Pseudoazurin mediates periplasmic electron flow in a mutant strain of *Paracoccus denitrificans* lacking cytochrome c_{550}

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Abstract A periplasmic protein able to transfer electrons from cytoplasmic membrane to the periplasmic nitrite reductase (cytochrome *cd*$_1$) has been purified from the anoxically grown cytochrome c_{550} mutant strain Pd2121 and shown to be pseudoazurin by several independent criteria (molecular mass, copper content, visible spectrum, N-terminal amino acid sequence). Under our assay conditions, the half-saturation of electron transport occurred at about 10 μM pseudoazurin; the reaction was retarded by increasing ionic strength.

Key words: Pseudoazurin; Nitrite reduction; Electron transport; *Paracoccus denitrificans*

1. Introduction

The denitrification pathway of *Paracoccus denitrificans* involves, in addition to the membrane-bound nitrate and nitric oxide reductases, two water-soluble enzymes that are located within the periplasmic space of the cell: nitrite reductase and nitrous oxide reductase [1]. The molecular basis for electron transport between the membrane and these periplasmic enzymes has not yet been completely resolved. Although a periplasmic *c*-type cytochrome designated cytochrome c_{550} is known to shuttle electrons in cell-free systems [2], phenotype characteristics of a cytochrome c_{550}-deficient mutant strain [3] disprove an obligatory role for this protein in denitrification and prompt speculations over a possible substitute. The participation of another *c*-type cytochrome has been suggested [4], based on the results of reconstitution experiments using subcellular fractions and on correlations between the cellular levels of proteins and enzyme activities. However, two other pieces of evidence favor electron transport via a copper protein: (i) a diminished activity of nitrite reduction in the cytochrome c_{550} mutant cells grown under copper deficiency [5] and (ii) an inhibition by the copper chelator diethyldithiocarbamate (DDC), which is more pronounced in the mutant than in the wild-type cells [6].

In view of the persisting uncertainty, the present work aims at resolving the nature of the periplasmic electron carrier.

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Abbreviations: AAS, atomic absorption spectroscopy; DDC, diethyldithiocarbamate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2. Materials and methods

2.1. Growth of cells

The cytochrome c_{550} insertion mutant strain Pd2121 was grown at 30°C in a synthetic medium containing 50 mM sodium succinate and 10 mM KNO_3 [7], supplemented with 50 μg/ml kanamycin. Precultures of 30 ml were used to inoculate 3 l flasks filled with the medium and the cultures were incubated statically for 22 h. Cells were harvested by centrifugation at 5500×*g* for 30 min and washed once in 0.1 M sodium phosphate buffer (pH 7.3).

2.2. Purification procedure

Step 1. Preparation of a periplasmic fraction. Periplasm was released from the freshly harvested cells (13×3 l of culture in total) by treatment with lysozyme followed by a mild osmotic shock as described [8], concentrated with Minitan filter plates (Millipore) to approximately 250 ml and clarified by centrifugation at 40 000×*g* for 40 min.

Step 2. First ion-exchange chromatography. The concentrated solution of periplasmic proteins was loaded onto a Sepharose Q (Pharmacia) column (16×300 mm) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.3) and eluted with a 600 ml linear gradient (0–1 M NaCl, pH 7.3) at a flow rate of 1.5 ml/min. Fractions of 5 ml were collected; those containing a reconstitutive activity were pooled and concentrated by ultrafiltration to a volume of 10 ml.

Step 3. Gel permeation chromatography. The sample was applied in two separate batches to a Superose 12 (Pharmacia) column (30×600 mm) equilibrated with 150 mM NaCl in 50 mM sodium phosphate (pH 7.3) and eluted with the same buffer, at a flow rate of 0.5 ml/min and a fraction volume of 5 ml.

Step 4. Second ion-exchange chromatography. After desalting with a Sephadex G25 column (5 ml), final purification of the pooled reconstitutively active fractions was achieved on a MonoQ Superose (Pharmacia) column (1 ml) equilibrated with 20 mM Na-HEPES (pH 7.2). After loading, elution was done with a 20 ml linear gradient (0–300 mM NaCl, pH 7.2) at 0.5 ml/min; individual peaks were collected.

2.3. Enzyme assays

Reconstitution of the succinate-nitrite reductase activity was carried out by adding samples (100 μl) to the reaction mixture (40 μl) containing Tris-HCl buffer (pH 7.3), 2 μmol; sodium succinate, 2.5 μmol; crude nitrite reductase, 0.3 mg protein and membrane vesicles, 0.35 mg protein, yielding a final volume of 140 μl. After 20 min of incubation at 30°C, the reaction was started with 7.5 μl of 10 mM $NaNO_2$ and terminated with 50 μl of saturated zinc acetate 30 min later. The disappearance of nitrite was followed colorimetrically [9] in the supernatants.

For the determination of peroxidase, a microplate assay was adopted: 5 μl of the sample was mixed with 200 μl of 0.1 M sodium acetate (pH 6.0) containing 0.2 mg/ml 3,3′,5,5′-tetramethylbenzidine dihydrochloride and 1.8 mM hydrogen peroxide and absorbance at 650 nm was recorded on a Multiscan microplate reader (Labsystem).

2.4. Analytical methods

Protein contents of fractions were measured by the method of Bradford [10] using bovine serum albumin as standard.

Absorption spectra were recorded at 25°C in an Ultrospec 2000 UV/Vis instrument (Pharmacia Biotech) linked to a PC.

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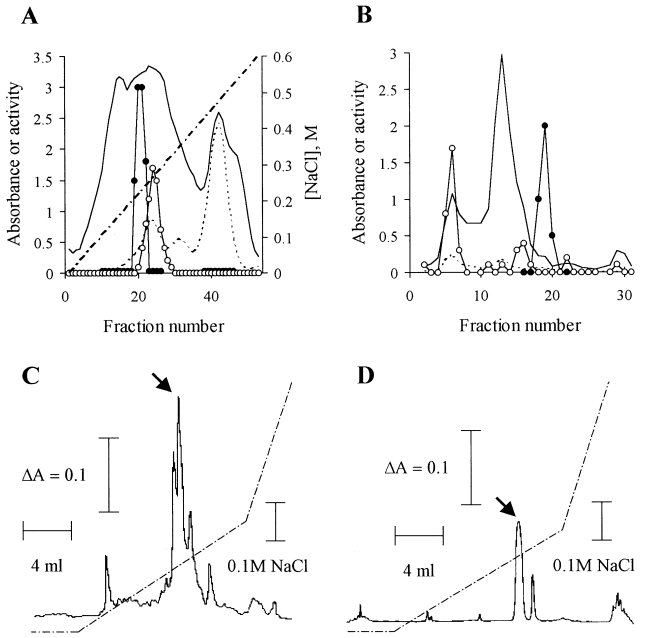

Fig. 1. Elution profiles for a typical purification run. Chromatographic conditions are specified in Section 2.2. A: Sepharose Q (step 2); B: Superose 12 (step 3); C: MonoQ Superose (step 4); D: a repeated MonoQ Superose (step 4). Solid lines, absorbance at 280 nm; broken lines, absorbance at 410 nm; dot/dash lines, NaCl gradient; solid circles, nitrite reductase activity after reconstitution, expressed in nmol NO_2^-/min per 1 μl of the eluate and multiplied by 120 (a scaling factor); empty circles, peroxidase activity expressed as $\Delta A_{650}/\Delta t$ (min^{-1}) per 1 μl of the eluate and multiplied by 1000. In C and D, the peaks containing reconstitutive activity are indicated by arrows.

Copper concentrations of purified protein preparations (20 μl, in triplicate) were analyzed by atomic absorption spectroscopy using a Perkin-Elmer 3030 AA spectrophotometer equipped with a HGA 74 graphite furnace.

SDS-PAGE was performed in 12.5% (w/v) acrylamide slab gels (6 cm×8 cm×0.75 mm) using a Mini Protean chamber system (Bio-Rad) [11]. SDS-PAGE standard proteins were obtained from Fluka. Gels were stained with Coomassie Brilliant Blue and destained in 40% methanol/10% acetic acid.

N-terminal amino acid sequence was determined by Edman degradation and subsequent analysis of the amino acids with a model 473A gas-phase amino acid Sequenator (Applied Biosystems). Protein sample was electrotransferred from PAGE to a poly(vinylidene difluoride) membrane (Bio-Rad) before sequencing.

3. Results

Initial studies indicated that reduction of nitrite by succinate in a cell-free system required the presence of a factor stemming from the bacterial periplasm. As documented in Fig. 1, the reconstitutive activity eluted in single peaks during successive chromatographic purification steps. There were differences in the elution volume between this activity and the components absorbing at 410 nm and exhibiting peroxidase activity. At the end of the purification procedure, approximately 2 mg of a reconstitutively active protein were obtained from a 39 l culture. SDS-PAGE of this preparation gave a single band at an M_r of 14 000 (Fig. 2). The same value of M_r was found by the column chromatography (not shown), indicating a monomeric form of the protein in the solution.

Effectiveness of the purified 14 000-M_r protein as an electron carrier in our assay system is demonstrated by the results of Fig. 3. A characteristic feature was the saturability of the reaction rate at relatively low mediator concentration. The saturation curve reminiscent of a Michaelis–Menten curve afforded the calculation of an apparent K_m as 9.7 μM. Additions of an inert electrolyte (NaCl) had a significant inhibitory effect on the reconstituted activity, 50% diminution being achieved at 0.2 M (results not shown).

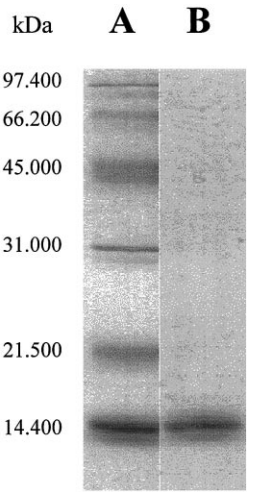

Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified protein. Lane A contained 50 μg of a mixture of standard proteins (Fluka) with the indicated molecular masses, lane B 4 μg of the final preparation.

Further work centered about the question of whether a heme or a copper prosthetic group is associated with the 14 000-M_r protein. The absorption spectrum of an oxidized but not reduced sample showed a pronounced maximum close to 590 nm (Fig. 4), which is typical for a family of type-1 copper proteins [12]. With atomic absorption spectroscopy, a content of 58 nmol of copper per mg of protein was determined, corresponding to 0.81 atoms of copper per molecule at 14 000-M_r. Definite identification of the protein became possible after N-terminal sequencing. The obtained sequence of 20 amino acids, ATHEVHMLNKGESGAMVFEP, is identical to that reported for *P. denitrificans* pseudoazurin [13].

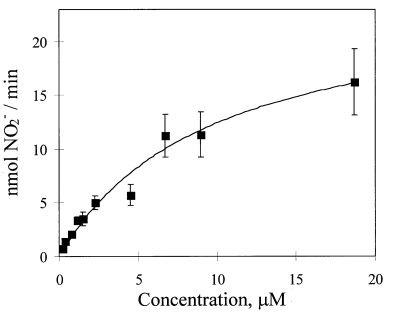

Fig. 3. Reconstitution of nitrite reductase activity brought about by the purified electron carrier. Reaction conditions were accomplished as described in Section 2.3 except that the reaction time was appropriately reduced. Each point shown is the mean of triplicate measurements ± S.D., the line represents the fit to the Michaelis–Menten equation (K_m = 9.7 μM, V_{max} = 35.0 nmol NO_2^-/min).

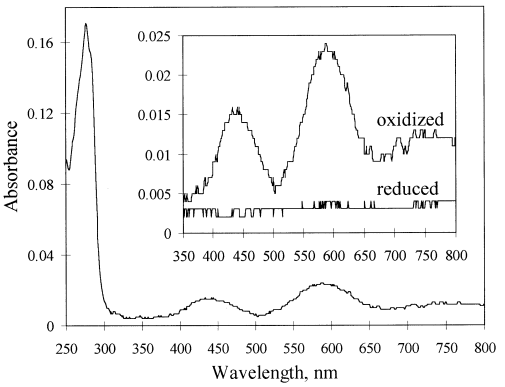

Fig. 4. Absorbance spectra of the purified protein. The sample cuvette contained 0.2 ml of 50 mM Na-HEPES buffer, pH 7.0; the protein concentration was 0.26 mg/ml. The inset shows the same spectrum in the region 350–800 nm and its appearance after reduction with a few crystals of ascorbic acid.

4. Discussion

Resolution and reconstitution experiments presented above have established that *P. denitrificans* grown anoxically contains a small soluble protein which is able to restore the activity of succinate-nitrite reductase when incubated with membrane vesicles and nitrite reductase. This redoxactive protein is now identified as pseudoazurin by its molecular mass, the content of copper, visible spectrum and N-terminal amino acid sequence. New conclusive identification corroborates earlier findings reached by indirect experimental approaches [5,6] and weakens an alternative interpretation according to which the effects of DDC result from a Cu-independent inhibition in combination with a direct electron donation to cytochrome *c* [14]. Disparity in the elution profiles of the reconstitutive activity, Soret absorption and (pseudo)peroxidase (Fig. 1) suggests that the presence of a *c*-type cytochrome in some previously studied redoxactive preparations [4] may be merely coincidental.

In their investigation of *Thiosphaera pantotropha* (a close relative to *P. denitrificans*) Moir and colleagues [15] found a Michaelis constant of 220 μM for the reaction of pseudoazurin with nitrite reductase and considered it to be telling about the actual concentrations of pseudoazurin needed in the periplasm. However, this is justified only if the control of electron flow resides exclusively in the terminal nitrite reductase step. At a low (rate-limiting) electron-transfer capacity for reduction of pseudoazurin by the respiratory chain the half-saturation concentration may differ. More detailed studies of pseudoazurin redox reactions should allow us to decide whether the 22 times lower value reported here (Fig. 3) reflects such kinetic factors or some interspecific differences between *P. denitrificans* and *T. pantotropha*.

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