Reassessment of pathways of electron flow to nitrate reductase that are coupled to energy conservation in *Paracoccus denitrificans*

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Received 11 January 1983

Electron flow from succinate dehydrogenase, or from non-physiological reductants including ascorbate plus phenazine methosulphate, to nitrate reductase in *Paracoccus denitrificans* is shown to be linked to energy conservation, contrary to previously accepted schemes for electron transport in this organism. The function of the *P. denitrificans* nitrate reductase is compared with its counterpart in *Escherichia coli*.

Proton electrochemical gradient *Fluorescence probe*

1. INTRODUCTION

Recently published schemes of electron transport in *Paracoccus denitrificans* show electron flow from succinate via ubiquinone to nitrate not to be coupled to energy conservation, or more specifically to the generation of a proton electrochemical gradient [1–3]. This conclusion has been reached because, whereas inverted membrane vesicles from this bacterium catalyse oxidative phosphorylation with P:2 e ratios as high as 1.8 for NADH⟶oxygen, 1.0 for NADH⟶nitrate and 0.6 for succinate⟶oxygen [4,5], almost no ATP synthesis could be detected to accompany electron flow from succinate via ubiquinone to nitrate [4]. In contrast, there is evidence that in *Escherichia coli* electron flow from reduced ubiquinone to nitrate is linked to the generation of a proton electrochemical gradient [6]. This apparent difference between the roles in oxidative phosphorylation of nitrate reductases in a denitrifying bacterium such as *P. denitrificans* and in non-denitrifiers including *E. coli* has been suggested to be related to a different membrane localisation of nitrate reductase in these two classes of respiratory nitrate reducing organisms [7,8].

This paper shows that, contrary to the accepted view, electron flow in *P. denitrificans* from succinate to nitrate is coupled to generation of a proton electrochemical gradient. This gradient can drive ATP synthesis, albeit with a low P:2 e ratio, but we discuss why measurement of a P:2 e ratio in membrane vesicles can be an unreliable indicator of whether a given segment of an electron transfer chain generates a proton electrochemical gradient.

2. MATERIALS AND METHODS

Paracoccus denitrificans (strain NC1B 8944) was grown anaerobically with succinate as carbon source and nitrate as electron acceptor [9]. Membrane vesicles were prepared as in [9], with the modification of [10], and were stored at 4°C under nitrogen as a suspension in 10 mM Tris–acetate

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; ANS, 1-anilinonaphthalene-8-sulphonate; DQH_2, durohydroquinone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; PMS, phenazine methosulphate

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

(pH 7.3), 1 mM magnesium acetate at ~15 mg protein/ml. Protein was determined as in [11].

Fluorescence measurements were carried out with a Baird Atomic spectrofluorimeter. Fluorescence of 8-anilinonaphthalene-1-sulphonate (ANS) was monitored by excitation at 372 nm and emission at 480 nm. 9-Amino-6-chloro-2-methoxyacridine (ACMA) fluorescence was measured using the excitation wavelength 422 nm and emission wavelength 500 nm.

ATP synthesis was measured spectrophotometrically by coupling it to production of NADPH. A cuvette, filled with a rubber stopper contained in 3 ml, 10 mM P_i–Tris (pH 7.3), 5 mM magnesium acetate, 300–600 μg vesicle protein, 10 mM glucose, 330 μM $NADP^+$, 12 μg rotenone and 4 μg piericidin A/mg protein, 4.2 units of glucose 6-phosphate dehydrogenase and 43 units of hexokinase. Succinate was then added to 5 mM and the solution allowed to become anaerobic while being gassed via vents in the rubber stopper with nitrogen. ADP was added to 40 μM and respiration initiated 5 min later by addition of 1 mM $NaNO_3$ from a 2 M stock solution. No ATP synthesis was observed when the nitrate was replaced by an equal volume of distilled water. The rate of nitrate reduction was measured by the appearance of nitrite which was determined as in [12].

Durohydroquinone was purchased from K & K Labs (via Kodak Ltd., Liverpool L33 7UF) and was prepared as a stock 50 mM solution in ethanol immediately before use. Piericidin A and ACMA were gifts from Dr P. John (University of Reading) and Dr K. van Dam (University of Amsterdam).

3. RESULTS AND DISCUSSION

In general the most convenient and direct method for demonstrating that an electron-transfer reaction generates a proton electrochemical gradient is to measure the uptake of a lipophilic ion and/or a weak acid (base) as respective indicator of membrane potential and pH gradient. However, for reasons that are not yet understood, uptake of either SCN^- or CH_3NH_2 into *P. denitrificans* vesicles is difficult to detect in the presence of nitrate even when NADH is the reductant [13]. Consequently, two fluorescent probes that are well known to give 'energy-linked' uncoupler-sensitive responses with energy-transducing membranes were chosen in order to investigate whether electron flow to nitrate is coupled to energy conservation.

Fig.1 shows the enhancement of the fluorescence of ANS in the presence of succinate together with either oxygen (dissolved initially in the reaction mixture) or nitrate as electron acceptor. In each case the fluorescence enhancement was abolished by the addition of NH_4^+ plus gramicidin D as uncoupler (fig.1). The enhancement in the presence of nitrate was drastically reduced by 0.1 mM azide (fig.1) which caused 97% inhibition of the rate of nitrate reduction, consistent with its action as a competitive inhibitor of nitrate reductase [14]. Fluorescence enhancements were not observed when succinate was omitted.

There is still some uncertainty as to the exact relationship of the uncoupler-sensitive enhancements of ANS fluorescence to proton electrochemical gradients [15]. Therefore a second fluorescent probe, ACMA, was also used. Fig.2 shows that ACMA fluorescence was quenched

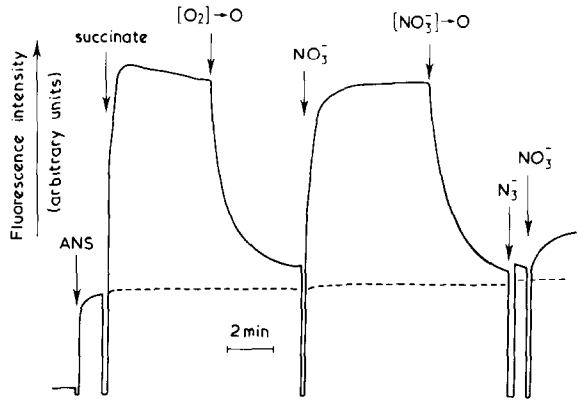

Fig.1. Uncoupler-sensitive fluorescence enhancement of ANS during electron flow from succinate to oxygen or nitrate (——). *P. denitrificans* vesicles (0.57 mg protein) were incubated in 3 ml of 10 mM P_i–Tris (pH 7.3), 5 mM magnesium acetate at 30°C. ANS (5 μM), sodium succinate (5 mM), $NaNO_3$ (0.66 mM) and NaN_3 (0.1 mM) were added as indicated. Gassing of the solution with nitrogen through vents in the rubber stopper of the cuvette was started with the addition of the succinate. (– – –) Fluorescence when uncoupler (3 μg gramicidin D and 30 mM ammonium acetate) was added to the vesicles initially.

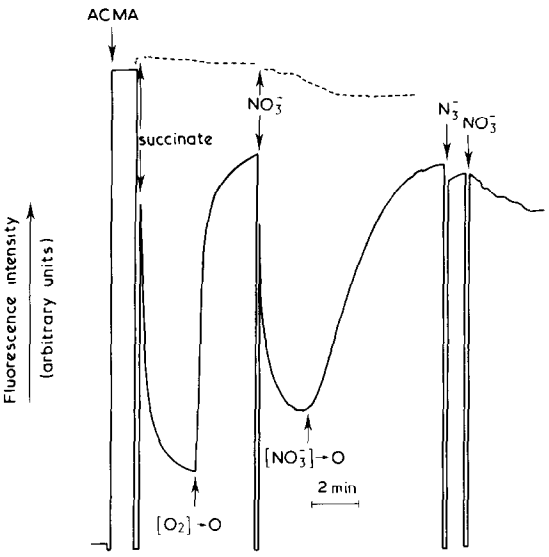

Fig.2. Energy-linked fluorescence quenching of ACMA (——). ACMA (5 μM) was added to 3 ml 10 mM P_i–Tris (pH 7.3) containing 5 mM magnesium acetate and *P. denitrificans* vesicles (0.57 mg protein). Sodium succinate (5 mM), $NaNO_3$ (0.66 mM) and NaN_3 (0.1 mM) were added as indicated. The temperature was 30°C. Gassing as in fig.1. (– – –) Fluorescence when uncoupler (3 μg gramicidin D and 30 mM ammonium acetate) was added to the vesicles initially.

when electrons flowed from succinate to either nitrate or oxygen and, as with ANS, the change in fluorescence was prevented by an uncoupler. The nitrate-dependent response was again substantially inhibited by 0.1 mM azide.

The experiments with the fluorescence probes establish that electron flow from succinate to nitrate is associated with energy conservation and that the extents of the responses of the probes with nitrate are only slightly less than those with oxygen (fig.1,2). These observations prompted a re-examination of the ATP synthesis associated with the succinate⟶nitrate reaction, even though the membrane vesicles used for the experiments shown in fig.1 and 2 had similar P:O ratios for NADH (~1.8) and succinate (~1) as the vesicles in which a very small P:2 e ratio was originally observed for the succinate⟶NO_3^- reaction [4]. With the vesicles used for the experiments of fig.1 and 2 an ATP synthesis rate of 0.12 μmol.min^{-1}.mg protein^{-1} was measured under anaerobic conditions in the presence of succinate and nitrate, corresponding to a P:2 e ratio of 0.16. This result thus adds to the evidence that contrary to the schemes shown in [1–3] the succinate to nitrate reaction is linked to proton translocation.

There is evidence that small changes in the magnitude of the proton electrochemical gradient are accompanied by large changes in the rate of ATP synthesis by *P. denitrificans* vesicles [16]. Thus very low P:2 e ratios can arise because the gradient generated by a given electron-transfer reaction is just too small to drive the ATP synthesis reaction at a rate that matches the electron-transfer rate. This can arise from a slow rate of proton pumping by electron transfer and/or a leakiness of the vesicles to protons. Evidence that effects of these types were important in determining the observed value of the P:2 e ratio came from comparing the sensitivities to an uncoupler of the rates of ATP synthesis associated with the succinate⟶oxygen and succinate⟶nitrate reactions. It was found that 85 nM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) inhibited by 50% the rate of ATP synthesis dependent on electron flow from succinate to nitrate, whereas 50% inhibition of the ATP synthesis driven by the succinate to oxygen reaction required 230 nM FCCP. This difference in sensitivity to FCCP is attributed to the succinate⟶nitrate reaction being less able to maintain a proton electrochemical gradient in the presence of a given amount of FCCP than the succinate⟶oxygen reaction. The latter is thought to pump protons at a faster rate than the succinate⟶nitrate reaction which is consistent with the rate of ATP synthesis being 5-fold greater when oxygen rather than nitrate is the electron acceptor with succinate as reductant.

The high sensitivity to FCCP of oxidative phosphorylation associated with the succinate⟶nitrate reaction indicates that the rate of ATP synthesis and hence the P:2 e ratio might be vanishingly small in a preparation of vesicles only slightly more intrinsically leaky to protons than the preparation used here. A factor of this kind is probably responsible for the extremely low P:2 e ratio reported in [4].

The experiments reported here reveal the danger of relying on measurements of ATP synthesis alone as an index of energy coupling. A similar

point was made earlier in respect of the question of whether the cytochrome *c*⟶oxygen reaction conserves energy in *P. denitrificans* [13]. In the intact cell both the latter and the succinate⟶NO_3^- reactions will contribute to ATP synthesis as their proton translocation will be reinforced by the simultaneous operation of other proton translocating segments of the electron-transfer chain. This consideration needs to be taken into account in studies of the stoichiometry of proton translocation [3] and of molar growth yields.

A remaining question is what is the maximum attainable P:2 e ratio for succinate⟶NO_3^-? By analogy with work on *E. coli*, the flow of two electrons from succinate to nitrate should translocate two protons via a redox loop comprising the ubiquinone/ubiquinol couple [6]. As at least three protons are probably required to drive the synthesis of one ATP molecule [13,17], the maximum P:2 e is no higher than 0.66. The maximum P:2 e for NADH⟶NO_3^- might be 3-fold greater because 4 $H^+/2\ e^-$ are believed to be translocated by the NADH⟶ubiquinone reaction [1–3] in addition to the $2\ H^+/2\ e^-$ suggested to be translocated by the ubiquinol⟶nitrate reaction.

As this work demonstrates a similar role for nitrate reductase of *P. denitrificans* in oxidative phosphorylation as in *E. coli*, some further common features were studied. Table 1 shows that both durohydroquinone (DQH_2) and ascorbate plus phenazine methosulphate (PMS) acted as reductants for nitrate reductase. Oxidation of these substrates along with succinate was inhibited by similar titres of 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) to those that inhibit nitrate reductase in *E. coli* [18]. Antimycin A at concentrations that inhibit electron flow to oxygen had no effect. Oxidation of both DQH_2 and ascorbate plus PMS by nitrate supported an uncoupler sensitive enhancement of ANS fluorescence, and quenching of ACMA fluorescence, although the extents of the changes were less than when succinate was the substrate. By analogy with work on the nitrate reductase of *E. coli* [6,18], an explanation of these findings is that both DQH_2 and the reduced form of PMS are oxidised by a *b*-type cytochrome in an HQNO-sensitive manner at the internal surface of the inverted vesicles of *P. denitrificans*. Electron flow across the vesicle membrane from cytochrome *b* to the active site of nitrate reductase at the external surface of the vesicles is then responsible for generating the membrane potential component of the proton electrochemical gradient.

Table 1

Nitrate reduction with succinate and artificial electron donors

Electron donor	HQNO (μM)	Nitrate reduction rate ($\mu mol.min^{-1}.mg\ protein^{-1}$)
5 mM succinate	0	0.70
	10	0.55
	20	0.51
1.66 mM DQH_2	0	0.35
	10	0.21
	20	0.13
10 mM iso-ascorbate + 10 μM PMS	0	1.29
	10	1.01
	20	0.71

P. denitrificans vesicles (0.6 mg protein) were incubated in 10 mM P_i–Tris (pH 7.3) containing 5 mM magnesium acetate, at 30°C. The appropriate electron donor was then added to give 3 ml final vol. Gassing with nitrogen was done as in fig.1 to ensure anaerobiosis. After 10 min, either the inhibitor or 1 mM $NaNO_3$ was added. If inhibitor was added, the vesicles were incubated for a further 3 min before the addition of 1 mM $NaNO_3$. Samples of 50 μl were withdrawn at intervals with a microsyringe and assayed for nitrite

ACKNOWLEDGEMENT

This work was supported by the UK SERC via a studentship to D.P. and a grant to S.J.F.

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