Catalytic and spectroscopic analysis of blue copper-containing nitrite reductase mutants altered in the environment of the type 2 copper centre: implications for substrate interaction

Miguel PRUDÊNCIO*[1], Robert R. EADY* and Gary SAWERS†[2]

*Department of Biological Chemistry, John Innes Centre, Colney Lane, Norwich NR4 7UH, U.K., and †Department of Molecular Microbiology, John Innes Centre, Colney Lane, Norwich NR4 7UH, U.K.

The blue dissimilatory nitrite reductase (NiR) from *Alcaligenes xylosoxidans* is a trimer containing two types of Cu centre, three type 1 electron transfer centres and three type 2 centres. The latter have been implicated in the binding and reduction of nitrite. The Cu ion of the type 2 centre of the oxidized enzyme is ligated by three His residues, and additionally has a co-ordinated water molecule that is also hydrogen-bonded to the carboxyl of Asp^{92} [Dodd, Van Beeumen, Eady and Hasnain (1998), J. Mol. Biol. **282**, 369–382]. Two mutations of this residue have been made, one to a glutamic acid residue and a second to an asparagine residue; the effects of both mutations on the spectroscopic and catalytic properties of the enzyme have been analysed. EPR spectroscopy revealed that both mutants retained intact type 1 Cu centres with $g_{\parallel} = 2.12$ ($A_{\parallel} = 0$ mT) and $g_{\perp} = 2.30$ ($A_{\perp} =$ 6.4 mT), which was consistent with their blue colour, but differed in their activities and in the spectroscopic properties of the type 2 centres. The D92E mutant had an altered geometry of its type 2 centre such that nitrite was no longer capable of binding to elicit changes in the EPR parameters of this centre. Accordingly, this mutation resulted in a form of NiR that had very low enzyme activity with the artificial electron donors reduced Methyl Viologen and sodium dithionite. As isolated, the EPR spectrum of the $Asp^{92} \rightarrow Asn$ (D92N) mutant showed no characteristic type 2 hyperfine lines. However, oxidation with iridium hexachloride partly restored a type 2 EPR signal, suggesting that type 2 copper is present in the enzyme but in a reduced, EPR-silent form. Like the $Asp^{92} \rightarrow Glu$ mutant, D92N had very low enzyme activities with either Methyl Viologen or dithionite. Remarkably, when the physiological electron donor reduced azurin I was used, both mutant proteins exhibited restoration of enzyme activity. The degree of restoration differed for the two mutants, with the D92N derivative exhibiting approx. 60 % of the activity seen for the wild-type NiR. These findings suggest that on formation of an electron transfer complex with azurin, a conformational change in NiR occurs that returns the catalytic Cu centre to a functionally active state capable of binding and reducing nitrite.

Key words: azurin, EPR spectroscopy, mutagenesis.

INTRODUCTION

Denitrification is an intrinsic step in the nitrogen cycle, in which micro-organisms reduce nitrate in a stepwise manner via nitrite to form nitrogen oxides and dinitrogen as part of their energy-generating metabolism [1]. One of the key steps in this process is the one-electron reduction of NO_2^- to NO catalysed by a dissimilatory nitrite reductase (NiR). It is a key step in denitrification because it is at this point that losses of fixed nitrogen from the soil to the atmosphere occur.

NiRs can be classified into two families: one utilizes a cd_1 haem prosthetic group; the other employs copper as a prosthetic group. The copper-containing enzymes isolated from different organisms have highly conserved amino acid sequences and are homotrimers of molecular mass approx. 109 kDa, containing three type 1 and three type 2 Cu centres. They are further subdivided into two classes based on the colour of the purified enzyme, which can be either blue or green, a colour arising from a Cu^{II} type 1 site. Green NiRs isolated from a number of species [2–4] have absorbance maxima at 460, 595 and in the 700–750 nm range, whereas blue copper NiRs isolated from *Alcaligenes xylosoxidans* [5] and *Pseudomonas aureofaciens* [6] lack the absorbance maximum at 460 nm. The X-ray crystal structures of the green enzymes from *Achromobacter cycloclastes* [7–9] and *Alcaligenes faecalis* S-6 [10] and of the blue enzyme from *A. xylosoxidans* [11–13] have been reported. The amino acid ligands to the type 1 centres of the green and blue enzymes are identical; the difference in colour arises from subtle differences in the angular geometry of the S-Met ligand to the type 1 Cu atom [12,13]. The type 2 Cu centre does not contribute to the visible spectrum but it can be detected by EPR spectroscopy.

Spectrosopic and crystallographic studies have shown that the type 1 centre functions as an acceptor of electrons from the physiological electron donor (a reduced cupredoxin), pseudoazurin in the case of green NiRs or azurin for blue NiRs. The type 2 centre is involved in the binding and reduction of substrate [14]. Consequently, the two sites are suggested to be redox coupled, with the electron being transferred from the type 1 to the type 2 centre during enzyme turnover. The centres in *Alcaligenes xylosoxidans* nitrite reductase (AxNiR) are connected through adjacent residues Cys^{130} and His^{129}, which are ligands to the type 1 and type 2 centres respectively [12,15]. The Cu centres are separated by approx. 12.6 Å. The type 1 centres are buried within the subunits of the protein, approx. 6 Å from the protein surface [12]. The three type 2 centres are formed at the interface between subunits; ligands from two distinct subunits

Abbreviations used: AxNiR, *Alcaligenes xylosoxidans* nitrite reductase; D92E, $Asp^{92} \rightarrow Glu$; D92N, $Asp^{92} \rightarrow Asn$; MV, Methyl Viologen; NiR, nitrite reductase.

[1] Present address: Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, 2300 RA Leiden, The Netherlands.
[2] To whom correspondence should be addressed (e-mail gary.sawers@bbsrc.ac.uk).

contribute to the composition of each centre. The type 2 centres of all copper-containing NiRs are liganded by three histidine residues and a water molecule. The water molecule is stabilized by a hydrogen bond to the nearby Asp^{92} residue (see Figure 1) and both structural studies and electron nuclear double resonance ('ENDOR') data have established that it is this water molecule that is displaced on substrate binding [12,16,17]. Indeed, the importance of this residue for enzyme activity with the artificial electron donor dithionite was verified in a recent mutagenesis study [18,19]. To demonstrate the importance of the Asp^{92} residue in the substrate binding process and catalysis by the type 2 Cu centre, we have performed a mutagenesis study of this residue and detailed spectroscopic and kinetic analyses of the resultant mutant proteins. Our findings establish that Asp^{92} is important not only for maintaining the geometry of the type 2 Cu centre but also in determining the accessibility and binding of nitrite to the copper atom.

EXPERIMENTAL

Bacterial strains, plasmids and growth media

The *Escherichia coli* strains used in this study were JM109 F′ *traD36 lacI*^{q} Δ(*lac Z*)*M15proA*$^+$*B*$^+$/e14$^-$(McrA$^-$) Δ(*lac-proAB*) *thi gyrA96*(Nal^r) *endA1 hsdR17* ($r_K^-m_K^-$) *relA1 supE 44recA1* [20], BL21(DE3) F$^-$ *ompT gal* [*dcm*] [*lon*] *hsdS*$_B$ (r^-m^-), an *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene [21], and Epicurian Coli™ XL1-Blue (Stratagene). The plasmids used in this study were pUC18 and pUC19 (Ap^R) [20], pBR322 (Ap^R) [22], pET28a (Kan^R) (Novagen) and pEnirsp-1, which is similar to pET28a but includes the *A. xylosoxidans nirA* gene under the control of the phage T7 ϕ *10* gene promoter [23]. Antibiotics were used at a final concentration of 50 μg/ml.

Bacteria were routinely grown in Luria broth [1 % (w/v) bactotryptone (Difco)/0.5 % yeast extract (Difco)/1 % (w/v) NaCl] at 37 °C. Small-scale cultures (up to 10 ml) for plasmid DNA isolation were grown aerobically in sterile conical flasks filled to approx. 10 % of their volume with growth medium. Large-scale growths (20 or 200 litres) used for the isolation of NiR were cultured in New Brunswick fermenters under the control of Bio-Command software, with 20 % air saturation. Media were supplemented with $CuSO_4$ (1 mM) and the appropriate antibiotic solution. Antibiotics were purchased from Sigma; stock solutions were prepared in water and filter-sterilized through sterile 0.2 μm syringe filters (Sartorius). Induction of high-level protein production was initiated when the cultures had reached the late exponential phase of growth, through the addition of 0.5 mM isopropyl β-D-thiogalactoside and 0.4 % glucose. Growth was continued for a further 90 min, after which the cells were harvested by centrifugation. Cells were used immediately for the isolation of NiR or were stored frozen at −80 °C until required.

Site-directed mutagenesis of the *nirA* gene

Site-directed mutagenesis of the *nirA* gene in plasmid vector pEnirsp-1 [23] was performed with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), in accordance with the instructions of the manufacturer. The oligonucleotides used to construct the *nirA* gene mutants were D92E-F (5′-CGCACAACGTCGAGTTCCACGGCGC-3′), D92E-R (5′-GCGCCGTGGAACTCGACGTTGTGCG-3′), D92N-F (5′-CGCACAACGTCAACTTCCACGGCGC-3′) and D92N-R (5′-GCGCCGTGGAAGTTGACGTTGTGCG-3′). The resulting plasmids were termed pEnirD92E and pEnirD92N, which encoded the NiR mutants $Asp^{92} \rightarrow$ Glu (D92E) and $Asp^{92} \rightarrow$ Asn (D92N) respectively. The complete DNA sequence of the mutant *nirA* genes was verified with the method of Sanger et al. [24].

Protein purification

NiR was purified from the periplasmic fraction of recombinant *E. coli* cells as described by Prudêncio et al. [23]. The purification characteristics of each of the mutant proteins were indistinguishable from the wild-type NiR enzyme. Reconstitution of the copper sites in recombinant NiR by dialysis against $CuSO_4$ was performed as described [23].

Azurin was isolated from *A. xylosoxidans* as described previously [25].

Metal determination

To determine the copper content in preparations of wild-type and mutant forms of NiR, as well as to investigate the presence of other metals (e.g. Zn), inductively coupled plasma emission ('ICP') was used. NiR samples were analysed by Southern Analytical (Brighton, Sussex, U.K.) after denaturation by wet ashing with concentrated H_2SO_4 and H_2O_2 before analysis [5].

Enzyme activity assays

NiR enzyme activity was determined with three independent methods. The discontinuous Methyl Viologen (MV) assay was performed as described by MacGregor [26] and Abraham et al. [5]. The reaction mixture contained, in a final volume of 2 ml, 250 mM potassium phosphate buffer, pH 7.1, and 0.1 mM $NaNO_2$. MV at a concentration of 0.5 mg/ml was used as the electron donor and the reaction was initiated by the addition of dithionite to a final concentration of 0.4 mg/ml, followed by gentle mixing. The mixture was then incubated at 25 °C for the duration required (typically 5 min) and the reaction was stopped by vortex-mixing to oxidize the residual dithionite. The amount of nitrite left in the reaction mixture was then determined colorimetrically [26]. One unit of enzyme activity is defined as the reduction of 1 μmol of nitrite/min.

The continuous spectrophotometric assay for NiR activity [27] used dithionite as the electron donor. The oxidation of dithionite was followed spectrophotometrically at 315 nm (ϵ_{315} 8000 $mM^{-1} \cdot cm^{-1}$). The reaction mixture contained, in a final volume of 300 μl, 100 mM potassium phosphate buffer, pH 7.1, 3 mM sodium dithionite and 1 mM $NaNO_2$. The reaction was performed under argon. Once a constant baseline rate had been attained, the reaction was initiated by the addition of NiR by injection through the rubber closure of the cuvette. The linear rate of decomposition of dithionite in the absence of enzyme was determined in a blank assay with water instead of protein solution, and subtracted from that obtained in the presence of NiR. One unit of enzyme activity is defined as the oxidation of 1 μmol of dithionite/min.

To study electron donation by the physiological electron donor, azurin, to NiR and mutant forms of NiR, azurin I from *A. xylosoxidans* was used. Reduction of azurin I (ϵ_{619} 6.27 $mM^{-1} \cdot cm^{-1}$) was performed in an anaerobic glove box (Miller Howe Ltd.; O_2 was at approx. 5 p.p.m.). Sodium dithionite was added to a final concentration of 1 mM to an approx. 0.7 mM solution of azurin I in 1 ml of 50 mM Mes buffer, pH 6.0. After incubation for 2 min, excess reductant was removed by passage of the protein through a Bio-Gel P-6 desalting gel (Bio-Rad) column (14 cm × 1 cm) equilibrated with degassed 100 mM Tris/HCl buffer, pH 7.1, at a flow rate of approx. 0.75 ml/min. After reduction and removal of the excess

dithionite, azurin I had a concentration of approx. 0.45 mM in a total volume of approx. 1.5 ml. The activity assay measured the reoxidation of reduced azurin from the increase in absorption at 619 nm. The degassed reaction mixture contained 22.5 μM reduced azurin I and 20 mM $NaNO_2$ in 100 mM Tris/HCl buffer, pH 7.1. The reaction was performed under argon and was started by injection of the NiR through the rubber closure of the cuvette. Oxidation of azurin I in the absence of enzyme was determined in a blank assay containing water instead of protein solution and this slope was subtracted from that obtained for the protein assays. One unit of enzyme activity is defined as the oxidation of 1 μmol of azurin/min.

Spectroscopic methods

UV–visible spectroscopy was performed at room temperature with a Hewlett-Packard 8452A diode-array spectrophotometer or a Perkin Elmer Lambda 18 spectrophotometer.

EPR data were collected at 60 K with a microwave power of 20 W, a microwave frequency of 9.3 GHz and a modulation amplitude of 4.637 G with a Bruker ER ER200 D-SRC spectrometer fitted with an ER042 MRH microwave bridge with an ER033C field frequency lock and an Oxford Instruments Ite^{503} temperature controller. EPR spectra were simulated with the program WINEPR–SimFonia (Bruker). Spin quantifications were made as described by Åasa and Vänngård [28] by comparing the area of the experimental curve under non-saturating conditions with that obtained, under the same conditions, for a sample of Cu^{II}–EDTA.

Analytical methods

Determination of the apparent molecular mass of mutant NiR proteins

The molecular masses of recombinant forms of NiR were compared with the wild type by analysing their retention times by FPLC chromatography on a Superdex 200 HR 10/30 (Pharmacia) gel-filtration column. The elution buffer used was 50 mM potassium phosphate, pH 7.0, containing 150 mM NaCl; the flow rate was 0.3 ml/min [5].

Electrophoresis of proteins

SDS/PAGE was used routinely to check the purity of protein samples throughout the purification of NiR and to check overproduction levels of recombinant NiR proteins. SDS/PAGE was performed by the method of Laemmli [29]. Typically, 12.5 % (w/v) or 15 % (w/v) polyacrylamide gels were run. For whole cell extracts, D_{600} was measured and a volume of $1.2/D_{600}$ (in ml) of cell suspension was centrifuged; the cell pellet was resuspended in 80 μl of SDS sample buffer.

Determination of protein concentration

Protein determinations were performed by the method of Lowry et al. [30]. For pure preparations of NiR the protein concentration was also estimated with a specific absorption coefficient at 280 nm (ϵ_{280}) of 1.54 ml·mg^{-1}·cm^{-1} (R. Eady, unpublished work).

RESULTS

Construction of D92E and D92N NiR mutants

The crystal structure of native AxNiR [12] shows that Asp^{92} forms a hydrogen bond with a water ligand to the type 2 Cu

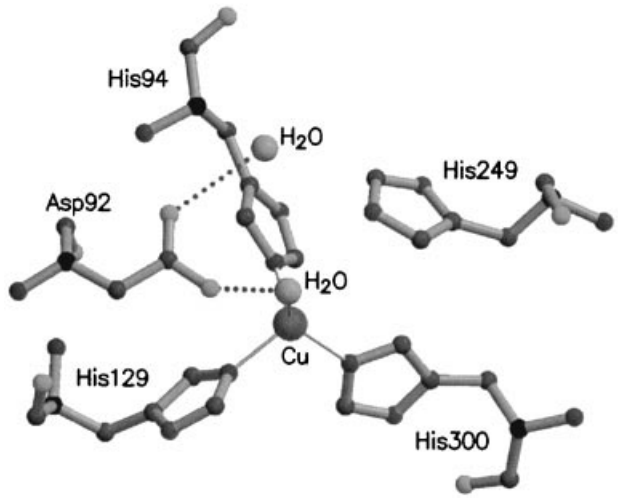

Figure 1 Amino acid residues and geometry of the type 2 Cu site in AxNiR

centre (Figure 1), in agreement with earlier findings of Adman et al. [8] for the green NiR enzyme from *Achromobacter cycloclastes*. This residue has been suggested to be important for catalysis because it also forms a hydrogen bond with the substrate nitrite when it binds to the type 2 Cu atom. We replaced Asp^{92} with a glutamic acid residue (D92E) and with an asparagine residue (D92N). The mutations introduced in this residue are likely to influence substrate-binding and catalysis (see also [18]). During the preparation of AxNiR the type 2 centres are labile; for both the wild-type enzyme from *A. xylosoxidans* [5,27] and the recombinant NiR from *E. coli* [23] they are routinely reconstituted by the addition of $CuSO_4$ (1 mM) before purification. In the present study, to determine the effects of the mutations that we introduced, we purified both mutant enzymes from untreated and from Cu-activated periplasmic extracts.

Both forms of each mutant protein, termed D92E, D92E^{Cu}, D92N and D92N^{Cu}, were purified from 200 g of cells (see the Experimental section). The superscript designates that these proteins were isolated from periplasmic fractions that had been incubated with 0.1 mM $CuSO_4$ before the start of enzyme purification. Co-chromatography with AxNiR as a mixture on an analytical Superdex 200 HR gel-filtration column gave a single peak. This is consistent with a trimeric nature for both forms of the D92E and the D92N proteins (results not shown).

Nature of the Cu centre

The UV–visible spectroscopic properties of both forms of both mutant NiRs were compared with those of the native recombinant enzyme. All samples were blue, with an absorption maximum in the 600 nm region, consistent with the presence of oxidized type 1 Cu centres in the proteins (results not shown). Because $\epsilon_{280}/\epsilon_{592}$ for purified NiR is a measure of the protein/type 1 Cu content, the values listed in Table 1 provide an indication of the type 1 Cu content of the proteins as isolated. All forms of the mutant proteins, with the exception of D92E, had $\epsilon_{280}/\epsilon_{592}$ values of approx. 12, which correspond to a full complement of type 1 copper. This is the ratio found for both the native wild-type [5] and recombinant wild-type enzyme (Table 1) [23]. In contrast, D92E protein isolated without incubation with copper had a ratio of 18.5, suggesting that this enzyme did not have a full complement of type 1 copper. However, incubation of the

Table 1 Metal contents of the D92E and D92N mutant proteins

The $\epsilon_{280}/\epsilon_{592}$ values were determined from the UV–visible spectra determined for each protein sample (see the Experimental section for details). Total Cu and total Zn were determined by inductively coupled plasma emission analysis. Abbreviation: WT^{Cu}, native recombinant NiR.

Parameter	Wild type	WT^{Cu}	D92E	$D92E^{Cu}$	D92N	$D92N^{Cu}$
$\epsilon_{280}/\epsilon_{592}$	12.2	12.5	18.5	12.1	11.4	11.2
Total Cu (atoms per protein trimer)	3.8	6.3	4.55	5.18	4.56	4.52
Total Zn (atoms per protein trimer)	2.52	2.54	2.15	1.79	0.78	0.96

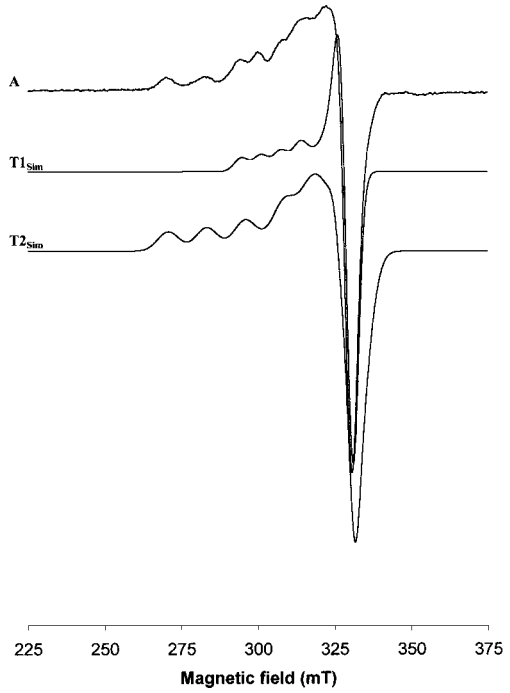

Figure 2 EPR spectroscopy of native recombinant AxNiR

Spectrum A, experimental spectrum recorded for native recombinant NiR at 4.8 mg/ml (135 μM monomer) and at 60 K at a microwave frequency of 9.312 Ghz; spectrum $T1_{Sim}$, simulation of the type 1 Cu signal; spectrum $T2_{Sim}$, simulation of the type 2 Cu signal.

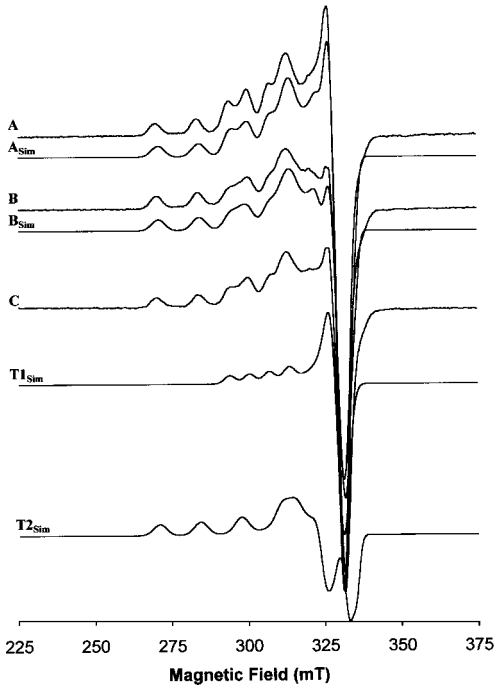

Figure 3 EPR spectra of the D92E mutant of NiR

Spectrum A, spectrum of $D92E^{Cu}$ protein purified from cells grown on medium supplemented with 1 mM $CuSO_4$, after reconstitution of the periplasmic fraction with $CuSO_4$ (12.5 mg/ml protein concentration). Spectrum B, spectrum of D92E protein purified from the same batch of cells and in which no reconstitution of the periplasmic fraction was performed (16.0 mg/ml protein concentration). Spectra A_{Sim} and B_{Sim} are the theoretical simulations of spectra A and B respectively. Spectrum C, spectrum of $D92E^{Cu}$ protein after incubation with a 20-fold molar excess of nitrite relative to the estimated type 2 Cu content. Spectrum $T1_{Sim}$, simulated spectrum after subtraction of spectrum B from spectrum A. Spectrum $T2_{Sim}$, spectrum A after subtraction of the type 1 Cu signal. The experimental spectra were recorded in 100 mM Tris/HCl, pH 7.1, at 60 K and at a microwave frequency of 9.41 GHz; they were normalized to the same protein concentration.

periplasmic fraction with copper before enzyme purification restored $\epsilon_{280}/\epsilon_{592}$ to a value that was of a fully complemented type 1 centre (Table 1).

Metal analysis of the proteins revealed that both mutant proteins had on average approx. 4.5 Cu atoms per trimer (Table 1). Thus, for those proteins with a full type 1 Cu content, the occupancy of the type 2 Cu centres was not complete. The presence of up to two Zn atoms (detected in the D92E mutant) makes it probable that Zn was present in a small proportion of the type 2 sites.

EPR spectroscopic properties of D92E NiR

Native recombinant NiR showed EPR spectral properties indistinguishable from the native AxNir enzyme [5,23,27], with g values for the type 1 centre of $g_\perp = 2.11$ ($A_\perp = 0$ mT) and $g_\parallel = 2.29$ ($A_\parallel = 6.4$ mT). The EPR spectrum of the type 2 centre has a small degree of rhombicity, with $g_x = 2.12$ ($A_x = 0$ mT), $g_y = 2.13$ ($A_y = 4.6$ mT) and $g_z = 2.41$ ($A_z = 12.5$ mT) (Figure 2). It was noted that isolation of NiR without preincubation with Cu resulted in an enzyme species lacking a full complement of type 2 copper (Table 1) [5,23,27]. A full complement of type 2 copper could be restored by incubation of the periplasmic fraction with 1 mM $CuSO_4$ before enzyme purification (Figure 2, spectrum A; Table 1).

EPR spectroscopy of the two forms of the D92E mutant demonstrated that they differed only in their type 1 Cu content, as shown by the difference between the spectra of the $D92E^{Cu}$ and the D92E proteins (Figure 3, spectra A and B respectively). The simulation of the difference spectrum generated by subtraction of spectrum B from spectrum A (Figure 3, spectrum $T1_{Sim}$) shows a type 1 Cu signal with $g_\parallel = 2.12$ ($A_\parallel = 0$ mT) and $g_\perp = 2.30$ ($A_\perp = 6.4$ mT). Double integration of the spectra showed this signal to contribute 26% to the total area of the spectrum of $D92E^{Cu}$. Assuming that this protein had a full complement of type 1 Cu, as indicated by $\epsilon_{280}/\epsilon_{592}$, and taking into account that the total Cu content was approx. 5 Cu atoms per trimer (Table 1), it can be estimated that approx. 1.25 Cu atoms were incorporated into the type 1 centre during incubation of the periplasmic fraction with $CuSO_4$. Thus the $D92E^{Cu}$ protein contains approx. 1.75 type 1 Cu atoms contributing to its EPR spectrum (Figure 3, spectrum A_{Sim}). Therefore by subtracting this contribution (1.75/1.25 = 1.4-fold the intensity of the type 1 Cu signal in spectrum $T1_{Sim}$) from the spectrum of the D92E protein, the resulting spectrum

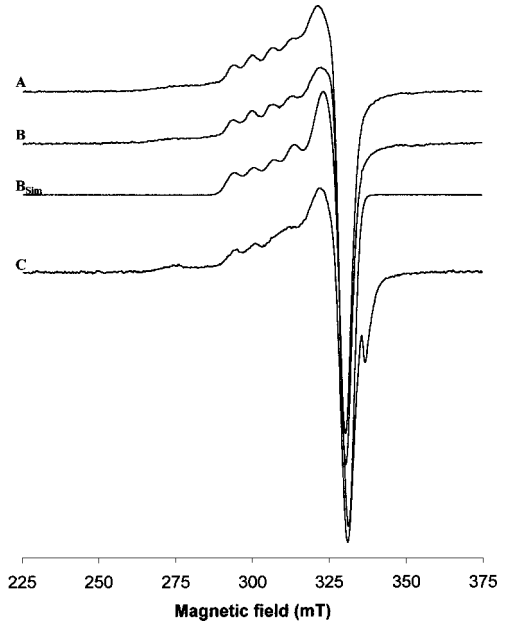

Figure 4 EPR spectra of the D92N mutant of NiR

Spectrum A, spectrum of D92N^{Cu} protein purified from cells grown on medium supplemented with 1 mM $CuSO_4$, after reconstitution of the periplasmic fraction with $CuSO_4$ (11.1 mg/ml protein concentration). Spectrum B, spectrum of D92N protein purified from the same batch of cells and in which no reconstitution of the periplasmic fraction was performed (8.5 mg/ml protein concentration). Spectrum B_{Sim}, simulation of the type 1 Cu signal in spectra A and B. Spectrum C, spectrum of D92N protein after oxidation with K_2IrCl_6. The experimental spectra were recorded in 100 mM Tris/HCl, pH 7.1, at 60 K and at a microwave frequency of 9.40 GHz; they were normalized to the same protein concentration.

(Figure 3, spectrum $T2_{Sim}$) represents the simulated type 2 Cu contribution to the overall signal. This simulation showed the type 2 signal in the D92E mutant to be rhombic, with $g_x = 2.10$ ($A_x = 0$ mT), $g_y = 2.18$ ($A_y = 2.1$ mT) and $g_z = 2.40$ ($A_z = 13.3$ mT). The spectra of the D92E^{Cu} and the D92E proteins were simulated (spectra A_{Sim} and B_{Sim} respectively in Figure 3) by the addition of the appropriate contributions of each of the simulated type 1 Cu and type 2 Cu EPR signals. It should be noted that the D92E protein did not contain partly reduced type 1 copper because oxidation of the protein with a 20-fold molar excess of K_2IrCl_6 did not result in a change in $\epsilon_{280}/\epsilon_{592}$.

D92N lacks oxidized type 2 Cu centres

The EPR spectra of the D92N mutant are shown in Figure 4. The spectra of the two forms of D92N are virtually identical, as indicated by the absence of a signal in the spectrum of the difference between the spectra of D92N^{Cu} and D92N (results not shown). In their forms as purified, both proteins displayed only a type 1 Cu signal. The simulation of this signal (Figure 4, spectrum B_{Sim}) showed that it had $g_{\|} = 2.12$ ($A_{\|} = 0$ mT) and $g_{\perp} = 2.30$ ($A_{\perp} = 6.4$ mT). The absence of type 2 Cu features from the EPR spectra of D92N^{Cu} and D92N was unexpected, considering the values obtained for the total Cu content of these proteins (see Table 1), which indicate that both forms of the D92N mutant contain approx. 4.5 Cu atoms per trimer. Assuming that the proteins had a full complement of type 1 Cu, as indicated by their $\epsilon_{280}/\epsilon_{592}$ values, this suggests that in this protein there might be a partial complement of reduced (and therefore EPR-silent) type 2 Cu. To test this hypothesis, the D92N sample was oxidized with a 20-fold molar excess over the estimated type 2 Cu, first with potassium ferricyanide and then with K_2IrCl_6. Ferricyanide had no effect on the signals; however, K_2IrCl_6 seemed to cause some oxidation of the D92N protein (Figure 4, spectrum C). Although the spectrum did have a weak signal with type 2 Cu features, there was also a change in the spectrum from an axial to a rhombic signal. It should also be noted that the double integration of the spectra showed that the intensity of the EPR signal in the oxidized sample was approx. 1.15-fold that of the sample as purified, suggesting that part of the Cu in the D92N protein was indeed in the reduced state. However, it is difficult to determine unequivocally the nature of this additional Cu, but it was apparently associated with a centre with a redox potential more positive than ferricyanide.

Table 2 Activity of the D92E and D92N mutants with three different electron donors

The activities of the native recombinant enzyme were 168 μmol of nitrite reduced/min per mg with MV as electron donor, 107 ± 11 μmol of dithionite oxidized/min per mg with dithionite as electron donor, and 45.2 ± 2.3 μmol of azurin oxidized/min per mg with azurin I as electron donor. The values shown are means $\pm$ S.D. Abbreviation: WT^{Cu}, native recombinant NiR.

	Activity (% of native recombinant NiR)					
Electron donor	Wild type	WT^{Cu}	D92E	D92E^{Cu}	D92N	D92N^{Cu}
MV	28 ± 2.4	100	0.13 ± 0.02	0.13 ± 0.02	0.04 ± 0.01	0.05 ± 0.01
Dithionite	51 ± 11	100	2.7 ± 0.2	2.8 ± 0.2	3.9 ± 0.4	5.3 ± 0.5
Azurin I	22.5 ± 3	100	24.2 ± 3.5	32.6 ± 4.1	61 ± 5.5	71.7 ± 6.1

NiR mutants have low activity with non-physiological electron donors

The NiR activity of the Asp^{92} mutants was determined by three different methods, with MV, dithionite and reduced azurin I as electron donors (see the Experimental section) and compared with that of native recombinant NiR. The results obtained are summarized in Table 2. Mutation of the Asp^{92} residue to either a glutamic acid or an asparagine residue resulted in an approximate three orders of magnitude decrease in MV-dependent NiR activity when compared with the wild type. This activity was the same for both forms of each mutant.

With dithionite as electron donor, the activity of both mutants was decreased to approx. 4% of wild type (Table 2). This low activity was presumably due to impaired nitrite binding in both mutants. Indeed, substrate binding to D92E, as monitored by EPR (Figure 3, spectrum C) revealed that no significant changes in the spectrum of the D92E^{Cu} mutant protein occurred on incubation with nitrite. This strongly suggests that the type 2 Cu site in D92E was not accessible to substrate and explains why the activity of this protein was so low when MV or dithionite was used as electron donor in the activity assay. As expected for an enzyme lacking type 2 copper features, no effect of nitrite on the EPR spectral properties of D92N was observed (results not shown).

Azurin functions as an effective electron donor to the wild-type and mutant NiR proteins

The significant increase in enzyme activity observed when reduced azurin I was used as an electron donor strongly suggests that the accessibility or reactivity of the type 2 site towards nitrite changed as a result of the interaction of the protein with its physiological electron donor (Table 2). For D92N the increase in enzyme

activity was very pronounced (between 60 % and 70 % of that of wild type). This activity found for D92N with azurin I as the electron donor was higher than would have been expected from the spectroscopic data for this protein. This suggests either that catalysis was possible at a metal-depleted type 2 site [31] or that Cu was indeed present in a spectroscopically silent form in the protein, as indicated by the K_2IrCl_6-oxidation studies (see Figure 4). However, for both mutants it seems clear that on interaction with its physiological electron donor, the D92E and the D92N proteins underwent a significant conformational change that facilitated catalysis, possibly by enhancing substrate accessibility to the catalytic site.

DISCUSSION

The crystal structures of several Cu-containing NiRs with nitrite bound at the type 2 Cu site [8,9,11,12] have identified amino acid residues in the substrate-binding pocket that are potential candidates for facilitating nitrite binding and reduction. The green and blue enzymes have very similar structures [12] and for both there are two residues, His^{255} and Asp^{92} (or equivalent), that have been identified as having a key role in maintaining the environment around the type 2 Cu centre of NiR. Both residues are clearly important targets in site-directed mutagenesis studies to probe proton delivery or nitrite binding to the active site of NiR.

Thus Olesen et al. [17] introduced a mutation at His^{287} of NiR of *Rhodobacter sphaeroides* and showed that the activity of the mutant protein was markedly diminished and the binding of substrate to its type 2 Cu centre was abolished. A recent report has shown that mutation of the Asp^{92} and His^{255} residues in the *A. xylosoxidans* [18] and *A. faecalis* [19] enzymes resulted in a loss of, or marked decrease in, activity and suggested that those residues are involved in substrate anchoring. In the latter case the crystal structure showed that in the D98N mutant reorientation of the Asn^{98} side chain disrupted the hydrogen bond to the water ligand on the Cu atom. These findings indicate that in the native structure, Asp^{98}, has a likely role in directing the binding and protonation of nitrite.

Previous mutagenesis studies have concentrated on the direct type 2 Cu ligands. For example, His^{135} of the NiR enzyme from *A. faecalis* when replaced by lysine was shown not to prevent type 2 Cu ligation but resulted in a catalytically inactive enzyme [32]. Our results have corroborated and extended these findings by clearly demonstrating that Asp^{92} has an important role both in substrate binding and in maintaining the geometry of the type 2 centre. The spectroscopic and metal content data collected on D92E and D92N indicate that the former contains a nearly full complement of both type 1 Cu and type 2 Cu, whereas the latter is at least partly depleted of type 2 Cu. This is an interesting finding in that it indicates that the charge of the residue at position 92 is important in the assembly of the type 2 Cu sites of NiR. However, the rhombic character found for type 2 Cu in D92E also indicates that the size constraints imposed by the presence of a bulkier amino acid residue at the type 2 site perturbs the geometry of this site, with possible implications for substrate accessibility and catalysis. The possibility that reduced type 2 Cu is present at the type 2 site of D92N was suggested by oxidation of the protein with K_2IrCl_6. Oxidation of the protein also introduced rhombicity to the type 2 EPR signal. Taken together, these findings strongly suggest that impairing the hydrogen-bonding network to the water molecule by introducing the amino group of Asn^{92} has a marked effect on the redox potential and geometry of the Cu in the type 2 site. A similar conclusion that the geometry of the type 2 site was altered in a comparable mutation of the green *A. faecalis* NiR was reached by Boulanger et al. [19] on the basis of EPR and structural studies.

The activity and substrate binding data obtained for the Asp^{92} mutant proteins provide interesting indications with regard to the catalytic ability of these mutants. For both proteins, enzyme activity was very low when artificial electron donors were used. Similar observations were made recently by Kataoka et al. [18] and Boulanger et al. [19]. For D92N this could be explained by an absence of Cu from the type 2 site or by the presence of Cu atoms with very high redox potentials. However, for D92E this is more difficult to rationalize owing to the presence of oxidized type 2 Cu. The EPR results obtained with the D92E protein give a clear indication that, although assembly of the type 2 Cu centres has not been prevented, substrate accessibility to the catalytic site has been greatly perturbed by the presence of a bulkier amino acid. Alternatively, it is feasible that the mutation does not prevent the substrate from entering the active site but affects its anchoring, as suggested by Kataoka et al. [18]. This would be consistent with our inability to detect a change in the EPR parameters of the type 2 Cu site in the presence of nitrite. The possibility that substrate accessibility and/or anchoring to the catalytic site in the D92N protein has also been perturbed cannot be excluded, although it is more difficult to monitor in the absence of type 2 Cu EPR features.

An extremely interesting result was obtained when the physiological donor, reduced azurin, was used to donate electrons to the Asp^{92} mutants. The activities determined under these conditions were significantly higher than those obtained when the artificial electron donors were used. Pulse-radiolysis experiments on AxNiR have shown previously that electron donation from radicals involves an initial reduction of the type 1 Cu site [15]. The difference in activity that we observe between reduced azurin and the radical electron donors $SO_2^{-\bullet}$ and $MV^{\bullet}$ might indicate that the interaction of azurin with D92E and D92N has an overall effect on the protein that modifies its catalytic ability. The most likely explanation for this enhancement of catalysis is that, on formation of a transient complex with azurin, the substrate accessibility to the catalytic site increases, possibly owing to a conformational change in the NiR protein. The D92E mutant has a specific activity with azurin that is approx. 25 % that of wild-type NiR. The reason why this value is not higher is not readily explained. Clearly, the type 1 Cu centre, which is the immediate electron acceptor from azurin, is not limiting, because $D92E^{Cu}$, which has a full complement of type 1 Cu, has an activity slightly higher than that of D92E (32 %). Rather, it is more likely that the altered geometry of the type 2 centre in the D92E enzyme restricts substrate binding or changes the redox potential of the Cu. The product of the reaction with azurin I as electron donor was not established. A possible product other than NO is N_2O, which wild-type NiR forms as a minor product of the reduction of nitrite when sufficient NO has accumulated [33]. Irrespective of the nature of the products, it is clear from our results that reduced azurin I is an effective electron donor to both Asp^{92} mutants described above.

For the D92N protein, the specific activity determined with reduced azurin as electron donor was approx. 60 % of that of NiR. Because the D92N protein has a full complement of type 1 Cu, the limiting factor for the activity of this protein is presumably its type 2 Cu content. As mentioned above, it is not clear whether D92N is depleted of type 2 Cu or whether type 2 Cu atoms in this protein are in an EPR-silent (presumably reduced) state. Thus the results obtained can be explained in two different ways: (1) on interaction with azurin, D92N undergoes a conformational change that might facilitate substrate accessibility to a Cu-

depleted type 2 site and thereby allow catalysis to occur at such a site; or (2) the D92N protein contains type 2 Cu atoms in a reduced, catalytically inactive state. On interaction with azurin, changes are induced in the geometry of the type 2 Cu site that modulate the potential of the type 2 Cu atoms, rendering them catalytically active. Substrate accessibility might also be enhanced by these changes. The first explanation requires that catalysis can occur at a metal-depleted site to an extent that is more than 50% of that seen in a fully loaded centre. Furthermore, it implies that electron transfer from the type 1 Cu would occur in the absence of type 2 Cu, a notion that is incompatible with the ordered mechanism suggested by Strange et al. [34]. Thus it seems that the second hypothesis provides a more likely explanation for the results. The determination of the metal content of D92N indicates that the protein might have approx. 50% Cu-loaded type 2 centres (assuming that this Cu is present in a spectroscopically silent state), in good correlation with the activity value determined when azurin was used as the electron donor.

A further unexpected finding of this study was that the D92E mutation influenced the occupancy of the type 1 Cu site in the enzyme that had not been incubated with copper before purification. It is possible that the altered geometry of the type 2 site in this enzyme affected the occupancy of the type 1 site, despite the fact that the sites were 12.5 Å apart [12]. Oxidation experiments with ferricyanide and iridium hexachloride ruled out a change in the redox potential of the Cu as providing an explanation for the altered EPR signal. Incubation of the periplasmic fraction with Cu ions relieved this deficiency in the type 1 site. It is currently unclear whether this resulted from a conformational change in the protein brought about by the occupancy of the type 2 site with Cu. However, our results with azurin I as electron donor to the enzyme, together with previous electron transfer studies [15], support the contention of coupling between the sites.

Thus the findings gathered for the two Asp^{92} mutant forms of NiR indicate that the interaction of this protein with azurin triggers important conformational changes that are likely to influence substrate accessibility to the catalytic site and to modulate the redox potential of the type 2 Cu atoms. This might help to provide an explanation of why electron transfer occurs between type 1 Cu and type 2 Cu of NiR in what seems to be an 'uphill' process [15,31] in the absence of the physiological electron donor. A similar observation of a change in the redox potential of the small copper-containing protein amicyanin has been shown to occur on complex formation with its redox partner methylamine dehydrogenase, to facilitate an otherwise thermodynamically unfavourable electron transfer reaction [35].

The results obtained for recombinant NiR suggest that MV is the most effective electron donor to this enzyme, followed by azurin and finally by dithionite. However, only dithionite seems to be able to donate electrons directly to the type 2 Cu site, as shown by a comparison of a kinetic analysis of the reduction of oxidized AxNiR with NiR depleted of type 2 Cu (F. Yousafzai and R. R. Eady, unpublished work). This is in agreement with the suggestion that $SO_2^{-\bullet}$ might act as an analogue of the NO_2^- ion and that its access to the catalytic site might therefore be facilitated, in contrast with either of the other two donors.

This study has brought to light a number of interesting questions that remain to be answered. For example, is catalysis possible at a Cu-depleted type 2 site? How does direct electron donation to the type 2 Cu proceed? What is the exact nature of the presumed conformational change that occurs on interaction of the protein with its physiological electron donor? The availability of the recombinant enzyme and its mutant derivatives should greatly facilitate answering these questions.

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