased upon addition of an uncoupler [14,15]. These aspects led Cobley [15] to propose scheme A (fig.1) for the coupling of electron flow to the generation of the proton electrochemical gradient. The novel feature was the suggestion that hydride is transferred from the nitrite oxidase to cytochrome *c*, using an unidentified component of the electron-transfer chain. Cobley's scheme accounted for the enhancement by the membrane potential of the rate of nitrite oxidation. Electrogenic movement of hydride or its equivalent would be enhanced by the membrane potential which would be relatively positive on the side of the membrane where cytochrome *c* is located.

In terms of energetics, scheme A predicts the translocation of one proton and also just one net positive charge out of the cell for each two electrons passing from nitrite to oxygen. ATP synthesis by the proton-translocating ATP synthase is believed to require the electrogenic movement of at least 2 protons into the cell [15,16]. Thus the scheme of Cobley (A) allows a maximum P : 2 e^- ratio of 0.5. Scheme B (fig.1) shows an alternative mechanism suggested by Mitchell [17] and by Aleem [7] in which hydrogen transfer is envisaged to occur across the membrane between the sites of nitrite oxidation and cytochrome *c* reduction. Energetically this scheme predicts the movement of both two protons and two net positive charges out of the cell for each two electrons flowing. Thus it

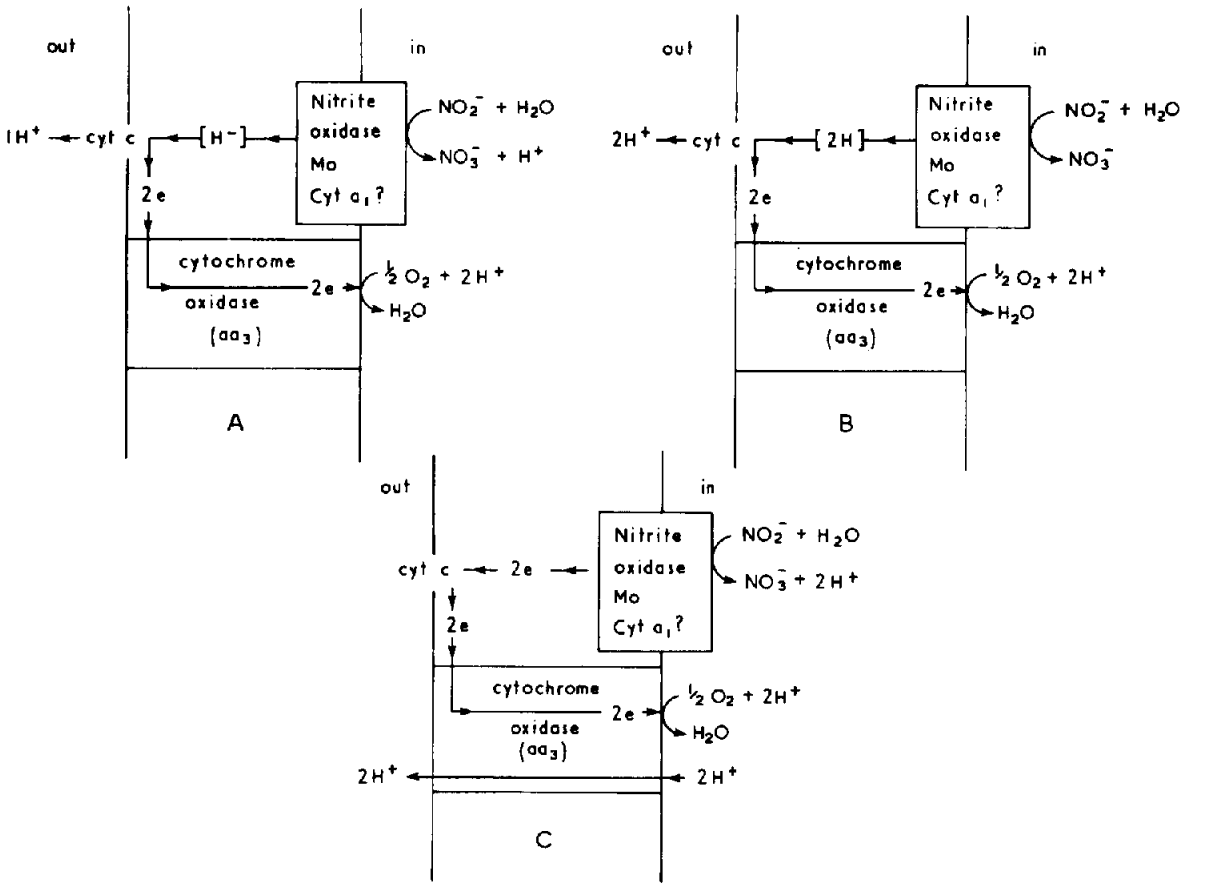

Fig.1. Schemes to account for the coupling of electron flow from nitrite to oxygen in *Nitrobacter* to the generation of a proton electrochemical gradient: 'in' refers to the cytoplasmic side of the plasma membrane of the cell; 'out' refers to the external side of the membrane. Cytochrome a_1 has not definitely been established as a component of the nitrite oxidase complex (see text).

would be consistent with claims of a P : O ratio = 1 for nitrite oxidation [18] (but note that lower values have been reported [13]). However, scheme B does not account [15] for the observation that dissipation of a membrane potential reduces both the rate of electron transfer [13] and the steady state extent of cytochrome *c* reduction [15].

Scheme A is an ingenious attempt to rationalise what is known about the oxidation of nitrite by oxygen, but has the defect of predicting a relatively low yield of ATP and requires the postulate of an unorthodox, although not impossible, hydride-translocating component in the respiratory chain. Since scheme A was proposed [15], the evidence has emerged that cytochrome aa_3 of the mitochondrial respiratory chain has a proton-pumping function [1–3]. In addition, several lines of evidence indicate that the aa_3-type oxidase of *Paracoccus denitrificans* is similarly a proton pump [19–21]. When this feature of aa_3-type oxidases is taken into account it is possible to rationalise energy coupling in *Nitrobacter* in terms of scheme C (fig.1).

The postulate (scheme C) of an electron-carrying process between nitrite oxidase and cytochrome *c* together with a proton-pumping function of cytochrome aa_3 (with proposed stoichiometry of 2 H^+/2 e^-) means that two protons and two net positive charges would be moved out of the cell for each two electrons flowing from nitrite to oxygen. The predicted P:O ratio is thus higher than in the scheme where trans-membrane hydride transfer was suggested (scheme A). Scheme C clearly does not require any novel type of respiratory chain component to act between nitrite oxidase and cytochrome *c*; simple electron transfer is all that is envisaged. The movement of electrons outward across the membrane from nitrite oxidase to cytochrome *c* will be favoured by a membrane potential, positive on the cytochrome *c* side. The electrochemical potential [22] of an electron on cytochrome *c* will be raised relative to that of nitrite oxidase by an amount equal to that of the membrane potential, provided that electron transfer between these two components takes place across the full width of the membrane. If the membrane potential is of the order of 150 mV (cytochrome *c* side relatively positive), a typical situation in bacteria [16], the redox equilibrium between nitrite oxidase and cytochrome *c* will be markedly displaced in favour of reduction of the latter. By analogy with mitochondrial systems, the rate of cytochrome *c* oxidation can be expected to depend on the steady state concentration of reduced cytochrome *c* [23], and therefore the rate of nitrite oxidation by oxygen in *Nitrobacter* will decrease if the membrane potential is collapsed. In comparison with scheme A, scheme C predicts a greater increase in the steady state reduction of cytochrome *c* for a given value of the membrane potential. This is because in scheme A only one negative charge is moved from nitrite oxidase for each two electrons transferred to cytochrome *c*, and so that the membrane potential would only be half as effective in enhancing the extent of reduction of cytochrome *c* as predicted by scheme C.

At present the cytochrome aa_3 of *Nitrobacter*, which in one case has been reported to be a three subunit enzyme [10] in contrast to other two subunit bacterial aa_3-type oxidases [24], has not been tested to determine whether it is a proton pump. Such experiments are now required. Discrimination between the schemes might also be made by studies with cells of whether oxidation of nitrite by electron flow via cytochrome *c* to ferricyanide is accompanied by release of 1 H^+/2 e^- (scheme A), 2 H^+/2 e^- (scheme B) or 0 H^+/2 e^- (scheme C) into the aqueous phase external to the cells.

Ingledew and Chappell [25] reported that ATP hydrolysis shifted the mid-point potential of cytochrome *c* from +274 mV to + 360 mV when measured in electron-transport particles which had the inside-out configuration. These observations are in qualitative agreement with scheme C because ATP hydrolysis would be expected to generate a membrane potential, interior of the vesicles relatively positive, and as the redox potential would have been measured in the aqueous phase external to the vesicles, a shift to higher measured potential of the *c*-type cytochrome would be expected (see [22]). The measurements of Ingledew and Chappell [19] suggest that ATP hydrolysis must have generated a membrane potential of −90 mV.

The site of nitrite oxidation is shown in fig.1 and in other publications [7,15] as being on the cytoplasmic surface of the cell membrane. The evidence for this location is that inside-out vesicles readily oxidise nitrite [13–15] and that therefore the site of nitrite oxidation is taken to be on their external surface. At first sight this location would

appear pointless because it requires that the cells must have an otherwise unnecessary mechanism for import of nitrite and export of nitrate. However, although an external location for nitrite oxidation would avoid the need for a transport system for nitrate and nitrite it would not be so satisfactory for linking the nitrite oxidation reaction ($E^{o'} = +420$ mV) to a cytochrome *c* with an $E^{o'}$ of +274 mV. If nitrite oxidase and cytochrome *c* were both on the same external surface of the membrane in the cell then the steady state extent of reduction of cytochrome *c* would be low because the membrane potential would have no influence on the position of equilibrium.

It has been pointed out that the redox span between mitochondrial cytocrome *c* and oxygen is sufficient to allow the pumping of 1 H^+/e^- against accepted values of the proton electrochemical gradient [1–3]. If this proton pumping did not occur, a less than maximal conservation of free energy available from redox reactions would occur [1–3]. When viewed from the standpoint of *Nitrobacter* it would be almost profligate for cytochrome aa_3 not to have a proton-pumping function with a stoichiometry of at least 1 H^+/e^-, as this type of bacterium effectively relies on electron flow from cytochrome *c* to oxygen alone to generate the proton electrochemical gradient that is needed for both ATP synthesis and reversed electron flow to NAD(P). A higher stoichiometry of proton pumping, and thus higher yield of ATP and NAD(P)H, might be thermodynamically feasible, depending on the actual *E*-values for the NO_3^-/NO_2^- and $\frac{1}{2}\ O_2/H_2O$ reactions in the cell and the magnitude of the proton electrochemical gradient.

The attraction of scheme C is that it does not involve any feature that has not already been documented in other systems. It is nevertheless worthwhile considering combining schemes A and C. The result of this would be a mechanism in which 3 H^+ and also three positive charges would move out of the cell per two electrons flowing. Whether this is thermodynamically possible would depend on the same factors as have been discussed above in connection with the possibility that cytochrome aa_3 might pump more than one proton per electron flowing to oxygen. The combination of schemes A and C also illustrates what could be a general mechanism for a proton translocation stoichiometry of three per two electrons flowing through a segment of a respiratory chain. Exactly such a stoichiometry has been suggested for the NADH dehydrogenase region of the mitochondrial respiratory chain [1–3].

In this article, cytochrome a_1 has been taken to be associated with nitrite oxidase and cytochrome aa_3 has been designated as the terminal oxidase, in agreement with previous proposals [7–9]. However, Ingledew [26] has suggested that cytochrome aa_3 may be absent from at least some *Nitrobacter* species and that cytochrome a_1 might be the terminal oxidase. If after further study this view should prove correct, scheme C could be retained, with the modification that cytochrome a_1 would be removed from its tentative position in the nitrite oxidase complex and placed as a proton-pumping terminal oxidase instead of cytochrome aa_3.

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