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A PERIPLASMIC LOCATION FOR METHANOL DEHYDROGENASE FROM *PARACOCCUS DENITRIFICANS*: IMPLICATIONS FOR PROTON PUMPING BY CYTOCHROME aa_3

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SUMMARY

Evidence is presented that methanol dehydrogenase from *Paracoccus denitrificans* has a periplasmic location. The implications for the mechanism of proton translocation during electron flow from methanol to oxygen via cytochromes c and aa_3, or to nitrite via cytochrome c and nitrite reductase, are discussed.

Bacteria that can grow on methanol as carbon source possess a methanol dehydrogenase which is not NAD(P) or flavin linked but uses a novel type of co-factor, a pyrrolo-quinoline quinone [1,2]. In *Paracoccus denitrificans* [3,4] and *Pseudomonas* AM1 [5] the evidence is that methanol dehydrogenase feeds electrons to c-type cytochromes thus providing a relatively rare example of a physiological donor to the terminal segment of a respiratory chain. Electron transfer from methanol to oxygen has been shown to drive proton translocation, but whereas $H^+/O = 3.5$ has been estimated for *P. dentrificans* [4], for other bacteria $H^+/O = 2$ has been obtained [6-8]. The component(s) of the respiratory chains responsible for proton translocation are unknown, but a prerequisite for understanding energy conservation accompanying methanol oxidation is to determine whether methanol dehydrogenase, a water soluble protein when isolated from cells [3], is located at the periplasmic or cytoplasmic side of the plasma membrane. A periplasmic location immediately suggests that protons released upon oxidation of methanol could directly contribute to the generation of a protonmotive force. This is of particular interest for *P. denitrificans* where the terminal oxidase for methanol oxidation is an aa_3 type cytochrome which has been purified in a form with fewer subunits than its mitochondrial counterpart,

Abbreviations: H^+/O, number of protons translocated/oxygen atom reduced; DCPIP, 2,6,dichlorophenol-indophenol.

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but has not yet been demonstrated to possess the proton pumping capability of the mitochondrial enzyme [9].

MATERIALS AND METHODS

P. denitrificans (NCIB 8944) was grown at 30°C in 2 liter batches in stoppered 2.5 liter bottles under conditions that were stagnant except that the cultures were swirled for approx. 30 sec each day to disperse clumps of cells. The growth medium contained, per liter, 6 g K_2HPO_4, 4 g KH_2PO_4, 1.6 g NH_4Cl, 10.1 g KNO_3, 0.5 g $NaHCO_3$, 0.2 g $MgSO_4.7\ H_2O$, 40 mg $CaCl_2.2\ H_2O$, 12 mg Fe^{2+}-EDTA, 0.1 ml modified Hoagland's solution [10], 0.1 g yeast extract (Oxoid) and 1% v/v methanol. Spheroplasts were prepared from 6 liters of cells, harvested when A_{550}^{1cm} = 0.45-0.6 after approx. 6 days growth. After washing [10] the cells were resuspended in 100 ml 0.2 M Tris-HCl, pH 7.3, 0.5 M sucrose and 0.5 mM EDTA. 25 mg of lysozyme (Hen egg, grade 1 from Sigma Ltd., U.K.) was added, a mild osmotic shock administered by dilution with an equal volume of H_2O, and after a 10 min incubation at 30°C the suspension was centrifuged [10] and the pellet of spheroplasts resuspended in approx. 10 ml 0.2 M sucrose, 0.15 M KCl, 10 mM magnesium acetate, 20 mM Tris-acetate, pH 7.3. 100 ml of the supernatant was dialysed [10] and then concentrated by removal of H_2O with aquacide, and cytochrome cd in the concentrate was determined by the pyridine haemochrome method [10]. The remaining supernatant (approx. 100 ml) was retained for tests of other enzymic activities. Spheroplasts were lysed by dilution of 2 ml of the suspension into 18 ml water at 0°C followed by 4 x 30 sec periods of sonication (output setting 6, Dawe Soniprobe type 7532A with $\frac{1}{2}$" tip) to ensure complete disruption of the spheroplasts. Deoxyribonuclease plus 10 mM magnesium acetate were added to reduce the viscosity of the resulting suspension, which was then centrifuged [10] to yield a supernatant, termed the lysate, and a pellet which was resuspended in approx. 7 ml of the spheroplast resuspension medium, and termed the lysed spheroplasts.

Malate dehydrogenase activity was determined as before [10]. Methanol dehydrogenase was assayed spectrophotometrically with 2,6-dichlorophenol-indophenol (DCPIP) as electron acceptor and phenazine methosulphate as mediator [3,11]. As the latter assay becomes non-linear as the reaction proceeds [11], the initial rate was measured over 30 secs starting approx. 15 secs after adding enzyme to the reaction mixture [11]. A considerable reduction of DCPIP in the absence of added methanol was usually observed, and this has been attributed to the presence of unidentified endogenous substrates [11]. It has been recommended that the rate of reaction in the absence of methanol should not be subtracted from the rate found with methanol present [11], but owing to the uncertainty as to the source of, and possible variation in concentration of, the endogenous substrates, in the present work methanol dehydrogenase activities were calculated both in terms of the total rate of DCPIP reduction (assay A) and also of the rate that was dependent on added methanol (assay B).

RESULTS AND DISCUSSION

Table 1 shows that a high proportion of the total methanol dehydrogenase activity was found in the supernatant fraction collected after lysozyme treatment of cells. In contrast malate dehydrogenase, a cytoplasmic enzyme, was predominantly detected in the lysate after disrupting the spheroplasts (Table 1). It was also found that a known periplasmic protein nitrite reductase [10,12],

Table 1

Location of methanol dehydrogenase and malate dehydrogenase in P. denitrificans

	Methanol dehydrogenase		Malate dehydrogenase
	% total recovery		
	Assay A	Assay B	
Supernatant from spheroplast preparation	67.7 ± 1.3	74.0 ± 2.9	6.3 ± 0.3
Lysate	19.0 ± 2.5	20.0 ± 2.9	89.3 ± 0.7
Lysed spheroplasts	13.3 ± 2.0	6.3 ± 3.2	4.3 ± 0.9

The results are presented as means ± standard error using data from three separate experiments.

cytochrome cd, could only be detected as a pyridine haemochrome in the supernatant fraction. Thus methanol dehydrogenase cofractionates principally with a periplasmic protein rather than with a cytoplasmic protein, and it is concluded that methanol dehydrogenase is located in the periplasm, or more probably, associated with the periplasmic side of the plasma membrane. This conclusion is valid irrespective of the method used for determining methanol dehydrogenase activity (Table 1). The retention of some methanol dehydrogenase activity by the spheroplasts, detected both in the lysate and in disrupted spheroplasts, is attributed to either insufficient breakdown of the cell wall or to the strength of the interaction between methanol dehydrogenase and a component of the respiratory chain. It was possible to release a higher proportion of the methanol dehydrogenase into the supernatant, but the necessary conditions (longer incubation with lysozyme) also caused greater leakage of malate dehydrogenase from the spheroplasts which clearly underwent spontaneous lysis as judged by the high viscosity of the suspension. A milder treatment of cells with lysozyme resulted in only slight release of methanol dehydrogenase, and no release

of malate dehydrogenase, thus parallelling our earlier conclusion [10] that mere treatment with lysozyme is not sufficient to release periplasmic enzymes from this bacterium.

Whereas it was possible to prepare relatively stable spheroplasts from cells grown under stagnant conditions, attempts to make analogous preparations from cells grown aerobically on methanol were unsuccessful as the spheroplasts always underwent considerable lysis. Thus although we are proposing that methanol dehydrogenase is a periplasmic enzyme, we cannot strictly exclude the unlikely possibility that the enzyme might have a different location in aerobically grown cells.

A location for methanol dehydrogenase on the periplasmic side of the plasma membrane is consistent with its electron acceptors being c type cytochromes which, particularly by analogy with their mitochondrial counterparts, are expected to be on the external surface of the membrane. A further point to consider is that the product of methanol oxidation, formaldehyde, is highly reactive, and therefore damage to cell components may be minimised by its generation outside the cytoplasm before diffusion into the cell for further metabolism. These considerations suggest that a periplasmic location for methanol dehydrogenase may be widespread amongst the methanol utilising bacteria, although for methane oxidising bacteria which have extensive intracytoplasmic membranes it is probable not meaningful to describe a periplasmic space. It is predicted in these organisms that methanol dehydrogenase will lie on the opposite side of the membrane to the F_1 segment of the H^+-ATPase which may be in contact with the aqueous phase where methanol is produced from methane by a soluble NAD(P)H dependent oxygenase [13].

Fig. 1 summarises some possible schemes for electron and proton movements associated with methanol oxidation. Scheme 1 shows how a periplasmic location for methanol dehydrogenase could inevitably lead to net proton translocation but without a requirement for H^+ pumping by the respiratory chain. The requirement that cytochrome aa_3 should accept H^+ for the reduction of O_2 from the cytoplasm

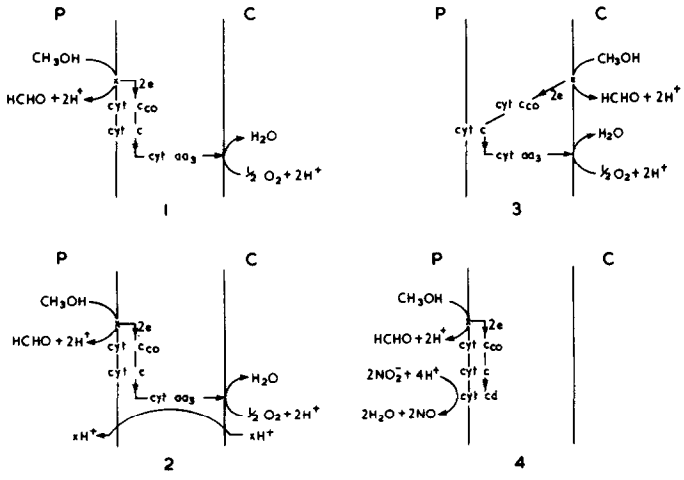

Figure 1 Topological schemes for methanol oxidation in P. denitrificans

X is methanol dehydrogenase, P the periplasm and C the cytoplasm. The location of the CO-binding c-type cytochrome (c_{CO}) associated with methanol oxidation [4] has not been established and only by inference can it be shown as the electron donor to cytochrome c. Other schemes in which the quinone cofactor of methanol dehydrogenase or a component of the respiratory chain (e.g. cytochrome aa_3) is proton translocating would be required to account for proton translocation if methanol dehydrogenase were cytoplasmic (cf. Scheme 3), or if, irrespective of the location of methanol dehydrogenase, water were formed using protons taken from the periplasm.

It is possible that N_2O rather than NO is the product of nitrite reductase, but this alteration would not affect the conclusion about the absence of proton translocation shown in scheme 4.

is credible both by analogy with the mitochondrial enzyme [14], and because of respiratory control and therefore generation of membrane potential in reconstituted vesicles containing P. denitrificans cytochrome aa_3 with external cytochrome c as electron donor [9]. Scheme 1 predicts $H^+/O = 2$ for methanol oxidation but the experimental value reported is 3.5 [4]. However, this could be consistent with scheme 2 in which a proton pumping capacity of cytochrome aa_3 is shown, although purified P. denitrificans cytochrome aa_3 could not be shown to act as a proton pump [9]. In other bacteria where an $H^+/O = 2$ has been measured for methanol oxidation [6,8] scheme 1 might be operative. If the methanol dehydrogenase were to have a cytoplasmic location then an H^+-pump, or other proton translocation mechanism perhaps using the quinone of methanol dehydrogenase would be needed, otherwise no conservation of energy as a proton-

motive force could accompany electron flow from methanol to oxygen (Scheme 3, Fig. 1).

The efficiency of energy conservation associated with methanol oxidation has implications for single cell protein production [7], and therefore it is important to find out whether P. denitrificans, which uses the ribulose bisphosphate pathway for growth on methanol, has a higher H^+/O and thus P/O ratio for methanol than the methylotrophs. The possibility of an H^+/O greater than 2 also suggests that the P/O in P. denitrificans could be greater than 1, the exact value depending on H^+/ATP which is at present uncertain. Comparison with mitochondrial ATP synthesis where non-integral P/O ratios are suggested, with the highest value for the terminal segment of the respiratory chain [15], is very relevant.

P. denitrificans grows poorly on methanol under anaerobic conditions in the presence of NO_3^- or NO_2^- [3], and it was concluded that methanol could reduce only NO_2^- but not NO_3^- [3]. Scheme 4 (Fig. 1) may provide at least a partial explanation for the poor growth, since if both methanol dehydrogenase and nitrite reductase are periplasmic then the transfer of electrons from methanol to nitrite is probably unable to generate a protonmotive force irrespective of the available redox energy. (A similar conclusion may also be drawn concerning electron flow from ethanol to N_2O, as N_2O reductase may be predicted to be periplasmic on the basis of its association with c-type cytochromes.)

In summary we conclude that the methanol/oxygen reaction may be catalysed in an obligatory chemiosmotic fashion, as discussed for other bacterial systems in [16], but depending on the outcome of further work on cytochrome aa_3 the generation of the protonmotive force may be supplemented by a proton pumping oxidase, at least in P. denitrificans.

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