Purification and characterization of the dissimilatory nitrite reductase from *Alcaligenes xylosoxidans* subsp. *xylosoxidans* (N.C.I.M.B. 11015): evidence for the presence of both type 1 and type 2 copper centres

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Dissimilatory nitrite reductase was isolated from extracts of *Alcaligenes xylosoxidans* subsp. *xylosoxidans* (N.C.I.M.B. 11015), after activation of crude extracts by the addition of copper(II) sulphate. The enzyme was purified by a combination of $(\text{NH}_4)_2\text{SO}_4$ fractionation and cationic-exchange chromatography to 93% homogeneity as judged by SDS/PAGE. SDS/PAGE and spray m.s. showed that the enzyme had a subunit M_r of 36.5 kDa. The copper content was 3.5 ± 0.8 Cu atoms/trimer of M_r 109 500. E.p.r. spectroscopy of nitrite reductase as isolated showed that both type 1 ($g_{\parallel} = 2.208$, $A_{\parallel} = 6.3$ mT) and type 2 ($g_{\parallel} = 2.298$, $A_{\parallel} = 14.2$ mT) Cu centres were present, in contrast with published data [Masuko, Iwasaki, Sakurai, Suzuki and

Nakahara (1984) J. Biochem. (Tokyo) **96**, 447–454], where only type 1 copper centres were reported. Our preparations had a specific activity of 150–300 μmol of NO_2^- reduced/min per mg of protein, 6–12-fold higher than reported previously. As isolated, the oxidized form of our preparations of the enzyme showed absorption maxima in the visible region at 460, 593 and 770 nm. The ratio of the absorption bands at 460 nm and 593 nm resulted in this protein having a strong blue colour, in contrast with the green colour of other purified copper-containing nitrite reductases. We conclude that, in contrast with previous reports, this 'blue' nitrite reductase requires both type 1 and type 2 copper centres for optimal activity.

INTRODUCTION

Dissimilatory nitrite reductase is a key enzyme in the stepwise reduction of nitrate to the gaseous products nitrous oxide and dinitrogen, since it is at this point in the denitrification pathway that significant losses of fixed nitrogen from the soil to the atmosphere become irreversible. There are two distinct classes of nitrite reductase, one containing cd_1 haems as the prosthetic groups and the other containing copper (see Zumft, 1992).

Copper-containing nitrite reductases have been characterized from a variety of denitrifying bacteria, but show considerable variation in their physicochemical properties. Differences include the number of subunits and native M_r , and also the visible and e.p.r. spectra (see Hochstein and Tomlinson, 1989). Nitrite reductase purified from *Alcaligenes xylosoxidans* subsp. *xylosoxidans* (previously *Alcaligenes* sp. N.C.I.M.B. 11015, *Achromobacter xylosoxidans*, or *Pseudomonas denitrificans*) has been reported to be tetrameric (Iwasaki et al., 1963) and dimeric with a subunit M_r of ~ 37000 (Masuko et al., 1984). The Cu content of the latter preparation of nitrite reductase was reported as two Cu atoms/dimer, and e.p.r. spectroscopy showed the presence of only type 1 Cu centres (Masuko et al., 1984). These preparations were of low specific activity compared with the nitrite reductase from the closely related organism '*Achromobacter cycloclastes*', which is reported to contain both type 1 and type 2 Cu centres (Liu et al., 1986). Here we describe a large-scale purification procedure for nitrite reductase from *Alc. xylosoxidans*, which yields protein with a 6–12-fold higher specific activity than that previously reported (Masuko et al., 1984). The enzyme is trimeric and contains 3–5 Cu atoms/trimer. It exhibits e.p.r. spectra consistent with the presence of both type 1 and type 2 Cu centres in approximately equal proportions. These properties show that the dissimilatory nitrite reductase of *Alc. xylosoxidans* described here is very similar to the enzyme from '*Achr. cycloclastes*',

although significant differences in the visible spectra result in solutions of the former enzyme being blue and the latter green.

MATERIALS AND METHODS

Cultivation of bacteria

Alcaligenes xylosoxidans subsp. *xylosoxidans* was obtained from the N.C.I.M.B. culture collection. Cultures were grown, from a 10% inoculum, anaerobically at 30 °C in a 200-litre pilot plant fermenter (New Brunswick Scientific) with a minimal stirring rate of 50 rev./min. The medium was as used previously (Iwasaki and Shidara, 1969), except that nutrient broth (8 g/l) was substituted for beef extract and peptone. The cultures were harvested after 40 h using a SA7 Westfalia multistack centrifuge. The cell paste was stored in liquid nitrogen until used for purification.

Assay for nitrite reductase activity

Nitrite reductase activity was assayed at 25 °C using Methyl Viologen reduced with an excess of dithionite as the electron donor, as described previously for the assay of nitrate reductase, except that nitrite (0.1 mM) was substituted for nitrate (MacGregor, 1978). Dithiothreitol was omitted from the assay mixture, since in its presence the non-enzymic reduction of nitrite has been reported under our assay conditions (Farnden and Robertson, 1980). The enzymic consumption of nitrite was determined by subtracting the amount of nitrite present in the reaction mixture from that in a control reaction mixture lacking enzyme. The amount of residual nitrite was determined by the method of Nicholas and Nason (1957), which involves the formation of a red azo-dye whose concentration can be measured spectrophotometrically at 540 nm. Care was taken to ensure that the activity was measured under conditions where the rate of utilization of

nitrite was linear with both time and enzyme concentration. Units of nitrite reductase activity are expressed in terms of 1 μmol of nitrite reduced/min at 25 °C. Specific activity is expressed as units/mg of protein.

Determination of protein concentration

Protein concentration was determined using the microbiuret method (Gornall et al., 1949) or the Folin phenol method (Lowry et al., 1951). A trichloroacetic acid-precipitation step was carried out before protein estimation of purified protein in order to obtain a linear colour development with increasing amounts of enzyme. This was necessary because of the effect on the colour development of various amounts of copper released from the protein. BSA was used as a calibration standard, an $A_{278}^{1\text{ mg/ml}}$ value of 0.0667 being used.

Determination of copper

Copper was determined on wet-ashed samples of nitrite reductase (0.4–1.4 mg of protein) using differential-pulse cathodic-stripping voltammetry (d.p.c.s.v.). This method involves the electro-deposition of the complex formed between Cu(II) and catechol at a hanging-mercury-drop electrode and subsequent quantification by d.p.c.s.v., as described in H.M.S.O. (1987), using a compact polarographic/voltammetric apparatus (Metrohm AG, CH-9100 Herisau, Switzerland). The digested protein (50 μl) was added to a solution (20 ml) containing 10 mM-Hepes buffer, pH 7.5, and catechol (1 mM). After analysis of the digest, three sequential additions of 50 ng of Cu from a standard solution were made to generate an internal calibration curve which had a slope of 2.3 ng of Cu/ μA .

M_r determination by SDS/PAGE

The M_r of the subunit of purified nitrite reductase was measured by the method of Weber and Osborn (1969), using 15 % acrylamide gels. Standard M_r markers were obtained from Bio-Rad, Hemel Hempstead, Herts., U.K.

Electrospray m.s.

A sample of nitrite reductase (0.7 mg/ml) in 10 mM-Tris/HCl buffer, pH 7.5, was prepared. Sample solutions (10 μl , 200 pmol) were injected into the carrier solvent stream [water/methanol (1:1, v/v), containing 10 % acetic acid, at a flow rate of 4 $\mu\text{l}/\text{min}$] of a V.G. BIO Q quadrupole mass spectrometer. The spectrometer scanned over a range of 700–1500 m/z and was calibrated with myoglobin. The results were analysed and data printed using software provided by the instrument manufacturer (V.G. Analytical, Manchester, U.K.).

E.p.r. measurements

E.p.r. spectra were recorded on a Bruker ESP 300 spectrometer fitted with a Bruker ER 35M n.m.r. gaussmeter and a Marconi Instruments 2440 microwave frequency counter. Integrated intensities were obtained by comparison with aqueous CuEDTA with a g -value correction applied as described by Åasa and Vänngård (1975). Simulations, performed using a computer program based on that of Lowe (1978), and data handling, were done on a MicroVAX II computer.

Amino acid composition

Nitrite reductase (0.6 mg) was subjected to SDS/PAGE to remove the minor bands observed on Coomassie Blue-stained

gels of samples of the purified enzyme, and the major band was recovered from the gel by electroelution into a volatile buffer (50 mM ammonium bicarbonate/0.1 % SDS). The samples were analysed using an Applied Biosystems 420H amino acid analyser with automatic hydrolysis and derivatization by the SERC Amino Acid Sequencing Service, University of Aberdeen, Aberdeen, Scotland, U.K.

M.s. of reaction products

For analysis of the products formed by the enzyme, assays were performed in 5 ml round-bottomed flasks equipped with a stopcock leading to a B10 socket which could be connected to the inlet of the mass spectrometer. Nitrite reductase (20 μg) was incubated with either the standard dithionite/Methyl Viologen assay mixture, with the NO_2^- correction increased to 10 mM, or the ascorbate-reduced phenazine methosulphate assay mixture described by Zumft et al. (1987). After the protein was injected, the reaction mixtures (2 ml) were incubated at $\sim 20^\circ\text{C}$ for different times before freezing in an ethanol bath cooled with liquid N_2 . The gas in the headspace was then analysed with a mass spectrometer (S.I.R.A. 10, VG Isogas Limited, Chester, Cheshire, U.K.). The assay mixtures were subsequently analysed for residual nitrite (as described above) and ammonia (Chaney and Marbach, 1962).

RESULTS

Purification of nitrite reductase

Unless otherwise stated, all purification procedures were carried out at 4 °C using 20 mM Mes buffer, pH 6.0. A summary is shown in Table 1.

Step 1: cell disruption and activation of crude extracts by added Cu(II)

Cell paste (~ 500 g) was resuspended in 500 ml of buffer, containing a small amount of deoxyribonuclease, and disrupted in a pre-cooled Manton–Gaulin homogenizer at 28 MPa. The resulting suspension, which had become alkaline ($\sim \text{pH } 8$), was then centrifuged at 25000 g for 1 h to remove cell debris and unbroken organisms.

The specific activity of nitrite reductase in these crude extracts was ~ 20 -fold lower than that expected from small-scale preparations of crude extracts from cells sampled just before harvesting. The reasons for this difference, which was observed consistently, are not clear. However, the addition of CuSO_4 to the crude extracts dramatically increased the nitrite reductase activity.

The following procedure gave optimum activation: the crude extract was adjusted to a protein concentration of 30 mg/ml by dilution with buffer and then CuSO_4 was added to give a final concentration of 1 mM. The extract was stirred gently for 30 min before incubation without stirring for 17 h. This treatment resulted in a 15–20-fold increase in nitrite reductase activity in the crude extract.

Step 2: $(\text{NH}_4)_2\text{SO}_4$ fractionation

Before $(\text{NH}_4)_2\text{SO}_4$ fractionation the activated extract was adjusted to pH 7.0 with 10 % acetic acid and treated with 1 % streptomycin sulphate (30 min), followed by centrifugation at 25000 g for 15 min. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 35 % (w/v) with gentle stirring. After 30 min the precipitated protein was collected by centrifugation, as described above, and discarded. The supernatant was then adjusted to 100 % (w/v) saturation by the addition of solid $(\text{NH}_4)_2\text{SO}_4$, and the procedure described above for the 0–35 % fraction repeated,

Table 1 Purification of nitrite reductase from *Alc. xylosoxidans*

Step no.	Fraction	Volume (ml)	[Protein] (mg/ml)	Total protein (g)	$10^{-3} \times$ Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
1	Crude extract supernatant						
	After centrifugation	870	30	26	4.2	0.2	—
	After activation	879	28	25	57	2.3	100
2	Redissolved $(\text{NH}_4)_2\text{SO}_4$ precipitate	400	26	10.5	53	5.4	93
3	CM-cellulose combined active fractions	182	1.2	0.2	37.5	173	63
4	S-Sepharose combined active fractions	50	2	0.1	24.4	244	43

except that the supernatant was discarded and the pellet of the 35–100% fraction containing the nitrite reductase activity was dissolved in a minimal volume of buffer and then dialysed against buffer (2×4 litres for 20 h).

Step 3: CM-cellulose chromatography

The dialysed solution from Step 2 was then chromatographed in batches containing ~ 3.5 g of total protein on a column of CM-cellulose ($12 \text{ cm} \times 5 \text{ cm}$) equilibrated with buffer (Figure 1). After the protein had been loaded, the column was washed with buffer (400 ml) to remove any unbound proteins and then developed by stepwise elution with buffer containing NaCl. On the addition of 50 mM NaCl, two blue bands appeared. The second blue band, which contained the nitrite reductase activity, was not eluted until the NaCl concentration was increased to 0.1 M. The fractions eluted at 0.1 M were pooled and dialysed against buffer (2×2 litres) in order to remove NaCl before Step 4.

Step 4: S-Sepharose chromatography

The dialysed fraction from Step 3 was allowed to warm to 20°C before loading directly on to a $10 \text{ cm} \times 2.5 \text{ cm}$ column of S-Sepharose equilibrated with standard buffer at 20°C . The column was developed at a flow rate of 180 ml/h with a linear gradient extending from 50 mM to 120 mM NaCl in a total volume of 300 ml. The blue fraction, eluted over the NaCl concentration range 105–120 mM, contained the majority of nitrite reductase activity and was collected (Figure 2).

The specific activity of nitrite reductase purified by this procedure was between 150 and 300 units/mg of protein. Incubation of our purified protein preparations for 48 h at 4°C with Cu^{2+} in 50 mM Tris, pH 7.5, did not change the specific activity.

The yield from 500 g wet weight of cells was 100 mg of enzyme, which is 3-fold higher than that achieved by the purification procedure of Masuko et al. (1984). The current procedure uses only four steps, and gives a higher percentage recovery of total units of enzyme activity with a 6–12-fold higher specific activity than Masuko et al. (1984). SDS/PAGE (Figure 3) showed that the protein was 93% pure, as judged by scanning densitometry of Coomassie Blue-stained gels. The major band of impurity probably results from proteolytic degradation of nitrite reductase, since hydrophobic-interaction chromatography, gel filtration and S-Sepharose chromatography at pH 8.0 failed to produce, remove or change the proportion of the minor component.

Proteolytic hydrolysis resulting in the nicking of nitrite reductase and causing the appearance of minor bands on SDS/

PAGE is well documented (Liu et al., 1986; Kakutani et al., 1981). Proteinase activity, as measured by the resorufin-labelled-casein assay, was separated from nitrite reductase activity during Step 3 of the purification described above. Two proteinase inhibitors (bestatin at $200 \mu\text{g/ml}$ and antipain dihydrochloride at 2 mg/ml) were shown to inhibit proteolytic activity by about 30–50% in crude extracts of *Alc. xylosoxidans*, but their use in the large-scale purification described here was prohibitively expensive. Phenylmethanesulphonyl fluoride was considered as an alternative but, at the concentrations used (1 mM), the propan-2-ol required to dissolve this inhibitor prevented binding of nitrite reductase to CM-cellulose.

M_r determination

The subunit M_r was determined on SDS-containing gels by comparison of the rates of migration with proteins of known M_r (Figure 4) and gave a value of 38800 ± 600 kDa. This value is in good agreement with the value of 37000 reported previously by others (Masuko et al., 1984). However, because an accurate assessment of the subunit M_r is essential to the assignment of the number of subunits in native nitrite reductase, electrospray m.s. was also utilized. This technique provides an independent and very precise value for the M_r of monomeric proteins (Fenn et al., 1989), and in the present case gave a value of 36523 ± 9.8 .

The native M_r was determined using gel-filtration chromatography on Superose-12 and gave a value of 75000 ± 3500 . This value, together with the subunit M_r data presented above, implies that, under these conditions, nitrite reductase from *Alc. xylosoxidans* is a dimer in solution, in agreement with the proposal of Masuko et al. (1984). However, a trimeric structure had been shown from the X-ray crystal structure of nitrite reductase from '*Achr. cycloclastes*' (Godden et al., 1991). X-ray solution scattering and sedimentation-equilibrium centrifugation experiments on our preparations of nitrite reductase from *Alc. xylosoxidans* gave values for the M_r consistent with the enzyme being a trimer (Grossmann et al., 1993). Comparison of the small-angle X-ray scattering pattern from a protein in solution with models based on crystallographic structure co-ordinates provides a very powerful method of defining molecular conformation in solution. When the scattering pattern of solutions of *Alc. xylosoxidans* nitrite reductase (5 mg/ml) was compared with models based on combinations of monomers derived from the crystal structure of the enzyme from '*Achr. cycloclastes*' (Godden et al., 1991), only a trimeric solution structure was consistent with the data. Furthermore in low-speed sedimentation-equilibrium measurements the plots of log absorbance versus the square of the radial distance implied an M_r of 103000 ± 5000 and were essentially

Figure 1 Elution profile for Step 3 of the purification procedure of nitrite reductase

The data (continuous line) show a typical protein elution profile when crude extracts are fractionated on a column of CM-cellulose using stepwise increases in NaCl concentration. Nitrite reductase was eluted as a blue band and is labelled 'NiR'.

Figure 2 Elution profile for Step 4 of the purification procedure of nitrite reductase

The data (continuous line) show a typical elution profile when combined active fractions from Step 3 are applied to a column of S-Sepharose and separated using a linear NaCl gradient from 50 to 120 mM (broken line), followed by a stepwise addition of 500 mM NaCl. Elution of nitrite reductase (stippled bars) activity is shown by the histogram.

linear, indicating very little heterogeneity, at concentrations down to 0.5 mg/ml. Thus these data also were *only* consistent with nitrite reductase from *Alc. xylosoxidans* being trimeric in solution. Consequently we conclude that nitrite reductase from *Alc. xylosoxidans* is a trimer of M_r 109 500 in solution. The reasons for the anomalous behaviour on gel filtration are not clear, but may be associated with the unusual trimeric conformation of the protein resulting in non-standard elution behaviour relative to the monomers or dimers used as standards.

Amino acid composition

The amino acid composition of nitrite reductase from *Alc. xylosoxidans* together with the data for '*Achr. cycloclastes*' is shown in Table 2. The degree of relatedness (SAQ) between the two nitrite reductases was estimated by using the method of Marchalonis and Weltman (1971). When analysed by this method, related proteins have SAQ values less than 50. The amino acid compositions in Table 2 give an SAQ value of 39,

Figure 3 Purification of nitrite reductase from *Alcaligenes xylosoxidans* subsp. *xylosoxidans*

The data show a typical pattern of fractions obtained during the purification procedure (Steps 1–4) described in the Results section. Fractions were subjected to SDS/15%–PAGE under the conditions described by Weber and Osborn (1969). Lane A, M_r markers (Bio-Rad) as indicated on the left of the photograph; lane B, crude extract after Step 1; lanes C–E, after Steps 2–4 respectively.

Figure 4 Determination of the subunit M_r of nitrite reductase by SDS/PAGE

Samples and gels were prepared as described in the Materials and methods section. Standard peptides (●) were run simultaneously as listed in order of descending M_r ($\times 10^{-3}$): rabbit muscle phosphorylase *b* (97.4), BSA (66.2), ovalbumin (45), bovine carbonic anhydrase (31), soya-bean trypsin inhibitor (21.5), and lysozyme (14.4). Nitrite reductase is indicated by the open circle (○).

suggesting that the amino acid sequence of nitrite reductase from *Alc. xylosoxidans* is likely to be similar to that from '*Achr. cycloclastes*'.

Products of reaction

The products of the reduction of nitrite by our preparations of nitrite reductase were analysed by m.s. and also by colorimetric determination of ammonia. When using dithionite-reduced Methyl Viologen as the electron donor, no nitric oxide was detected, but ammonia was produced. However, when ascorbate-reduced phenazine methosulphate was used as electron donor to nitrite reductase, nitric oxide, but no ammonia, was detected. The amount of nitric oxide detected was proportional to the incubation time. These results are in agreement with those published previously (Masuko and Iwasaki, 1984) for preparations of the enzyme which apparently contained only type 1 copper centres.

Metal content

The copper content determined from seven preparations of nitrite reductase by d.p.c.s.v. was 3.5 ± 0.8 (S.D.) Cu atoms/trimer, ranging from 2.3 to 4.6 Cu atoms/trimer. In one

Table 2 Comparison between the amino acid composition of the nitrite reductase of *Alc. xylosoxidans* and that of '*Achr. cycloclastes*'

The experimental conditions for hydrolysis and analysis are given in the Materials and methods section. Data for '*Achr. cycloclastes*' are from Fenderson et al. (1991)

Amino acid	Composition (residues/monomer)	
	<i>Alc. xylosoxidans</i>	' <i>Achr. cycloclastes</i> '
Asx	26	34
Glx	25	28
Ser	9	6
Gly	38	34
His	15	15
Arg	11	12
Thr	40	28
Ala	33	32
Pro	29	23
Tyr	11	13
Val	23	34
Met	7	9
Cys	N.d.*	1
Ile	11	12
Leu	29	24
Phe	13	13
Trp	N.d.	4
Lys	21	17
Total	341	339
Proportion of Glx + Asx (%)...	15	18
Proportion of Lys + Arg (%)...	9	8

*N.d., not determined.

preparation with 4.6 Cu/trimer, as measured by d.p.c.s.v., the Cu(II) content calculated from the integration of the e.p.r. spectrum was only 2.8 Cu atoms/trimer, but after the enzyme was oxidized with H_2O_2 (see below) the integrated intensity of the e.p.r. spectrum increased to 90% of the value obtained by d.p.c.s.v. The value determined by Masuko et al. (1984) is at the lower end of the range reported here, whereas the upper end of our range is in agreement with the value published for '*Achr. cycloclastes*' when recalculated for the trimeric enzyme shown from the X-ray structure (Godden et al., 1991) (see Table 3).

Table 3 Comparison of properties for nitrite reductases from *Alc. xylosoxidans* and '*Achr. cycloclastes*'

For ease of comparison, the copper contents and absorption coefficients have been recalculated on the basis of trimeric enzyme molecules. References: ^aMasuko et al., 1984; ^bLiu et al., 1986 (M_r assumed to be 109200); ^cIwasaki and Matsubara, 1972; ^dGodden et al., 1991; ^eIwasaki et al., 1975.

Property	<i>Alc. xylosoxidans</i>		' <i>Achr. cycloclastes</i> ' ^{b-e}
	This present paper	A previous paper ^a	
Specific activity (units/mg)	240	23	150 ^b 300 ^c
$10^{-3} \times M_r$	109.5 (3 × 36.5)	70 (2 × 37)	109.2 (3 × 36.4) ^d
Cu(II) atoms/trimer	3.5 ± 0.8	2.4	4.3 ^b
Absorption maxima (ϵ , $\text{mM}^{-1} \cdot \text{cm}^{-1}$)	280	282	283 ^d
	—	—	400 (2.4) ^c
	460 (1.6)	470 (0.96)	464 (3.9) ^c
	593 (6.3)	594 (5.5)	590 (3.0) ^c
	770 (3.0)	780 (2.4)	700 (2.6) ^c
Colour	Blue	Blue	Green
E.p.r. parameters ($g_{\parallel}$, $A_{\parallel}$)			
Type 1	2.208, 6.3 mT	2.22, 6.5 mT	2.195, 7.3 mT ^e
Type 2	2.298, 14.2 mT	Not detected	2.262, 17.5 mT

Figure 5 U.v. and visible absorption spectra of nitrite reductase as isolated

Spectra of nitrite reductase were recorded in 50 mM Hepes buffer, pH 7.5, at room temperature. The main spectrum was recorded at 0.9 mg/ml protein and that of the inset spectrum at 2.5 mg/ml.

Visible absorption spectrum

The visible spectrum of nitrite reductase purified by the procedure described above is shown in Figure 5 and is similar to that published previously by Masuko et al. (1984) with absorption maxima at 460, 593 and 770 nm. Table 3 shows that, based on a native M_r of 109.5 kDa, the absorption coefficients of our preparations of nitrite reductase do not differ greatly from the preparations described previously (Masuko et al., 1984). In both

Figure 6 E.p.r. spectra of nitrite reductase

The continuous line shows the e.p.r. spectrum of a sample of 62 mg/ml of nitrite reductase recorded at 120 K and 10 mW microwave power at 9.37 GHz using 0.52 mT field modulation at 100 kHz. The broken line is a simulation, using the parameters in Table 3, comprising 60% type 1 and 40% type 2 copper by integrated intensity. The stick diagrams above the spectra show the positions of the features corresponding to each of the four values of the nuclear spin quantum number, I , for the type 1 and type 2 spectra.

Figure 7 Effect of H_2O_2 on the e.p.r. spectra of nitrite reductase

The sample was 58 mg/ml and spectra were recorded under the same conditions as those described for Figure 6, except that the field modulation was 1.03 mT. Sample treatments were as follows: (a) nitrite reductase alone; (b) thawed and refrozen; (c) plus 10 mM H_2O_2 for 15 min; (d) plus 10 mM H_2O_2 for 1 h; (e) plus 10 mM H_2O_2 for 16 h. The arrow indicates a feature (not seen in Figure 6) which derives from an additional type 2 e.p.r. centre. We have been unable, using a single additional species, to deconvolute the overall spectral envelope to identify unequivocally the other components of this species.

cases, the ratio of the 460 nm absorption intensity to that at 593 nm is significantly different from that of nitrite reductase of '*Achr. cycloclastes*', resulting in nitrite reductase from *Alc. xylosoxidans* having a distinctly blue colour rather than the more commonly observed green colour of nitrite reductases isolated from other sources (summarized in Denariáz et al., 1991). Recent studies on the molecular redesign of Cu/Zn-superoxide dismutase show that this difference in colour is an important indication of changes in the nature and/or spatial arrangement of the ligands forming the type 1 Cu sites (Sanders-Loehr, 1992; Valentine et al., 1992).

E.p.r. spectrum

Figure 6 shows the e.p.r. spectrum of our nitrite reductase preparation with the highest specific activity (300 units/mg), together with a simulation made up of 60% (by integration) type 1 and 40% type 2 Cu spectra, using the parameters given in Table 3. The total amount of e.p.r. detectable copper was, by comparison of double integrals with a CuEDTA standard, 2.8 g of atoms Cu/protein trimer compared with a Cu analysis of 4.6 atoms of Cu/protein trimer.

Figure 7(a) shows the e.p.r. spectrum of an average-activity preparation (150 unit/mg). The $g_{\perp}$ (high-field) region of this spectrum shows that, compared with the spectrum shown in Figure 6, additional species are present, although we were unable to deconvolute these from the experimental spectra; features corresponding to the type 1 and type 2 copper centres of Table 3 can be seen in the $g_{\parallel}$ (low-field) region. Fig. 7(b) shows that a freeze-thaw cycle had no effect on the spectrum and therefore is unlikely to be the cause of the observed heterogeneity. We were concerned at the relatively low copper levels detected by e.p.r. integration compared with those measured by total Cu analysis and therefore oxidized our preparations with H_2O_2 to render any Cu(I) present e.p.r.-visible. Figures 7(c)–7(e) show the time course of exposure to 10 mM H_2O_2 at 21 °C for 15 min, 1 h and 16 h; no further changes occurred after 16 h. The main effects of H_2O_2 treatment were: (1) an increase in integrated intensity from 2.8 Cu/trimer in Figure 7(a) to 4.1 Cu/trimer in Figure 7(e) (cf. a voltammetric analysis of 4.6 Cu/trimer); (2) an increase in the relative intensity of features corresponding to type 1 copper; (3) a change in the form of the low-field $g_{\parallel}$ peak consistent with the formation of an additional type 2 copper species with an increased $A_{\parallel}$ at the expense of the type 2 copper of Figure 7(a). This last effect might indicate binding of H_2O_2 to a type 2 Cu site.

DISCUSSION

The dissimilatory nitrite reductase from *Alc. xylosoxidans* has been considered unusual, because it was reported to contain only type 1 copper centres. It was thus an example of a protein in which blue copper centres showed an enzymic function.

Using the purification procedure described, we have isolated nitrite reductase from *Alc. xylosoxidans* with at least a 6-fold higher specific activity than previously found for preparations from the same organism (Masuko et al., 1984). Our preparations show some similarities to those of Masuko et al. (1984): the subunit M_r and the protein's behaviour on gel filtration are similar, and the visible spectra are comparable and typical of blue type 1 Cu sites. However, e.p.r. spectroscopy revealed that all our preparations contain type 2 Cu in addition to the type 1 Cu centres reported by Masuko et al. (1984). The correlation between the presence of type 2 Cu and the increased specific activity suggests that type 2 Cu sites are required for optimal nitrite reductase activity. Recently, Libby and Averill (1992)

have reported that the activity of nitrite reductase from '*Achr. cycloclastes*' is proportional to the type 2 Cu centre content.

We observed a 2-fold variation in copper content and specific activity between preparations, but a comparison of the total copper content with specific activity of nitrite reductase from seven preparations did not reveal any correlation between activity and total copper content. The reason for such variations is not yet clear; however, the copper centres appear to be rather unstable, and may be lost during the purification procedure. We presume that the cation-exchange chromatography used in the purification would have removed adventitiously bound copper cations.

Only one other blue nitrite reductase, namely that from *Pseudomonas aureofaciens*, has been isolated to date, and this, too, was initially thought to contain only type 1 Cu centres (Zumft et al., 1987). They reported changes in the e.p.r. spectra with the appearance of type 2 Cu centres which were ascribed to the disruption of type 1 sites after certain purification procedures. However, it is important to note that this enzyme preparation had a very low specific activity (3 units/mg). We also observed some variability in the e.p.r. spectra, particularly between that of our best preparation (sp. activity 300 units/mg) and average preparations (sp. activity 150 units/mg). The e.p.r. of average preparations had similar characteristics to those seen by Zumft et al. (1987). All of our preparations of nitrite reductase from *Alc. xylosoxidans* contain more than one species of e.p.r.-observable type 2 copper centre.

The absorption coefficient of the 593 nm band in the visible spectrum of our preparations was a good measure of the intensity of the type 1 e.p.r. spectrum. This observation implies that there is only one type 1 Cu environment in our preparations. Differences in the amounts of type 1 and type 2 Cu observed in the e.p.r. spectra may be due to the nitrite reductase being isolated in a partially reduced form (Figure 7).

The nitrite reductase from *Alc. xylosoxidans* described here shares many physicochemical properties with the much-studied nitrite reductase from '*Achr. cycloclastes*': the specific activities are similar, and both enzymes contain both type 1 and type 2 Cu centres. Statistical analysis of the amino acid compositions shows that the sequences of these two proteins are likely to be similar. We have also demonstrated (Grossman et al., 1993) that our nitrite reductase has a trimeric structure similar to that elucidated by crystallographic methods for nitrite reductase from '*Achr. cycloclastes*'. However, the visible spectra of these two proteins are quite different: nitrite reductase from '*Achr. cycloclastes*' is green, but that isolated from *Alc. xylosoxidans* is blue. This difference in the type 1 Cu centres may reflect a difference in ligand character or co-ordination geometry and is currently under investigation using resonance Raman spectroscopy.

It is clear from this work that both types 1 and 2 copper centre are required for optimal nitrite reductase activity. Further work is necessary to understand the structure of the metal centres and the role of the different types of copper in nitrite reduction.

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