Measurement of the oxidation-reduction potentials of amicyanin and *c*-type cytochromes from *Paracoccus denitrificans*

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The oxidation-reduction potentials of four periplasmic electron carrier proteins from *Paracoccus denitrificans* have been determined. Their midpoint potentials are: amicyanin, 294 ± 6 mV; cytochrome *c*-550, 253 ± 5 mV; cytochrome *c*-551i, 190 ± 4 mV; and cytochrome *c*-553i, 148 ± 5 mV. Although rapid amicyanin-mediated transfer of electrons from methylamine dehydrogenase to cytochrome *c*-551i was observed, reduced amicyanin did not reduce oxidized cytochrome *c*-551i in the absence of methylamine dehydrogenase.

Amicyanin *Cytochrome c* *Methylamine dehydrogenase* *Methylotrophic bacteria* *Redox potential* (Paracoccus denitrificans)

1. INTRODUCTION

Paracoccus denitrificans is capable of growth on methanol or methylamine as a sole source of carbon and energy [1]. Each of these substrates is oxidized to formaldehyde by specific methanol and MADHs, pyrroloquinoline quinone-containing enzymes [2], which function in the periplasmic space [3,4] of this Gram negative bacterium. The natural electron acceptor for MADH in *P. denitrificans* is a periplasmic type I blue copper protein, amicyanin [4,5]. Amicyanin is induced only during growth on methylamine [4]. Also present in the periplasm of methylamine-grown cells are three *c*-type cytochromes [6], a constitutive cytochrome *c*-550 and two inducible cytochromes, *c*-551i and *c*-553i, which are present during growth on methanol or methylamine, but absent during heterotrophic growth. None of these cytochromes accepts electrons directly from MAHD, but amicyanin-mediated electron transfer from MADH to each cytochrome was observed, with cytochrome *c*-551i functioning most efficiently [6]. To understand better the pathways of electron transport from methylamine and methanol to the membrane-bound respiratory chain, the oxidation-reduction potentials of amicyanin and cytochromes *c*-550, *c*-551i and *c*-553i were measured.

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Abbreviations: E_m, midpoint potential; MADH, methylamine dehydrogenase

2. EXPERIMENTAL

Purification of amicyanin [4] and the *c*-type cytochromes [6] from *P. denitrificans* (ATCC 13543) was as previously described. Electrochemical titrations were performed as described [7] using an optically transparent gold electrode in a thin-layer cell. Absorption spectra were recorded

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in 20 mM potassium phosphate buffer, pH 6.7, at either 20°C or 5°C with an Aminco DW-2a UV-Vis spectrophotometer. Control titrations, performed with horse heart cytochrome *c*, methyl viologen and spinach ferredoxin, gave E_m values within 5 mV of literature values.

3. RESULTS AND DISCUSSION

All of the cytochrome and amicyanin titrations (figs 1,2 and table 1) were fully reversible. For all four proteins, identical E_m values were obtained over a 5-fold range of mediator concentrations. For the three cytochromes, identical E_m values were obtained from titrations at 5°C and 20°C. For amicyanin, data were reported only for titrations that were performed at 5°C because some denaturation of amicyanin was observed during titrations at 20°C. The E_m value of *P. denitrificans* amicyanin was calculated from the reductive and oxidative titrations (fig.2) to be 294 ± 6 mV (n = 0.95). This value is consistent with values reported for three other amicyanins which have been isolated from taxonomically divergent sources: *Pseudomonas* sp. strain AM1, E_0 = 280 mV [8]; *Thiobacillus versutus*, E_0 = 260 mV [9]; and organism 4025, E_0 = 294 mV [10]. Cytochrome *c*-550 is presumably identical to the cytochrome *c*-550 which was isolated previously from *P. denitrificans* (formerly *Micrococcus denitrificans*) [11,12]. The measured E_m (fig.2) of 253 ± 5 mV (n = 0.99) is in good agreement with the E_m value previously reported for that protein of 250 mV [11]. The E_m values of the inducible cytochromes *c*-551i and *c*-553i are 190 ± 4 mV (n = 0.92) and 148 ± 5 mV (n = 1.15), respectively (figs 1,2).

Based on thermodynamic considerations, it is possible that electrons from these inducible cytochromes could enter the membrane-bound respiratory chain in favorable reactions via the constitutive cytochrome *c*-550. The precise physiological roles of the inducible cytochromes are not known. That they are induced during growth on methanol or methylamine implies a role

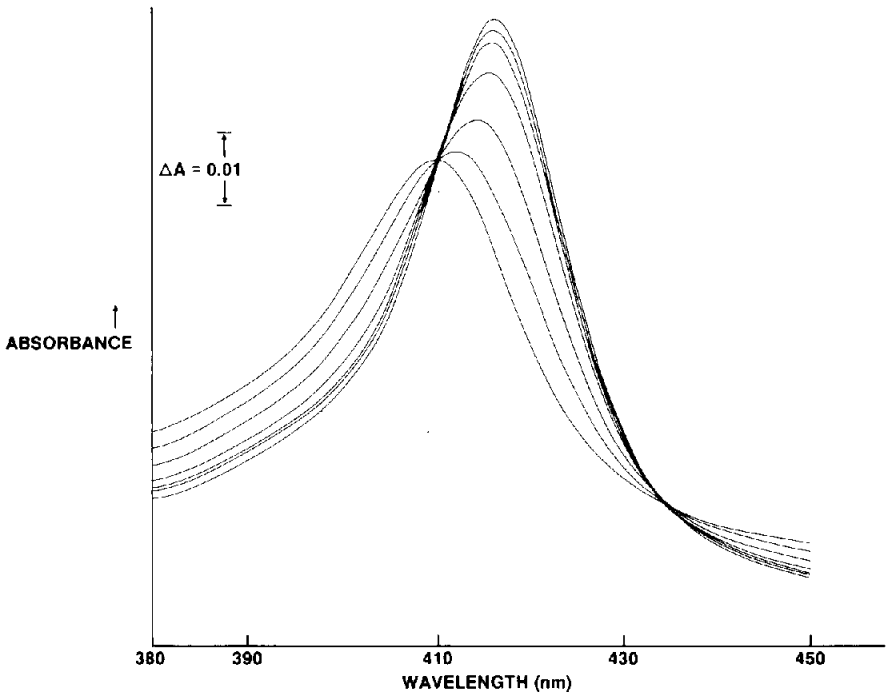

Fig.1. Potentiometric titration of *P. denitrificans* cytochrome *c*-551i. Cytochrome *c*-551i (45 μM) was titrated at 5°C in the presence of 50 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), 5 μM phenazine methosulfate (PMS) and 20 μM potassium ferricyanide. The applied potentials were (from top to bottom) 46, 96, 116, 146, 176, 206 and 266 mV. The optical pathlength was 0.3 mm.

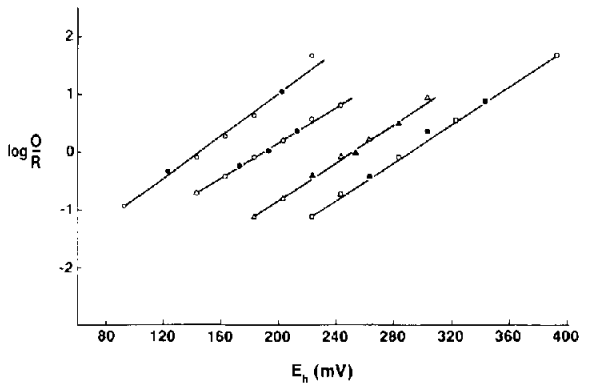

Fig.2. Oxidation-reduction potentials of *P. denitrificans* electron carrier proteins. (○) Cytochrome *c*-551i (30 μM) was titrated in the presence of 50 μM TMPD, 5 μM PMS, 5 μM phenazine ethosulfate and 10 μM duroquinone. ( ) 25 μM cytochrome *c*-551i and (△) 30 μM cytochrome *c*-550 were titrated in the presence of 50 μM TMPD, 5 μM PMS and 20 μM potassium ferricyanide. (□) 130 μM amicyanin was titrated in the presence of 10 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine, 5 μM PMS, 10 μM hydroquinone and 10 μM 1,2-naphthoquinone. Titrations were conducted at 5°C and the oxidation states of each protein were determined by monitoring a ΔA at two wavelengths using an isosbestic point as a reference. The wavelengths used were 418 and 442 nm for cytochrome *c*-553i, 416 and 435 nm for cytochrome *c*-551i, 415 and 435 nm for cytochrome *c*-550, and 595 and 500 nm for amicyanin. Open symbols represent points taken during reductive titrations and closed symbols represent points taken during oxidative titrations. The straight lines represent the best fits as determined by a least-squares program run on an Apple II computer.

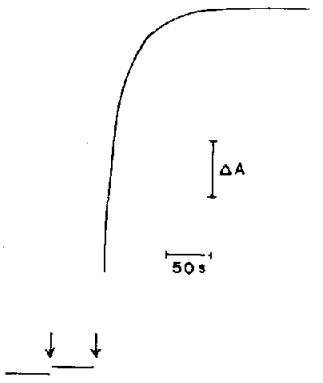

Fig.3. Methylamine dehydrogenase-dependent reduction of cytochrome *c*-551i by amicyanin. The assay mixture (1.1 ml) contained 1.3 nmol of cytochrome *c*-551i in 0.1 M potassium phosphate, pH 6.7. The first arrow indicates addition of 50 μl containing 2.2 nmol of reduced amicyanin. The second arrow indicates addition of 14 pmol of MADH and 4 μmol of methylamine. Changes in absorbance were monitored at 420 nm. The small increase in absorbance after the first addition is due to absorbance by amicyanin.

Table 1

Redox properties of *P. denitrificans* electron carrier proteins

Protein	E_m value (mV)	*n* value
Amicyanin	294 ± 6	0.95 ± 0.04
Cytochrome *c*-550	253 ± 5	0.99 ± 0.02
Cytochrome *c*-551i	190 ± 4	0.92 ± 0.07
Cytochrome *c*-553i	148 ± 5	1.15 ± 0.04

E_m and *n* values represent the average of three independent titrations

in methanol- and methylamine-dependent respiration. Soluble cytochromes *c*, which are thought to be involved in methanol- and methylamine-dependent respiration, and which were designated c_H and c_L on the basis of isoelectric point, have been isolated from *Pseudomonas* sp. strain AM1 [13] and *Methylophilus methylotrophus* [14]. These cytochromes have E_m values which range from 256 to 373 mV [13,14]. The inducible *P. denitrificans* cytochromes, although exhibiting significantly different physical properties [6] and redox potentials, may be functionally similar to the cytochromes *c* of those methylotrophs.

It has been previously demonstrated [4,6] that amicyanin, but not cytochrome *c*-551i, is directly reduced by MADH, and that amicyanin efficiently transfers electrons between MADH and cytochrome *c*-551i. The thermodynamic data presented here, however, are inconsistent with the latter finding. To examine this apparent discrepancy, amicyanin was reduced with sodium dithionite and then added to cytochrome *c*-551i in the absence of MADH (fig.3). Under these conditions, consistent with the thermodynamic data, no reduction of the cytochrome was observed. On addition of methylamine and MADH, which do not by

themselves reduce cytochrome *c*-551i [6], rapid reduction of the cytochrome occurred. One explanation for these observations is that the E_m value of amicyanin is shifted when the protein forms a complex with MADH. This possibility is currently under investigation.

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