

Copper-containing nitrite reductase from *Pseudomonas chlororaphis* DSM 50135

Evidence for modulation of the rate of intramolecular electron transfer through nitrite binding to the type 2 copper center

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The nitrite reductase (Nir) isolated from *Pseudomonas chlororaphis* DSM 50135 is a blue enzyme, with type 1 and type 2 copper centers, as in all copper-containing Nirs described so far. For the first time, a direct determination of the reduction potentials of both copper centers in a Cu-Nir was performed: type 2 copper (T2Cu), 172 mV and type 1 copper (T1Cu), 298 mV at pH 7.6. Although the obtained values seem to be inconsistent with the established electron-transfer mechanism, EPR data indicate that the binding of nitrite to the T2Cu center increases its potential, favoring the electron-transfer process. Analysis of the EPR spectrum of

the turnover form of the enzyme also suggests that the electron-transfer process between T1Cu and T2Cu is the fastest of the three redox processes involved in the catalysis: (a) reduction of T1Cu; (b) oxidation of T1Cu by T2Cu; and (c) reoxidation of T2Cu by NO₂⁻. Electrochemical experiments show that azurin from the same organism can donate electrons to this enzyme.

Keywords: copper nitrite reductase; EPR; redox-titration; type 1 copper; type 2 copper.

Several microorganisms reduce nitrate in a stepwise manner via nitrite to form sequentially NO, N₂O, and eventually dinitrogen as part of their energy-generating metabolism, in a process known as denitrification. Nitrite reductase (Nir) plays a key role among the four dissimilatory reductases of the denitrifying pathway, as this is the step where losses of 'fixed' nitrogen from soil into the atmosphere become irreversible. In denitrifying bacteria two rather different types of Nir have been found, one of which is a cytochrome *cd₁*, while the other contains copper, and no iron [1].

Copper-containing nitrite reductases present a trimeric structure [2–5] and contain both type 1 (T1Cu) and type 2

(T2Cu) copper centers. Each subunit has a molecular mass of ≈ 40 kDa and, although the total number of copper atoms found in enzymes from different organisms varies considerably depending on the purification and storage process, six copper atoms have been found in all the crystal structures determined so far. Cu-Nirs have been classified in two groups, according to the spectroscopic properties of their T1Cu centers. Blue reductases (e.g. from *Pseudomonas aureofaciens* or *Alcaligenes xylosoxidans*) exhibit a very intense absorption band at ≈ 590 nm and axial EPR signals. Green reductases (e.g. from *Achromobacter cycloclastes* or *Alcaligenes faecalis*) present two intense absorption bands (≈ 460 and 600 nm) and rhombic EPR signals. The T1Cu site is bound by four ligands (His95, His145, Cys136 and Met150 in the *Ac. cycloclastes* numbering) and the geometry is an axially flattened tetrahedron in green Nir or an axially distorted tetrahedron in blue Nir [4,5]. The T2Cu site is coordinated by a water molecule and three His residues, two from one monomer (His100 and His135) and another from the adjacent monomer (HisB306), and shows a distorted tetrahedral geometry. T1Cu center is involved in the intramolecular electron transfer [6], while the T2Cu is the catalytic center [7]. The study of this protein therefore entails the investigation of three redox processes: the reduction of T1Cu by an external electron donor, the intramolecular electron transfer from T1Cu to T2Cu, and the reduction of nitrite at the T2Cu center.

In *Ac. cycloclastes*, electron transfer between pseudoazurin and nitrite reductase has been investigated by cyclic voltammetry [8]. Pseudoazurin accepts the electrons

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Abbreviations: Az-iso2, azurin iso-2 from *Methylomonas* sp.; *cd₁*-Nir, cytochrome *cd₁* nitrite reductase; Cu-Nir, copper-containing nitrite reductase isolated from *Pseudomonas chlororaphis* DSM 50135; cyt., cytochrome; DDC, diethyldithiocarbamate; *k_{app}*, apparent rate constant; MADH, methylamine dehydrogenase; NHE, normal hydrogen electrode; pAz, pseudoazurin; T1Cu, type 1 copper; T2Cu, type 2 copper.

Enzyme: nitrite reductase (EC 1.7.2.1).

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from the electrode and donates them to the Nir, in the presence of nitrite. Under these conditions, the shape of the voltammogram becomes sigmoidal, with an increase of the cathodic current (catalytic current) due to the regeneration of oxidized pseudoazurin in the diffusion layer near the electrode. In *Al. xylosoxidans* GIFU 1015, however, the voltammetric response is unaffected in the presence of Nir and nitrite, which indicates a slower electron transfer process [9]. Unlike earlier reports [10], recent studies seem to indicate that cytochrome *c*₅₅₁ could be the physiological electron donor to the aforementioned Nir [11].

The electrons donated by the donor to the T1Cu center are transferred to the catalytic T2Cu center through a chemical path involving the residues Asp98 and HisB255 (*Al. xylosoxidans* GIFU1051 numbering). Studies of site-directed mutagenesis showed that both amino acids control the intramolecular electron transfer process through the formation of a hydrogen bond network, which is involved in the proton supply for substrate reduction [12,13]. The intramolecular electron transfer rate changes smoothly with pH in the absence of nitrite, but decreases very sharply with increasing pH when nitrite is present [14], suggesting that nitrite binding to the enzyme breaks the hydrogen bond network surrounding the T2Cu center. The shape of this dependence is identical to the pH dependence of the enzyme activity, which suggests that the catalytic process and the intramolecular electron transfer are closely linked.

The most recent model for the catalytic mechanism of Cu-Nir supposes that nitrite binds to the oxidized form of the T2Cu center, displacing a solvent molecule and forming a hydrogen bond between one of its oxygen atoms and the Asp98 residue. After reduction of the T2Cu center with an electron from the T1Cu center, the proton of this hydrogen bond is transferred from the Asp98 residue to the oxygen atom of the substrate, yielding an O=N–O–H intermediate. The N–O bond in this oxygen atom is then broken, yielding the product NO, whose release re-establishes the original coordination of the active center. HisB255 could be involved in the formation of additional hydrogen bonding, stabilizing the deprotonated form of the Asp98 residue [15] or the O=N–O–H intermediate itself [16].

In order to study the electron flow in the denitrifying pathway of the Gram-negative bacterium *Pseudomonas chlororaphis* DSM 50135, we have purified and characterized its nitrite reductase (Cu-Nir). This blue copper-containing enzyme is able to accept electrons from the azurin isolated from the same strain at moderate rates. Spectroscopic characterization allowed, for the first time, the determination of the redox potentials of both copper centers. Our studies suggest that the presence of substrate plays an important role in the modulation of the redox potential of the T2Cu center influencing the intramolecular electron-transfer rate between both copper centers.

Materials and methods

Organisms and growth

Ps. chlororaphis DSM 50135 was grown in microaerobic conditions at 28 °C and pH 7.0 in a well-defined medium,

continuously stirred during growth. The composition of the growth medium was (g·L⁻¹): KH₂PO₄, 2; di-hydrated trisodium citrate, 5; MgSO₄·7H₂O, 1; CaCl₂·2H₂O, 0.05; NaCl, 1; NaNO₃, 4.5; NH₄Cl, 3; KCl, 0.75. The medium was also supplemented with oligoelements (μM): FeCl₃, 74; CuCl₂, 1; ZnSO₄, 1; MnSO₄, 1; (NH₄)₆Mo₇O₂₄, 0.2; Ni(NO₃)₂, 0.03; Na₂SeO₃, 0.03; CoCl₂, 0.6 and Na₂B₂O₇, 0.1. The optical density, the concentrations of nitrate [17] and nitrite [18], the pO₂ and the pH of the medium were monitored, and the composition of the gaseous phase in the fermentor was analyzed by mass spectrometry. At the end of the exponential growth phase, after the depletion of both nitrate and nitrite in the medium, the cells were harvested at 4 °C, using a Sharples centrifuge, at 9900 g, with a 60 L·h⁻¹ flow. Cell yield was 1.7 g wet weight per L. Cell paste was stored at -20 °C.

Protein purification

Cu-Nir was isolated by chromatographic procedures from *Ps. chlororaphis* strain DSM 50135. All steps were performed at 4 °C. In every step, all fractions were dialyzed and concentrated by ultrafiltration on Diaflo cells (Amicon Corp., Danvers, MA, USA), using YM30 membranes, and analyzed by electronic spectroscopy (spectral ratio: *A*₂₈₀/*A*₆₀₀). Activity staining of the enzyme in native electrophoresis gels was also used to follow the protein during purification. Five hundred and seventeen grams of cells (wet weight) were suspended in 10 mM Tris/HCl pH 7.6, supplemented with 10 μM CuSO₄ (standard buffer) and lysed with a Manton Gaulin press at 9000 MPa. Cell debris and intact cells were removed by centrifugation (20 000 g for 30 min, at 4 °C) and the membrane fraction was separated by ultracentrifugation (180 000 g for 90 min, at 4 °C). The soluble fraction was then applied to a DEAE-cellulose 52 column (5 × 40 cm) equilibrated with standard buffer. A linear gradient was then applied onto the column, from standard buffer to 400 mM Tris/HCl pH 7.6. Several fractions with nitrite reductase (Nir) activity eluted from the column, up to an ionic strength of ≈200 mM. All these fractions were sequentially applied onto an ionic exchange column, Source 15Q (1.6 cm × 30 cm), equilibrated in standard buffer, and eluted at ionic strengths between 20 and 50 mM Tris/HCl pH 7.6. Finally, the protein was submitted to a gel filtration in a Sephadex 75 column (2.6 × 60 cm) equilibrated with 0.3 M Tris/HCl pH 7.6 buffer. An electrophoretically pure sample (SDS/PAGE) with a spectral ratio, *A*₂₈₀/*A*₆₀₀, of 18.8 was concentrated, frozen in liquid nitrogen and stored at -70 °C until use. Azurin from the same strain was purified as described before [19].

Protein and copper determination

Protein was assayed with the microbiuret method [20]. Bovine serum albumin was used as standard. The copper content was determined by atomic absorption spectroscopy on a PerkinElmer spectrophotometer, Model 5000, equipped with a copper hollow cathode lamp. The standard solutions in the concentrations 0, 0.5, 1.0, 1.5 and 2.0 p.p.m. were prepared in water by appropriate dilution from a 1000 mg·L⁻¹ Cu stock solution (Titrisol, Merck).

Electrophoresis, activity stain, molecular mass and isoelectric point determination

Purity of the proteins was established by polyacrylamide gel electrophoresis. Molecular mass was determined by SDS/PAGE, according to the method of Laemmli [21], using the Pharmacia low molecular mass kit as standards for calibration (values in kDa): phosphorylase *b* (94.0), albumin (67.0), ovalbumin (43.0), carbonic anhydrase (30.0), trypsin inhibitor (20.1) and α -lactalbumin (14.4).

Nitrite reductase activity was detected directly in the native gel as a clear band of oxidized methylviologen after addition of nitrite [22]. After electrophoresis, the gel was placed for 10 min in a degassed solution containing 100 mM potassium phosphate buffer pH 7, 4 mM methylviologen and 10 mM sodium nitrite. Then 3 mL of sodium dithionite 70 mg·mL⁻¹ in 0.1 M NaHCO₃ were added to the reaction mixture. The gel was shaken slowly until clear bands appeared against the dark blue gel background, and placed in 2.5% triphenyltetrazolium chloride (TTC) to fix the bands. Afterwards, the gel was washed in water and kept in 50% ethanol before drying.

The molecular mass of the purified proteins was also estimated by gel filtration using a Superdex 75 HR 10/30 column (Pharmacia) equilibrated with 50 mM Tris/HCl buffer pH 7.6, 100 mM KCl, with a flow rate of 0.5 mL·min⁻¹. Albumin (66.0 kDa), carbonic anhydrase (29.0 kDa), chymotrypsinogen A (25.0 kDa), ribonuclease A (13.7 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa) were used as calibration markers. The void volume was determined with Dextran blue.

The isoelectric point (pI) of Cu-Nir was determined by isoelectric focusing with a Pharmacia Ampholine® PAG-plate gel, with polyacrylamide matrix total monomer concentration (T) 5% and cross-linking factor (C) 3% and pH values between 3.5 and 9.5. The focusing conditions were: constant power, $P = 10$ W; focusing time, 1 h 30 min, until equilibrium ($V \approx 1500$ V and $I \approx 0$); temperature, 10 °C; anodic and cathodic solutions, 1 M H₃PO₄ and 1 M NaOH, respectively. A 20 μ L sample was used, with ≈ 5 μ g protein. Low-pI Pharmacia standards were used to calibrate the gel: amyloglucosidase (3.50), methyl red (3.75), glucose oxidase (4.15), soybean trypsin inhibitor (4.55), β -lactoglobulin (5.20), bovine carbonic anhydrase (5.85) and human carbonic anhydrase (6.55).

Activity assays and protein handling

Nitrite reductase activity was measured using dithionite-reduced benzylviologen as the electron donor. The assays were performed at room temperature, in a degassed rubber-sealed UV-visible cell with 100 mM phosphate buffer pH 7.0, 0.5 mM benzylviologen and a sample aliquot, in a 2 mL total volume. Dithionite (10–20 μ L, 50 mM) was added to reduce the benzylviologen until $A_{540} \approx 1.2$. The reaction was initiated with the addition of nitrite in a final concentration of 50 mM. The time-course assay monitored the oxidation of benzylviologen at 540 nm. The specific activity was calculated using the value 13.1 mm⁻¹·cm⁻¹ for the reduced benzylviologen molar absorptivity (ϵ_{540}) [23]. The activity values were expressed in U·mg⁻¹ total protein (1 U = 1 μ mol NO₂⁻ min⁻¹), after correction for the slow

nonenzymatic oxidation of benzylviologen. Dithionite-reduced azurin from the same organism was also used as electron donor, in a final concentration of 40 μ M, in 0.1 M phosphate buffer pH 7.0 with 50 mM nitrite. Azurin oxidation was followed at 625 nm ($\epsilon = 3.86$ mm⁻¹·cm⁻¹) [19]. Inhibition assays were performed as described, with addition of the inhibitors (azide, cyanide and the copper chelator diethyldithiocarbamate, DDC) to a final concentration of 500 μ M.

Ascorbate and dithionite reduction of the enzyme and addition of nitrite to reduced samples were performed under an inert atmosphere. Buffer exchange was accomplished by simultaneous dilution and concentration in centricon systems (Amicon).

Spectroscopy

UV-visible optical spectra were recorded on a Shimadzu UV-2101PC split-beam spectrophotometer using 1-cm quartz cuvettes. Time-course activity assays were performed on an HP 8452 A Diode-Array spectrophotometer.

Variable-temperature EPR measurements at X-band were performed on a Bruker EMX spectrometer equipped with a rectangular cavity (Model ER 4102ST) and an Oxford Instruments' continuous flow cryostat. EPR spectra were simulated using the program WIN-EPR SIMFONIA 1.2 (Bruker Instruments). Spin quantifications were performed under nonsaturating conditions by double integration of the spectra and comparison to a copper-EDTA standard.

Anaerobic redox titration of nitrite reductase was carried out as follows. An 81 μ M enzyme solution in 75 mM Tris/HCl buffer, pH 7.6, was poised at different redox potentials in the presence of redox mediators (2 μ M). The mediators and their respective potentials were: potassium ferricyanide (430 mV), tetramethylphenylene diamine (260 mV), 2,6-dichloro-4-[4-(hydroxyphenyl)imino]-2,5-cyclohexadien-1-one (217 mV), 1,2-naphthoquinone (118 mV), 1,4-naphthoquinone (60 mV), 5-hydroxy-1,4-naphthoquinone (30 mV), duroquinone (5 mV), indigo tetrasulfonate (-46 mV), indigo carmine (-111 mV), phenazine (-125 mV), 2-hydroxy-1,4-naphthoquinone (-145 mV), anthraquinone-2-sulfonate (-225 mV), phenosafranin (-275 mV), safranin O (-280 mV), neutral red (-325 mV), methylviologen (-436 mV) and triquat (-550 mV). Ascorbate/dithionite-reduced enzyme was oxidatively titrated with ferricyanide. After a suitable equilibration time, samples were frozen and kept in liquid N₂.

Electrochemistry

The electron transfer process between the azurin and the nitrite reductase was studied by cyclic voltammetry in the presence of substrate. The electrochemical experiments were performed with a modified gold electrode (1.6 mm diameter, Bioanalytical Systems) arranged in a two-compartment nylon cell designed for small volumes of material. The side arm, containing the reference electrode [Ag/AgCl (3 M KCl), Bioanalytical Systems], was connected to the working compartment by a Luggin capillary. A platinum wire served as counter electrode. Voltammetry was performed with an Autolab 10 electrochemical analyzer (Eco Chemie, Utrecht, the Netherlands) controlled by GPES 4.0 software. The

potentials are referred to the normal hydrogen electrode (NHE). The electrode surface was polished for 15 min with 0.3 μm alumina (Buehler) on a polishing cloth and then cleaned for about 5 min in Millipore water using an ultrasonic pool. Electrode modification was performed by dipping the freshly polished electrode surface into 1 mM 4,4'-dithiodipyridine solution for 4 min. Excess modifier was then removed by rinsing thoroughly with Millipore water. The working electrode shows, after modification, an effective surface area of 0.018 cm^2 . Before each measurement, both the cell and the sample (70–100 μL) were flushed with argon for 15 min. During the measurements the solution was kept under a flow of argon. The voltammetric experiments were performed at room temperature, in the presence of 0.1 mM 4,4'-dithiodipyridine and an excess of nitrite. The electrochemical response of the azurin was measured in the absence and presence of nitrite reductase. Data were analyzed according to Nicholson & Shain [24], as described by Hoogvliet *et al.* [25].

Results and discussion

Isolation of nitrite reductase from the cells

A blue protein with nitrite reductase activity was isolated from the soluble extract of *Ps. chlororaphis* DSM 50135 cells, grown under denitrifying conditions. The purification process yielded ≈ 40 mg of active and electrophoretically pure enzyme (Fig. 1).

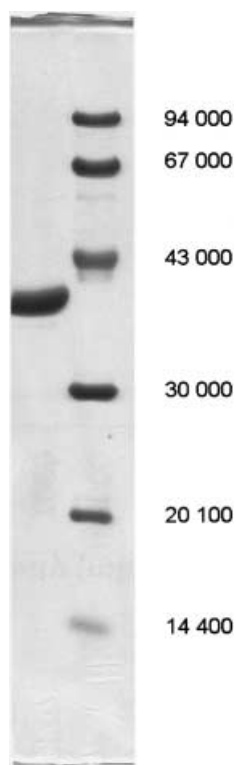


Fig. 1. SDS/PAGE of Cu-Nir. *Ps. chlororaphis* DSM 50135 nitrite reductase (left lane); molecular mass (in Da) markers (right lane).

Biochemical characterization of the protein

The molecular mass of the protein was determined by gel filtration (107 kDa) and by SDS/PAGE (37.7 kDa). These results are consistent with a homotrimeric structure for the protein, as found in other copper-containing nitrite reductases reported in the literature [1].

When submitted to isoelectric focusing, the protein migrated to form a smear, at pH values located between 5.4 and 6.2. As the protein is highly pure (cf. SDS/PAGE results), this smear probably does not result from the existence of any contaminants; rather, it may be due to some heterogeneity of the oxidation states of the metal centers in the protein. The pI of other copper nitrite reductases described in the literature are also acidic, except for the *Al. xylooxidans* NCIB 11015 protein [1]. The pI determined for *Ps. aureofaciens* Cu-Nir is 6.05, which is clearly inside the interval obtained for the Cu-Nir in study.

Copper quantification yielded 3.2 Cu per trimer. According to the crystallographic structure of several copper nitrite reductases [2,26], two Cu per monomer, i.e. six copper centers for each protein molecule are to be expected, three T1Cu and three T2Cu. However, the values reported in the literature seldom exceed 4.6 Cu per holoenzyme [27], which reflects losses during the purification process, especially of the more labile T2Cu [7,28]. Regeneration of the demetallated centers was attempted by incubating a protein aliquot with CuSO_4 , followed by extensive dialysis against Tris/HCl 100 mM pH 7.6 buffer. EPR spectroscopy confirmed partial regeneration of T2Cu centers under these conditions: the T1Cu/T2Cu ratio increased from 1 : 0.37 (as isolated) to 1 : 0.50 (regenerated sample). All electrochemical and kinetic studies were performed with the regenerated aliquot, while spectroscopic studies were performed with as-isolated protein.

Enzymatic assays

Preliminary assays for the determination of kinetic parameters of the enzyme were performed with dithionite-reduced benzylviologen as electron donor. A specific activity of 130 $\text{U}\cdot\text{mg}^{-1}$ protein was determined in presence of a large excess of substrate (50 mM nitrite, pH 7.0), i.e. a turnover number of 243 (reduced NO_2^-) $\text{s}^{-1}\cdot(\text{Cu-Nir})^{-1}$. The purified enzyme represents only $\approx 2\%$ of the total enzyme activity in the cell extract. This low yield cannot be explained by the usual protein losses during a purification process only, but is probably also due to the high lability of the T2Cu center, whose content ($< 50\%$ of the stoichiometric value as observed by EPR) greatly influences the enzyme activity [7]. As expected, the protein is inhibited by DDC and cyanide [1].

Electronic absorption spectroscopy

The UV-vis spectrum of the native form of the Cu-Nir (Fig. 2) exhibits absorption maxima at 280, 411, 460 and 598 nm and also a broad band at ≈ 780 nm, with molar absorptivity values of $\epsilon_{460} = 4.89 \text{ mm}^{-1}\cdot\text{cm}^{-1}$, $\epsilon_{598} = 9.87 \text{ mm}^{-1}\cdot\text{cm}^{-1}$ and $\epsilon_{780} = 4.63 \text{ mm}^{-1}\cdot\text{cm}^{-1}$, assuming a molecular mass of 113 kDa. The 598 nm band is a $\text{S}_{\text{Cys}} \pi \rightarrow \text{Cu } d_{x^2-y^2}$ charge-transfer band, typical of type 1

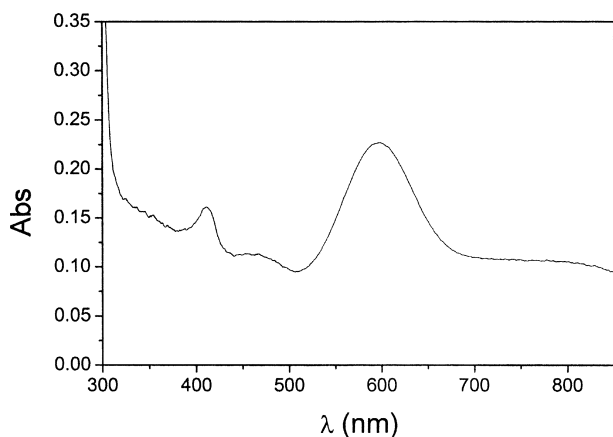


Fig. 2. Electronic absorption spectrum of the as-purified (blue) form of the *Ps. chlororaphis* DSM 50135 nitrite reductase. [Cu-Nir] = 23 μM in 20 mM Tris/HCl buffer pH 7.6.

copper centers, while the 460 nm band originates in a second $S_{\text{cys}} \rightarrow \text{Cu}$ transition [29]. In the literature, an increase in the intensity of this second band has been correlated with a higher rhombic distortion of the T1Cu EPR signal, and with the presence of a green, rather than blue, color [30]. This is observed for *Ps. chlororaphis* DSM 50135 Nir, which exhibits a blue color and an A_{460}/A_{598} ratio of 0.496, while the green nitrite reductases present A_{460}/A_{598} ratio values above unity [31]. These different spectroscopic characteristics reflect different orientations of the axial methionine side chain in the blue and green reductases [4,5]. According to Dodd *et al.* [4], structure comparison of the blue Nir from *Al. xylosoxidans* NCIMB 11015 with the green Nir from *Al. faecalis* S-6, reveals that the deviation of the $S_8(\text{Met}150)$ atom from the axial position of the NNS plane formed by two $N_8(\text{His}95)$ and $\text{His}145)$ atoms and one $S_8(\text{Cys}136)$ atom causes the different colors in the enzymes.

The spectrum of Cu-Nir also exhibits a small peak at 411 nm, probably due to a minor cytochrome contamination, evaluated in less than 2% (mass/mass) of the total protein (based on known cytochrome *c* extinction coefficients).

Azurin-Cu-Nir electron transfer

It has been suggested that blue Cu-Nirs receive the electrons needed for nitrite reduction from cognate blue copper-containing proteins [4]. We have therefore studied the electron transfer between these two proteins by spectrophotometric and electrochemical methods.

In the spectrophotometric assay the reoxidation of the dithionite-reduced azurin was followed at 625 nm, under an argon atmosphere. Under these conditions, no reoxidation was observed unless both nitrite and nitrite reductase were present in the assay vial. The oxidation curve observed for the azurin was biphasic, with an initial linear region, followed by an extensive nonlinear phase (not shown). Using 25 μM azurin, at pH 7.0, in the presence of 50 mM nitrite, a specific activity of 0.33 $\text{U}\cdot\text{mg}^{-1}$ Cu-Nir was determined, which is equivalent to a turnover number of 0.62 (reduced

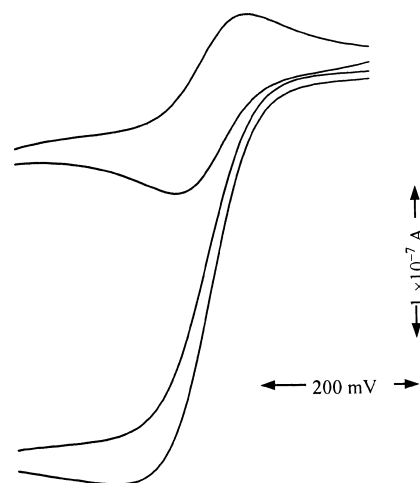


Fig. 3. Effect of the addition of *Ps. chlororaphis* DSM 50135 Cu-Nir on the electrochemical response of azurin from the same organism, in the presence of nitrite. Upper trace: 400 μM azurin in 50 mM Mes buffer pH 6.2 and 50 mM KCl. Lower trace: addition of 6.5 μM Nir. [nitrite] = 50 mM. Scan rate, 2 $\text{mV}\cdot\text{s}^{-1}$.

NO_2^-) s^{-1} $(\text{Cu-Nir})^{-1}$, considerably higher than the values reported for *Al. xylosoxidans* NCIB 11015 azurins I and II, 0.07 and 0.06 (reduced NO_2^-) s^{-1} $(\text{Cu-Nir})^{-1}$, respectively (values calculated from data presented in reference [10]).

The electron transfer between azurin and nitrite reductase from *Ps. chlororaphis* was also studied by cyclic voltammetry (Fig. 3). In the presence of Cu-Nir and nitrite, the cyclic voltammograms of azurin exhibit a sigmoidal shape, with enhanced cathodic currents and decreased anodic currents, particularly at low scan rates. This behavior is consistent with a reaction mechanism involving an initial heterogeneous electron transfer reaction at the electrode, followed by an irreversible homogeneous chemical reaction in solution. The measured catalytic current is independent of the scan rate and proportional to the square root of the enzyme concentration (not shown), which indicates that the enhanced cathodic current is due to the catalytic regeneration of the azurin reoxidized by Nir [24]. The theory describing this kind of mechanism has been developed by Nicholson and Shain [24] and by Savéant and Vianello [32] and is frequently applied to kinetic studies of reactions between redox enzymes and mediators. Second order rate constants (k) were calculated as described in [25]: the kinetic parameter λ was calculated from the catalytic efficiencies (the ratio of cathodic current in the presence and absence of substrate) using the values computed by Nicholson and Shain, and plotted vs. the inverse of the scan rate. The plots yielded straight lines, confirming the applicability of the Nicholson and Shain theory to the present system, and the variation of the pseudo-first order rate constant with CuNir concentrations (between 1 and 4 μM) yielded a value of $k = (2.9 \pm 0.9) \times 10^4 \text{M}^{-1}\cdot\text{s}^{-1}$ for the rate constant between reduced azurin and nitrite reductase.

The treatment described above is valid when the rate of recycling of $\text{Cu-Nir}_{\text{ox}}$ [expressed by Eqn (1)] is not a limiting factor for the catalytic current, i.e. when the limiting step of the mechanism is the electron transfer between azurin and Cu-Nir, whose rate is expressed by Eqn (2).

Table 1. Electron transfer second order rate constants determined by cyclic voltammetry in physiologically relevant systems.

Organism	Electron transfer	k ($10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$)
<i>Al. faecalis</i> ^a	pAz → Cu-Nir	18
<i>Ac. cycloclastes</i> ^b	pAz → Cu-Nir	7.3
<i>Al. xylosoxidans</i> ^c	Cyt. <i>c</i> ₅₅₁ → Cu-Nir	4.0
<i>Ps. nautica</i> ^d	Cyt. <i>c</i> ₅₅₂ → <i>cd</i> ₁ -Nir	4.7
<i>Methylomonas</i> sp. ^e	Az-iso2 → MADH	1.0
<i>Ps. chlororaphis</i> ^f	Az → Cu-Nir	0.29

^a [40]; ^b [8]; ^c [11]; ^d [41]; ^e [42]; ^f this work.

$$V_1 = k_{\text{app}}[\text{Cu-Nir}] \quad (\text{constant pH, large excess of } \text{NO}_2^-) \quad (1)$$

$$V_2 = k[\text{azurin}][\text{Cu-Nir}] \quad (2)$$

The applicability of the Nicholson and Shain treatment implies that $V_2 < V_1$, which allows the determination of a minimal value of 13 s^{-1} for k_{app} . This value is clearly smaller than the observed turnover number of 243 s^{-1} , which confirms the applicability of the referred treatment to this system.

Table 1 gathers several electron transfer constants obtained by cyclic voltammetry in other physiologically relevant systems. These data suggest that the electron transfer between azurin and the Cu-Nir in *Ps. chlororaphis* occurs at lower rates than those observed for other systems, which raises the question whether the azurin is the physiological reductant of Cu-Nir in this organism or not. *Ps. chlororaphis* azurin reacts with its cognate Cu-Nir at much higher rates than observed in similar experiments with *Al. xylosoxidans* NCIB 11015 Cu-Nir and cognate azurins [10], which have been reported as its electron donors. However, the relevance of this comparison is hard to ascertain, as recent results [9,11] suggest that, in the related strain *Al. xylosoxidans* GIFU 1051, Cu-Nir may accept electrons from cytochrome *c*₅₅₁ rather than from azurins I and II.

EPR spectroscopy

EPR spectra of *Ps. chlororaphis* nitrite reductase in the as-purified, ascorbate-reduced, dithionite-reduced and turnover forms are presented in Fig. 4. The turnover form was obtained by brief incubation of the dithionite reduced-form with substrate under anaerobic conditions, and probably corresponds to an enzyme form involved in the catalytic cycle.

The as-purified form spectrum of Cu-Nir exhibits two magnetically isolated components in a 3 : 1 ratio (Fig. 4A). Addition of ascorbate reduces one of the components, which becomes EPR silent, while the other component remains in the oxidized form (Fig. 4B). The latter shows a rhombic spectrum ($g_z = 2.350$, $g_y = 2.110$, $g_x = 2.040$) with hyperfine structure with the copper nucleus ($I = 3/2$) at the g_z region (${}^{\text{Cu}}A_z = 10.7 \text{ mT}$). These properties identify the system as a T2Cu center. Subtraction of this semi-reduced spectrum from the native spectrum yielded an almost axial spectrum ($g_z = 2.220$, $g_y = 2.052$, $g_x = 2.036$),

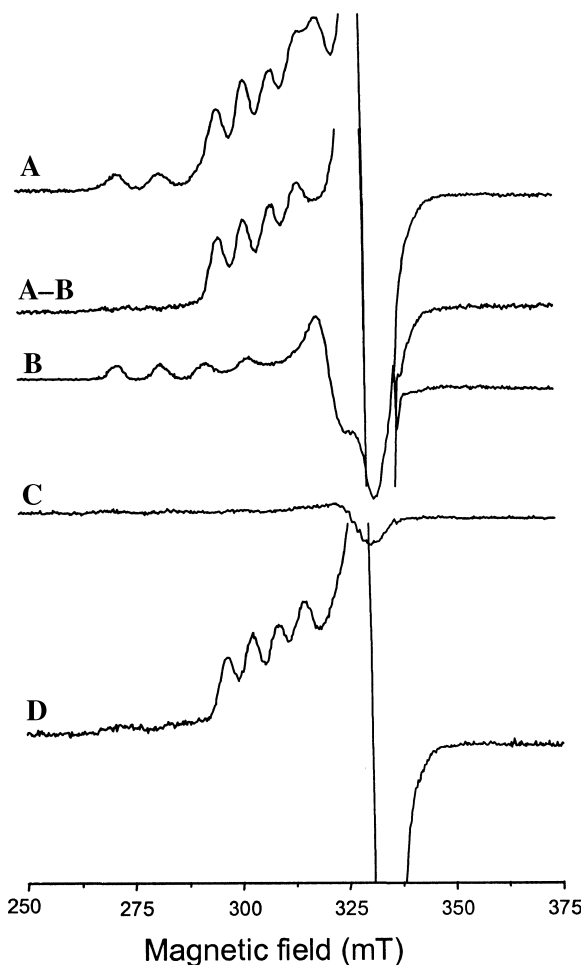


Fig. 4. Electron paramagnetic resonance spectra of *Ps. chlororaphis* DSM 50135 nitrite reductase. (A) Native, (B) ascorbate-reduced, (C) dithionite-reduced (the observed spectrum is a weak background from the cavity), and (D) 'turnover' forms (see text for details); (A–B), difference spectrum, native minus ascorbate-reduced. Cu-Nir was $340 \mu\text{M}$ in 300 mM Tris/HCl pH 7.6. Instrument conditions: microwave frequency, 9.49 GHz ; microwave power, 2 mW ; modulation amplitude, $0.4 \text{ mT}_{\text{pp}}$; modulation frequency, 100 kHz and temperature, 40 K .

showing also hyperfine structure at g_z (${}^{\text{Cu}}A_z$ of 5.8 mT), which identifies it clearly as originating from a T1Cu center (Fig. 4A–B). EPR parameters obtained by simulation are given in Table 2. EPR spectra of both T1Cu and T2Cu centers are consistent with a $d_{x^2-y^2}$ ground state, with $g_{\parallel} > g_{\perp} > 2$ [33].

Table 2. EPR parameters for the type 1 and type 2 Cu centers in the nitrite reductase from *Ps. chlororaphis* DSM 50135. ND, not determined. g -values ± 0.001 , A -values ± 0.1 .

Cu center	g_z	g_y	g_x	${}^{\text{Cu}}A_z$ (mT)
T1Cu	2.220	2.052	2.036	5.8
T2Cu				
As-purified	2.350	2.110	2.040	10.7
With NO_2^-	2.34	ND	ND	12.0

Upon reduction with excess of dithionite, T2Cu is also completely reduced to an EPR-silent form (Fig. 4C). Reoxidation of the protein with nitrite under anaerobic conditions yielded a partially oxidized ('turnover') form of the enzyme (Fig. 4D). The EPR spectrum shows that in this form the reoxidation of T1Cu center is much more complete than that of T2Cu, which suggests the T1Cu → T2Cu electron-transfer is faster than both the reoxidation of T2Cu by nitrite and the reduction of T1Cu by dithionite, i.e. is the fastest of the three redox processes involved in the catalysis (cf [14]). The g_z value and hyperfine coupling constant of the spectrum of the turnover form of the T2Cu center are slightly different from that of the as-purified form (cf. Table 2) which suggests the existence of modifications in the catalytic center of the enzyme. These modifications may be attributed to either the presence of the substrate and/or a product in the vicinity of the T2Cu active center rather than to disruption of this center, as reoxidation by ferricyanide is complete and originates a spectrum virtually identical to that observed in the native form (data obtained during the redox titration). Changes in the EPR spectrum of the *Al. xylosoxidans* NCIB 11015 Nir upon addition of nitrite have also been reported [34].

Redox titration

The development of appropriate models for the Cu-Nir mechanism requires the determination of the potentials of both copper centers, especially in order to understand the relationship between redox catalysis and intramolecular electron transfer. However, the redox potentials of copper nitrite reductases are not fully characterized. Information available has been provided by electrochemical titrations monitored by UV-vis spectroscopy [35], that only follow the oxidation state of the T1Cu center [36]. T2Cu center potentials have only been indirectly estimated from pulse-radiolysis kinetic studies, and not by electrochemical studies under equilibrium conditions (cf. Table 3) [6,14,36,37].

Unlike UV-vis spectroscopy, EPR enables the simultaneous monitoring of both type 1 and T2Cu centers. Therefore, in order to obtain reliable equilibrium redox potentials, we performed for the first time an EPR monitored redox-titration for a copper-containing Nir. The protein was fully reduced with dithionite and reoxidized stepwise with ferricyanide. The spectra of the initial as-purified and the final reoxidized forms are virtually identical, which demonstrates that the whole

process does not affect the integrity of the protein sample. The results of the titration are presented in Figs 5 and 6. As inferred in other Cu-Nirs (cf. Table 3), the active center redox-potential in *Ps. chlororaphis* DSM 50135 Cu-Nir (E_m T2Cu = 172 ± 5 mV) is lower than the electron-transfer center redox-potential (E_m T1Cu = 298 ± 7 mV). Therefore, a slow electron transfer rate between T1Cu and T2Cu centers should be expected under these conditions, as it occurs *against* the electric

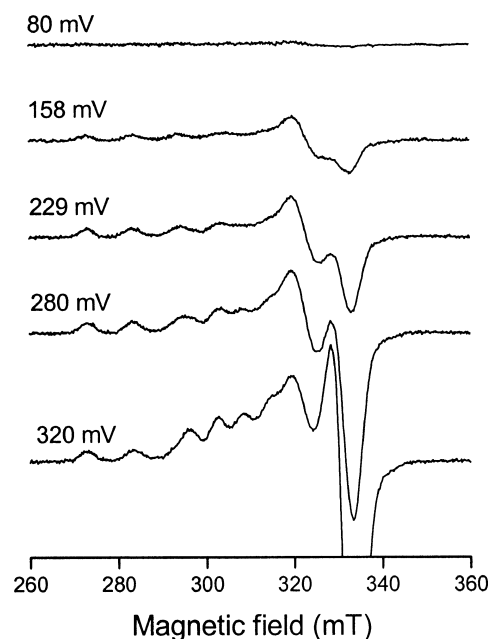


Fig. 5. EPR spectra observed during the redox titration of the *Ps. chlororaphis* DSM 50135 nitrite reductase. Instrument conditions: temperature, 20 K; microwave frequency, 9.49 GHz; microwave power, 0.63 mW; modulation frequency, 100 kHz and modulation amplitude, 1.0 mT_{pp}.

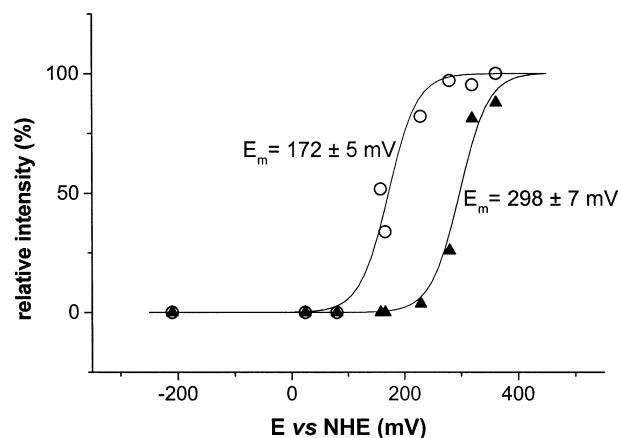


Fig. 6. Redox titration of the *Ps. chlororaphis* DSM 50135 nitrite reductase, at pH 7.6, monitored by EPR spectroscopy. ▲, oxidized T1Cu signal intensity; ○, oxidized T2Cu signal intensity. Experimental data were normalized to the respective maximum intensities obtained from Nernst equation fits (solid lines). EPR conditions given in Fig. 5.

Table 3. Redox potentials for the copper centers in some nitrite reductases.

Organism	E_m T1Cu (mV)	E_m T2Cu (mV)
<i>Ac. cycloclastes</i> IAM 1013 ^{b,d}	240	250 ^a
<i>Al. xylosoxidans</i> GIFU 1051 ^{b,d}	280	280 ^a
<i>Al. xylosoxidans</i> NCIB 11015 ^{b,e}	260	240 ^a
<i>Rhodobacter sphaeroides</i> 2.4.3 ^{b,f}	247	< 200
<i>Ps. chlororaphis</i> DSM 50135 ^{c,g}	298	172

^a Indirectly estimated value; ^b pH 7.0; ^c pH 7.6; ^d [14]; ^e [36]; ^f [35]; ^g this work.

potential. However, addition of nitrite to the reduced form of the enzyme causes more complete reoxidation of the T1Cu than of the T2Cu center (cf. Fig. 4), indicating that the electron transfer process is kinetically and thermodynamically favored in the presence of NO_2^- . Several explanations for these apparent contradictions can be found in the literature. The binding of nitrite to the oxidized form of T2Cu may increase its redox-potential, thus making the electron transfer more spontaneous, as suggested by ENDOR experiments performed in *Rh. sphaeroides* Cu-Nir [38]. Based on observations of *Al. xylosoxidans* NCIB 11015 Cu-Nir, Prudêncio *et al.* [39] have suggested that *in vivo* the T2Cu redox potential may be increased considerably (even in the absence of nitrite) by conformational changes induced by the interaction of Cu-Nir with its natural electron donor. In *Al. xylosoxidans* GIFU 1051 Nir, the T2Cu potential depends on pH, and it is about 100 mV higher in the protonated than in the deprotonated form of the enzyme [14], while the T1Cu potential is pH-independent. Supposing a similar dependence of the E_m T2Cu with pH in the *Ps. chlororaphis* Nir, it would be expected that at lower pH values (the titration was performed at pH 7.6) the electron transfer in the presence of nitrite should be even more favored.

Conclusions

The nitrite reductase isolated from *Ps. chlororaphis* DSM 50135 is a blue protein with two types of copper-containing centers, T1Cu and T2Cu, like other described Cu-Nirs. Enzyme assays and electrocatalysis studies have shown that the Cu-Nir from *Ps. chlororaphis* DSM 50135 accepts electrons carried by the azurin purified from the same organism. The direct determination of the redox potentials of both copper centers yielded values (E_m T1Cu = 298 mV and E_m T2Cu = 175 mV vs. NHE) which seem not to be consistent with the proposed electron transfer pathway (from electron donor to T1Cu to T2Cu to nitrite). However, the EPR data indicate that nitrite binding to the T2Cu increases the redox potential of this center, thereby making the intramolecular electron-transfer more favorable, as proposed by Veselov *et al.* [38]. EPR studies with the 'turnover' form of the enzyme also suggest that, in the presence of nitrite, the electron transfer between T1Cu and T2Cu is the fastest of the three redox processes involved in the catalysis: (a) reduction of T1Cu; (b) oxidation of T1Cu by T2Cu; and (c) reoxidation of T2Cu by NO_2^- (cf [14]). Moreover, as it has been recently observed in *Al. xylosoxidans* GIFU 1051 Nir [14] that the T2Cu potential increases at low pH, it is likely that at that pH the intramolecular electron transfer in the presence of nitrite will be even more favorable. Further studies on the changes in enzyme activity and both copper centers' redox potentials with pH will probably shed further light on the mechanisms underlying the relieving of the apparent thermodynamic impediments to the electron transfer to the substrate.

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