Reduction of Cytochromes by Nitrite in Electron-Transport Particles from *Nitrobacter winogradskyi*

PROPOSAL OF A MECHANISM FOR H⁺ TRANSLOCATION

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1. A novel component in the respiratory chain of *Nitrobacter winogradskyi* was identified. This component absorbs maximally at 552.5 nm when in its reduced form, has an E_0' (pH 7.0) value of -110 mV and undergoes reduction by a mechanism involving the transfer of a single electron. 2. Degrees of reduction of cytochromes *c* and *a*₁ in electron-transport (ET) particles were monitored during the course of NO₂⁻ oxidation, and the effects of ADP together with P_i, oligomycin and of carbonyl cyanide phenylhydrazone were determined. 3. The influences of ionophorous antibiotics, NH₄Cl and cyclohexylamine hydrochloride on the reductions of cytochromes *c* and *a*₁ by NO₂⁻ indicate that the flow of reducing equivalents from cytochrome *a*₁ (+350 mV) to cytochrome *c* (+270 mV) is facilitated by $\Delta\psi$, the electrical component of the protonmotive force. 4. Cytochromes *c* and *a*₁ in ET particles are reduced by the non-physiological reductant KBH₄ in a manner similar to that observed with the physiological reductant NO₂⁻. 5. To account both for the observed cytochrome reductions and for the translocation of H⁺ ions which accompanies NO₂⁻ oxidation, a mechanism is proposed which involves the transfer of a hydride equivalent (H⁺ plus 2e) inward across the membrane of the ET particle in response to $\Delta\psi$.

Although NO₂⁻ is a weak reductant [for the NO₂⁻/NO₃⁻ couple E_0' (pH 7.0) = +420 mV; Krebs & Kornberg, 1957], the oxidation of NO₂⁻ with O₂ in *Nitrobacter winogradskyi* involves cytochromes (Lees & Simpson, 1957) with absorption bands at 550 nm and 589 nm, and an absorption shoulder at 605 nm. For the cytochrome *c*, an E_0' (pH 7.0) value of +270 mV has been determined (Sewell *et al.*, 1972), and it has been shown by low-temperature spectroscopy (Butt & Lees, 1958) and by oxidation–reduction titration (Sewell *et al.*, 1972; Cobley, 1973) that two cytochromes *a*₁ of different E_0' (pH 7.0) values contribute almost equally to the absorption measured at 589 nm after complete reduction. The 605 nm shoulder is due to a cytochrome *a*+*a*₃ terminal oxidase, which forms a photodissociable CO complex (Sewell *et al.*, 1972).

During the course of NO₂⁻ oxidation by ET particles, cytochrome reduction has been observed, but on depletion of O₂ or on the addition of uncoupler reoxidation of cytochrome *c* occurred (Kiesow, 1967). The reduction of cytochrome *c* by NO₂⁻ therefore appears to involve the expenditure of conserved energy. The data on cytochrome reduction obtained in the present investigation have been used to construct a model which allows for both energy-dependent reduction of cytochrome *c* by

NO₂⁻ and for the overall oxidation of NO₂⁻ to result in the net phosphorylation of ADP.

Methods

Growth of the organism, preparation of ET particles and the determination of protein were as previously described (Cobley, 1976).

The double-beam (dual-wavelength) measurements were made with a spectrophotometer constructed by Professor J. B. Chappell on the principles described by Chance (1957). Potentiometric titrations were conducted as described by Dutton (1971), except that mixing in the gas-tight cuvette was performed by means of a stirrer (mini-stirrer, Series E1; Electrothermal Engineering Ltd., 270 Neville Road, London E.7, U.K.), which was inserted into the cuvette through a silicone rubber bung [size number E21; Esco (Rubber) Ltd., London E.C.3, U.K.]. All experiments were conducted at 25°C and in a cuvette of 1 cm light-path.

Materials

KBH₄ was dissolved at a concentration of 10 mM in ice-cold 50 mM-sodium tetraborate, pH 9.1. Under these conditions, evolution of H₂ gas from this stock solution was negligible. All other reagents were as previously described (Cobley, 1976).

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Results

Identification of a novel low-potential component in the respiratory chain of *N. winogradskyi*

Potentiometric titrations at the wavelength pairs 550–540nm and 553–540nm are illustrated in Fig. 1. The curves obtained indicate that in addition to the previously identified cytochrome *c* with E_o' (pH 7.0) = +270mV, there is a low-potential component which, in the reduced form, absorbs maximally at a wavelength longer than that of cytochrome *c*. Assuming complete reduction of cytochrome *c* (the high-potential component) at 0mV, the E_o' (pH 7.0) value of the low-potential component has been calculated to be –110mV, and its reduction shown to involve the transfer of a single electron. By comparing a fully reduced sample with a sample at $E_h = -110$ mV (where E_h is the voltage generated between the solution under investigation and the standard hydrogen electrode), the wavelength of maximum absorption for the low-

Fig. 1. Oxidation-reduction titrations of ET particles by using the wavelength pairs 550–540nm (open symbols) and 553–540nm (closed symbols)

ET particles were suspended in 50mM-KCl/50mM-Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid] pH 7.0, at a final protein concentration of 2mg/ml; 65 μ M- $K_3(FeCN)_6$ was added initially to cause complete oxidation and then endogenous compounds caused slow reduction until E_h = approx. 0mV. Small portions of a freshly prepared sodium dithionite solution were then added to cause further reduction. The mediating dyes were added during the reductive phase of the titration (circles) at the E_h values indicated: 20 μ M-diaminodurene (DAD), 10 μ M-phenazine ethosulphate (PES), 10 μ M-phenazine methosulphate (PMS), 5 μ M-pyocyanine (Pyo) and 10 μ M-2-hydroxy-1,4-naphthoquinone (2-OHNO). The suspension was then reoxidized by additions of $K_3(FeCN)_6$ (triangles). The temperature was 25°C. The units on the abscissa are log[oxidized cytochrome]/[reduced cytochrome].

potential component has been measured to be 552.5nm (Fig. 2).

Throughout this paper the term cytochrome *c* refers specifically to the high-potential component of the titration in Fig. 1. Reduction of the low-potential component was not discernible under any of the experimental conditions subsequently described.

Degrees of reduction of cytochromes during NO_2^- oxidation; effects of ADP together with P_i , of oligomycin and of carbonyl cyanide phenylhydrazone

Reduction of up to 35% of the cytochrome *c* in ET particles was observed on the addition of NO_2^- , and a transient reoxidation could be brought about by the subsequent addition of a limiting amount of ADP in the presence of P_i (Fig. 3a). A similar reoxidation cycle was observed on a second addition of ADP and the duration of these cycles was proportional to the amount of ADP added. Oligomycin either prevented or reversed these ADP-induced

Fig. 2. Fully reduced spectrum of ET particles minus the spectrum measured at $E_h = -110$ mV

The conditions were as described in Fig. 1. A suspension of ET particles poised at $E_h = -110$ mV was scanned with reference to the absorption at 540nm. After reduction with excess of sodium dithionite the suspension was again scanned with reference to the absorption at 540nm. The first spectrum was then subtracted from the second.

Fig. 3. Reduction of cytochromes *c* and *a*₁ in ET particles during NO_2^- oxidation

ET particles were suspended in 100 mM-choline chloride, 20 mM-Tricine [*N*-tris(hydroxymethyl)methylglycine], 10 mM- MgCl_2 , 10 mM-tetramethylammonium phosphate and 1.6 mg of bovine serum albumin/ml at a final particle protein concentration of 1.2 mg/ml and at pH 7.8. The temperature was 25°C and the final volume 1.25 ml. The additions were 4.8 mM- NaNO_2 , ADP (each addition 40 nmol), oligomycin (Oligo; 10 μg) and 200 μM -carbonyl cyanide phenylhydrazine (CCP). Vigorous agitation with a Teflon paddle was used to add oxygen to anaerobic suspensions (Stir). (a) Measurement of cytochrome *c* (550–540 nm); (b) measurement of cytochromes *a*₁ (589–574 nm).

effects. When the extent of reduction of cytochromes *a*₁ was followed in an otherwise identical experiment, ADP-induced transient reoxidation was again recorded. Although these were, for a given amount of ADP, identical in duration with those measured at 550–540 nm, the extent of cytochrome *a*₁ reoxidation was less than that of cytochrome *c* (Fig. 3b). When suspensions of ET particles oxidizing NO_2^- became anaerobic, an almost complete oxidation of cytochrome *c* took place, and on stirring to re-introduce O_2 the extent of reduction measured before anaerobiosis was regained. The addition of uncoupler at this point resulted in complete and irreversible reoxidation of cytochrome *c* (Fig. 3a). The responses of cytochromes *a*₁ to anaerobiosis and to the addition of uncoupler were similar to those of cytochrome *c*, but again the changes were less extensive (Fig. 3b).

Fig. 4. Reduction of cytochromes *c* and *a*₁ in ET particles during NADH oxidation

ET particles were suspended under the conditions described in Fig. 3 at a final particle protein concentration of 0.2 mg/ml. The additions were 2 mM-NADH, 100 μM -ADP and 200 μM -carbonyl cyanide phenylhydrazine (CCP). (a) Measurement of cytochrome *c* (550–540 nm); (b) measurement of cytochromes *a*₁ (589–574 nm).

In preparations where the reduction of cytochrome *c* by NO_2^- was less extensive than illustrated in Fig. 3(a), the reoxidation cycles induced by ADP were still clearly discernible. When the cytochromes *a*₁ in such preparations were monitored, reduction by NO_2^- was almost as extensive as in Fig. 3(b), but the effects of ADP together with P_i and of carbonyl cyanide phenylhydrazine were either less extensive or non-existent.

Degrees of reduction of cytochromes during NADH oxidation; the effects of ADP together with P_i and of carbonyl cyanide phenylhydrazine

Immediately after the addition of NADH to ET particles extensive reduction of cytochrome *c* was observed, but this amount of reduction was not maintained. After 1 min, when a more oxidized and steady level was reached, the addition of ADP resulted in further reduction (Fig. 4), which could be prevented by oligomycin (not shown). Even in the presence of excess of ADP the addition of carbonyl cyanide phenylhydrazine increased the extent of cytochrome *c* reduction. Qualitatively similar changes were recorded when the responses of cytochromes *a*₁ to ADP and carbonyl cyanide phenylhydrazine were monitored (Fig. 4). Complete reduction of cytochromes *c* and *a*₁ by NADH was observed under anaerobic conditions.

Influence of dianemycin and of valinomycin on the reduction of cytochromes by NO_2^-

In the presence of dianemycin (4 $\mu\text{g}/\text{ml}$) together with 4 mM-KCl the reduction of cytochrome *c* by

NO_2^- was 25% more extensive than that observed in the control experiment. In the presence of valinomycin (4 $\mu\text{g}/\text{ml}$) together with 4 mM-KCl, however, the reduction of cytochrome *c* by NO_2^- was both less extensive (by 40%) and less rapid. Although valinomycin did not prevent ADP from causing cytochrome *c* reoxidation, cyclic changes in response to ADP addition were no longer observed. In the presence of both dianemycin and valinomycin, reduction of cytochrome *c* by NO_2^- was barely discernible. The reduction of cytochromes a_1 by NO_2^- was unaffected even by the combined action of valinomycin and dianemycin.

*Influence of NH_4Cl , cyclohexylamine hydrochloride and oligomycin on the reduction of cytochrome *c* by NO_2^-*

The addition of oligomycin to ET particles oxidizing NO_2^- with O_2 resulted in a slow increase in the reduction of cytochrome *c*. After a few minutes a new steady level was reached where cytochrome *c* was 55% more reduced than in a control experiment. NH_4Cl and cyclohexylamine hydrochloride also caused an increased reduction of cytochrome *c* (35% increase), but the response of this cytochrome to the addition of these compounds was immediate. Although the effects of NH_4Cl and of cyclohexylamine hydrochloride on cytochrome *c* reduction by NO_2^- were not additive, the actions of both NH_4Cl and of cyclohexylamine hydrochloride were found to be synergistic with the action of oligomycin; in the presence of NH_4Cl and oligomycin together, cytochrome *c* reduction by NO_2^- was increased more than twofold.

*Reduction of cytochromes *c* and a_1 during the simultaneous oxidation of NO_2^- and NADH*

The reduction of cytochrome *c* observed on the addition of NO_2^- to ET particles already oxidizing NADH was more rapid and extensive than the reduction observed when NO_2^- was added before NADH. When the extent of reduction of cytochromes a_1 was monitored in an otherwise identical experiment, the increased reduction brought about by NO_2^- addition during NADH oxidation was smaller than the reduction of cytochromes a_1 which occurred with NO_2^- as sole reductant.

*Comparison of BH_4^- and NO_2^- as reductants of cytochromes *c* and a_1 in ET particles*

When KBH_4 was added to ET particles the reduction of cytochrome *c* observed was identical in rate and extent with the reduction of cytochrome *c* by

NO_2^- (Fig. 5a). At 35 μM - KBH_4 , the reduction of cytochrome *c* was half as extensive as the reduction measured at saturating concentrations of KBH_4 . In these experiments (which were conducted at

Fig. 5. Comparison of the reduction of cytochromes in ET particles by NO_2^- with the reduction caused by KBH_4 .

ET particles (final protein concentration 0.9 mg/ml) were suspended in 50 mM-choline chloride, 50 mM-sodium tetraborate and 5 mM-MgCl₂ at pH 8.6 and at 25°C. The additions were KBH_4 (A, 200 μM ; B, 100 μM ; C, 25 μM), 1.2 mM- NaNO_2 , oligomycin (Oligo; 4 $\mu\text{g}/\text{ml}$), 5 mM-cyclohexylamine hydrochloride (Amine) and 70 μM -carbonyl cyanide phenylhydrazon (CCP). (a) Measurement of cytochrome *c* reduction (550–540 nm); (b) measurement of cytochromes a_1 reduction (589–574 nm).

pH 8.6 to prevent the production of H_2 from KBH_4) the addition of ADP to ET particles reduced with either KBH_4 or NO_2^- did not cause cytochrome *c* reoxidation. However, under these conditions with either KBH_4 or NO_2^- as reductant, both oligomycin and cyclohexylamine hydrochloride were effective in causing increased reduction of cytochrome *c*, and uncouplers were effective in causing cytochrome *c* reoxidation.

Reduction of cytochromes a_1 by KBH_4 under these conditions was similar to that observed with NO_2^- as reductant; with either reductant the changes induced by oligomycin, cyclohexylamine hydrochloride or carbonyl cyanide phenylhydrazone were small (Fig. 5b).

Although 5 mM-potassium ferrocyanide and 5 mM-tetramethylammonium ascorbate together with $50 \mu\text{M}$ - $\text{NNN}'\text{N}'$ -tetramethylphenylenediamine reduced the cytochromes in ET particles, these reactions were completely independent of amines, oligomycin and uncouplers.

Discussion

Evidence has been presented (Aleem *et al.*, 1965) that when NO_2^- is oxidized by *N. winogradskyi* the oxygen atom appearing in NO_3^- is derived from water. It is therefore reducing equivalents derived from this water that flow by means of a respiratory chain to molecular oxygen. Cytochrome *c* ($E_0' + 270 \text{ mV}$) is reduced during NO_2^- oxidation to an extent greater than would be predicted from thermodynamic calculation (Fig. 3). The energy required to bring about this extensive reduction by NO_2^- is available during the coupled oxidation of NO_2^- or NADH with O_2 . As the reduction of cytochromes a_1 was little affected by uncoupling agents, it is concluded that cytochrome a_1 (+140 mV) is at most only slightly reduced during NO_2^- oxidation and that NO_2^- rapidly reduced cytochrome a_1 (+350 mV) in an energy-independent manner.

Since NO_2^- oxidation with O_2 is activated by a membrane potential, $\Delta\psi$, which this oxidation itself generates across the membrane of the ET particle (Cobley, 1976), experiments were conducted to determine where in the cytochrome chain of *N. winogradskyi* a membrane potential influences the rate of electron flow. The rationale behind the use of ionophorous antibiotics, amines and oligomycin to vary the relative contributions of a pH gradient and a membrane potential to the protonmotive force has been discussed elsewhere (Cobley, 1976). Under conditions which favour the conservation of energy in the form of an electrical potential, cytochrome *c* reduction by NO_2^- was increased. Under conditions which tend to favour the conservation of energy as a pH gradient, cytochrome *c*

reduction by NO_2^- was decreased. This indicates that the flow of reducing equivalents from cytochrome a_1 (+350 mV) to cytochrome *c* is subject to exactly those factors which control the rate of NO_2^- oxidation with O_2 .

Mitchell (1966) has proposed that H^+ translocation can be achieved by the alternation of hydrogen and electron carriers within the membrane, and has tentatively considered that NO_2^- oxidation might occur by means of the loop illustrated in Fig. 6(a) (Mitchell, 1970). However, this loop does not account for the stimulation of NO_2^- oxidation by $\Delta\psi$, the electrical component of the protonmotive force. In Fig. 6(b) a loop is shown which both allows H^+ translocation to accompany the flow of reducing equivalents, and permits the action of an electrical potential to influence the rate of the overall oxidation (the passage of H^+ plus $2e$ would be electrophoretically accelerated). I suggest that this latter loop can be used to create a model which fully explains the known properties of the nitrite oxidase system in ET particles from *N. winogradskyi*. This specific model is illustrated in Fig. 6(c).

Support for the model comes from the observation that cytochrome reduction by NO_2^- is closely mimicked by KBH_4 , a compound thought to reduce by means of hydride transfer (see Edmondson *et al.*, 1972). Evidence has been presented (Ingledew *et al.*, 1974) that two equivalents of cytochrome a_1 (+350 mV) are reduced by one hydride equivalent. This reduction of cytochrome a_1 (+350 mV), however, is complicated, since it occurs by single-electron transfer.

A prediction from the model is that the ratio of H^+ ions translocated to NO_2^- ions oxidized is unity. This prediction is, however, difficult to test because the apparent K_m values for both NO_2^- and O_2 are high (approx. 1 mM and $35 \mu\text{M}$ respectively; Cobley, 1973) and because NO_2^- oxidation is inhibited under conditions which maximize the contribution of the pH gradient to the protonmotive force, a condition essential for the meaningful determination of H^+/O or H^+/NO_2^- stoichiometries.

The coupling of NO_2^- oxidation to the phosphorylation of ADP can be explained if the mechanism of Fig. 6(c) operates in conjunction with a proton-translocating ATPase (adenosine triphosphatase). If the ATPase present in ET particles from *N. winogradskyi* (Cobley, 1973) translocates protons with the H^+/ATP stoichiometry of 2, as has been reported for mitochondria and chloroplasts (Moyle & Mitchell, 1973; Carmeli, 1970), the ATP/NO_2^- stoichiometry would be 0.5. Such a low ATP/NO_2^- value might well explain why in *N. winogradskyi* cells the assimilation of 1 mol of CO_2 requires the oxidation of 70 g-ions of NO_2^- (see Wezernak & Gannon, 1967).

Fig. 6. Chemiosmotic mechanisms to explain H^+ translocation in ET particles oxidizing NO_2^- with O_2

(a) An oxidation-reduction loop which translocates $2H^+$ (see Mitchell, 1970). (b) An oxidation-reduction loop which translocates $1H^+$. (c) The oxidation-reduction loop of (b) specifically applied to the oxidation of NO_2^- with O_2 in ET particles from *N. winogradskyi*.

A simple chemiosmotic loop explains the stimulation of NO_2^- oxidation by amines, the inhibition caused by uncoupling agents, the energy-dependent reduction of cytochrome c (+270mV) and the coupling of NO_2^- oxidation to the phosphorylation of ADP. It is difficult to see how a chemical hypothesis of energy conservation could provide an equally simple explanation.

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