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The Importance of Asn⁴⁷ for Structure and Reactivity of Azurin from *Alcaligenes denitrificans* as Studied by Site-directed Mutagenesis and Spectroscopy*

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To study the importance of a rigid copper site for the structure and function of azurin, a mutant with a reduced number of internal hydrogen bonds around the copper has been prepared and characterized. To this purpose, the previously cloned *azu* gene from *Alcaligenes denitrificans* (Hoitink, C. W. G., Woudt, L. P., Turenhout, J. C. M., Van de Kamp, M., and Canters, G. W. (1990) *Gene* (Amst.) 90, 15–20) was expressed in *Escherichia coli* and an isolation and purification procedure for the azurin was developed. The azurin obtained after heterologous expression in *E. coli* appears spectroscopically indistinguishable from azurin derived from *A. denitrificans*. The hydrogen bonding network around the copper site was altered by replacing Asn⁴⁷ by a leucine by means of site-directed mutagenesis. Asn⁴⁷ is a conserved residue in all blue copper proteins of which the primary structure has been reported. Characterization of the mutant protein with UV-visible, electron spin resonance, and NMR spectroscopy, and comparison with the wild type azurin revealed that the structure of the copper site as well as the overall structure of the protein have been largely retained. The redox activity as measured by the electron self-exchange rate appears not to have changed either. However, the mutant differs from the wild type azurin with respect to stability and midpoint potential. Midpoint potentials of mutant and wild type azurin amount to 396 and 286 mV, respectively. The difference is due to sizable entropic and enthalpic contributions which to a large extent cancel. Possible explanations for the outcome of these experiments are discussed.

Azurins are small copper-containing proteins (14.6 kDa), located in the periplasmic space of bacteria where they function as electron carriers. For *Pseudomonas aeruginosa* it has been shown that azurin is produced under denitrifying conditions (Parr *et al.*, 1976).

Azurins have been classified as type I blue copper proteins on the basis of their spectroscopic properties: an intense blue color due to a sulfur to copper charge transfer band near 600 nm, and a characteristic EPR spectrum (Adman, 1985). Because azurins are stable proteins with a single metal in their

active site, they provide good systems for studying the mechanism of biological electron transfer. Recombinant DNA techniques and in particular site-directed mutagenesis have opened up the possibility to look specifically into the importance of a single amino acid for the structure and function of a protein by substituting it with another residue. Experiments with mutants of *P. aeruginosa* azurin have shown, for example, which residues are involved in electron transfer of the azurin with its physiological redox partners (Van de Kamp *et al.*, 1990a).

The crystal structures of a number of blue copper proteins have been solved, namely those of azurin from *Alcaligenes denitrificans* (Baker, 1988), azurin from *P. aeruginosa* (Nar *et al.*, 1991), azurin from *P. denitrificans* (Korszun, 1987), pseudo-azurin from *Alcaligenes faecalis* (Petratos *et al.*, 1988), plastocyanin from *Enteromorpha prolifera* (Collyer *et al.*, 1990), cucumber basic blue protein (Guss *et al.*, 1988), and poplar plastocyanin (Guss and Freeman, 1983). The copper sites of these proteins appear to have a number of features in common. The copper ion is coordinated by four or five ligands arranged in a distorted tetrahedron or an elongated trigonal bipyramid. Only very small differences in the coordination sphere can be detected upon reduction of the copper (Shephard *et al.*, 1990; Guss *et al.*, 1986). This property, which is unusual from an inorganic chemical point of view, has been ascribed in part to the influence of a hydrogen bonding network around the active site (Baker, 1988). In fact, a fixed copper site geometry has been proposed as an important prerequisite for fast electron transfer because it may lead to a low reorganization energy (Marcus and Sutin, 1985; Williams, 1971).

In order to investigate how important a rigid copper site is for the structure and function of azurin, we decided to construct a mutant with a less extended hydrogen bonding network around the metal site. Among the possible amino acids to be substituted Asn⁴⁷ appeared to be a good candidate (Baker, 1988). The side chain of Asn⁴⁷ forms hydrogen bonds that link the loops containing the copper ligands (Fig. 1). The importance of this residue is illustrated by the observation that it is one of the very few strictly conserved residues in all blue copper proteins sequenced thus far (Chothia and Lesk, 1982; Ryden and Lundgren, 1976; Ambler and Tobari, 1985; Bergman *et al.*, 1977; Van Beeumen *et al.*, 1991). As a first attempt, leucine was chosen to replace Asn⁴⁷.

In the following the construction of the N47L *azu* gene, its heterologous expression in *Escherichia coli*, and the isolation and purification of the mutated protein is described. Also the heterologous expression of the wt¹ gene and the purification

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¹ The abbreviations used are: wt, wild type; IEF, isoelectric focusing; *azu*, the azurin-encoding gene from *Alcaligenes denitrificans*; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase(s); bp, base pair(s); MOPS, 4-morpholinepropanesulfonic acid; NaOD, deuterated sodium hydroxide; HDO, water with one hydrogen replaced by the deuterium.

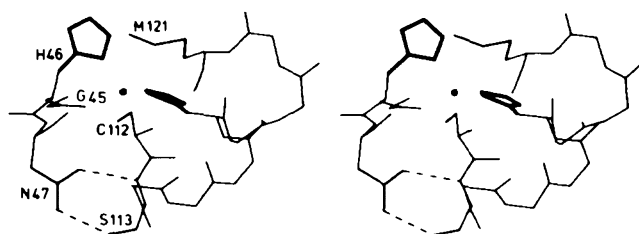


FIG. 1. Stereo view of the copper site of azurin from *A. denitrificans* (Baker, 1988). The ligands and the conserved residues Asn⁴⁷ and Ser¹¹² are indicated. The dashed lines represent the hydrogen bonds Asn⁴⁷ Oδ1..HN Ser¹¹² and Asn⁴⁷ Nδ2..OG Ser¹¹². Not shown is the hydrogen bond Cys¹¹² Sγ..HN Asn⁴⁷.

of the wt protein are dealt with. Furthermore, the N47L azurin has been characterized and the effect of the mutation on structure and function of the protein are reported and commented upon.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media

E. coli K12 strain JM101 with relevant genotype $\Delta lac-proAB$, F+ (*proAB*, *lacI^s*, *lacZΔ M15*) was used for cloning and heterologous expression of the *A. denitrificans* *azu* gene. Cells were grown in LB medium supplied with ampicillin (50 μ g/ml culture volume) (Sambrook *et al.*, 1989). Plasmids pCH1 and pUC19 are described elsewhere (Hoitink *et al.*, 1990; Yanisch-Perron *et al.*, 1982).

Cell Growth

E. coli JM101 cells freshly transformed with pCH5 (see below) were inoculated in LB medium supplemented with ampicillin (50 μ g/ml) and grown for 8 h. The culture was diluted 1/100 in LB medium and grown in 2-liter flasks under aeration at 37 °C until the optical density was about 1.0. Subsequently isopropyl- β -thiogalactopyranoside was added up to a concentration of 100 μ M, and the cells were grown for another 4 h. Cells were harvested by centrifugation, which resulted in 5 g of cell paste per liter of culture.

Isolation of Azurin from *E. coli*

Step 1—The bacterial paste was suspended in one-tenth of the culture volume in 30 mM Tris-HCl, 1 mM EDTA, pH 8.0, and stirred for 15 min at room temperature, after which the cells were centrifuged. We noticed that this procedure was sufficient to quantitatively transfer the azurin to the supernatant. Cupric sulfate and potassium ferricyanide were added to the azurin containing supernatant, up to a concentration of 1 and 0.1 mM respectively, in order to obtain stable oxidized holoazurin. Subsequently, the pH was lowered to pH 4.5 by stepwise adding 1 M acetic acid. The solution was cleared from precipitated proteins by centrifugation (10 min at 5000 \times g). Further treatments were performed at 4 °C.

Step 2—The azurin-containing supernatant was diluted with an equal volume of distilled water and applied to a Pharmacia CM fast flow column (30 \times 2.5 cm) equilibrated with 50 mM acetate buffer, pH 4.5. Azurin bound to the top of the column. The column was developed by a gradient with 50 mM acetate buffer (2 liters) from pH 4.5 to 6.0. Blue fractions were collected.

Step 3—The blue fractions were pooled and the buffer was exchanged with 5 mM Tris buffer, pH 9.0, by ultrafiltration. The azurin solution was reduced with ascorbic acid and applied to a Pharmacia DEAE fast flow column (30 \times 2.5 cm) equilibrated with 5 mM Tris buffer, pH 9.0. The column was developed by a gradient with 5 mM Tris buffer from pH 9.0 to 8.0 (3 liters). Azurin-containing fractions were identified by measuring A_{280} and A_{620} after oxidation by potassium ferricyanide. Usually the azurin was pure after this stage, as judged by an A_{620}/A_{280} ratio of 0.30. When necessary, a second CM column treatment identical to the first one was used to remove residual impurities (*i.e.* repetition of step 2).

Protein Electrophoresis

Protein electrophoresis was performed with a PhastSystem (Pharmacia, Sweden). Discontinuous denaturing SDS-polyacrylamide gel electrophoresis was performed with 20% polyacrylamide gels. IEF

focussing was performed with polyacrylamide gels in which a pH gradient of 3–10 was established. Gels were calibrated with pI marker proteins (Pharmacia).

Immunological Techniques

Rabbit anti-azurin serum was obtained by immunization of white rabbits as described by Den Blaauwen *et al.* (1989). Western blotting of protein gels to nitrocellulose filters was carried out with a Phast Transfer system (Pharmacia, Sweden).

Incubation with anti-azurin and staining of the filters were done as described by Den Blaauwen *et al.* (1989).

DNA Techniques

DNA cloning experiments were carried out using standard methods (Sambrook *et al.*, 1989). Site-directed mutagenesis was performed by applying the method of Kunkel (1985).

Redox Titrations

Redox titrations of reduced azurin with potassium ferricyanide were performed in 0.1 M phosphate, pH 7.0, at 298 K. Sample preparation and measurements were performed as described by Van de Kamp *et al.*, 1990a). Data were analyzed with a slightly modified form of the equation used by Goldberg and Pecht (1976; Van de Kamp *et al.*, 1990a), which yielded values for the midpoint potential as well as for the molar absorption coefficient.

Cyclic Voltammetry

Cyclic linear sweep voltammetry was performed with 1 mM azurin solutions in 100 mM phosphate buffer, pH 7.0. A platinum wire and a saturated calomel electrode were used as counter and reference electrode, respectively. As working electrodes a porous glassy carbon disc electrode of 5-mm diameter and a gold wire modified with pyridine-4-carboxaldehyde thiosemicarbazone was used (Hill *et al.*, 1985). Measurements were made at room temperature with a scan rate of between 0.5 and 20 mV/s with a potentiostat of Autolab. Voltammograms were analyzed with GPES software.

EPR Spectroscopy

Spectra were recorded with a JEOL JESRE2X spectrometer operating at X-band at 77 K and interfaced with an ES-PRIT330 data manipulation system. Parameters for recording EPR spectra were typically 12.5 mT/min sweep rate, 0.32 mT field modulation width, 9.099 GHz frequency and 4 mW microwave power. The magnetic field was calibrated with α,α' -diphenyl- β -picrylhydrazyl.

Thermal Denaturation

The stability of wt azurin was compared to that of the N47L azurin by following the tyrosine absorbance at 287 nm as a function of temperature for both proteins. A cuvette containing 5.9×10^{-5} M protein in 20 mM HEPES, pH 8.0, placed in a Cary photospectrometer, was heated with a speed of 1 centigrade degree per min. Absorbance was measured against a reference protein solution which was kept at 25 °C.

NMR Experiments

NMR samples with a protein concentration of 1 to 2 mM were made up in 20 mM potassium phosphate, pH 8.7, in 99.95% D₂O with ultrafiltration equipment (Amicon). Reduction of azurin was achieved by adding small aliquots of a 0.1 M solution of sodium dithionite in 0.1 M NaOD. Argon was bubbled through the solution before and after the reduction to prevent reoxidation by oxygen. The pH of the samples was adjusted by adding small amounts of NaOD or DCl solutions (0.01–0.1 M). A microelectrode MI-412 combination pH probe and a Radiometer PHM-84 research pH meter were used to measure the pH. Meter readings have not been corrected for the deuterium isotope effect. Partially oxidized samples were obtained by adding to a reduced sample small amounts of an oxidized protein solution with about the same protein concentration as the reduced solution. A special sample holder allowed the absorbance of the protein in the NMR tube to be measured at 619 nm on a spectrophotometer (Philips PU8700, The Netherlands). The concentration of the oxidized protein was determined directly before and after the recording of the NMR spectrum. The averaged value was used in the calculations.

All ¹H NMR spectra were recorded on a Bruker WM-300 spectrom-

eter. The HDO resonance was suppressed by presaturation. Chemical shifts are quoted in ppm downfield from sodium 2,2-dimethylsilapentane 5-sulfonate. Free induction decays were accumulated in 8K memory, deconvoluted by Gaussian multiplication, zero-filled to 16K data points, and Fourier-transformed. Line widths at half height were measured in spectra that were obtained by Fourier transformation without Gaussian multiplication.

RESULTS

Expression of the Azurin Gene in *E. coli*—The *azu* gene from *A. denitrificans* has been identified before on a 1.8-kb *Cl*I DNA fragment. This fragment has been cloned in the *Acc*I site of pUC19 (Yanisch-Perron *et al.*, 1979) resulting in plasmid pCH1 (Hoitink *et al.*, 1990). *E. coli* cells transformed with pCH1 did not produce quantities of azurin that could be detected with immunoblotting. This is probably caused by insufficient transcription due to terminator-like structures in front of the open reading frame of the *azu* gene (Hoitink *et al.*, 1990). In order to achieve sufficient expression of the gene, part of the upstream region in front of the *azu* gene was deleted. For this purpose a *Pst*I site was introduced 50 nucleotides upstream of the start codon for azurin by site-directed mutagenesis (Kunkel, 1985). To prevent the synthesis of a fusion protein of azurin with LacZ, also a stop codon in frame with the reading frame for LacZ was introduced. The DNA sequence between nucleotide 240–250 (Hoitink *et al.*, 1990) has changed by these mutations from CGGCAGC-GAC to CTGCAGCTGAC. The newly created *Pst*I site and the stop codon have been underlined. Finally, the *Sma*I restriction site originating from the polylinker of pUC19 was removed. This was done by cutting plasmid DNA with *Bam*HI and *Eco*RI (for which there are unique sites in the polylinker), filling in the resulting sticky ends with deoxyribonucleotides and circularizing by ligation. The resulting construct, pCH5 (Fig. 2) contains two unique sites in the *azu* gene, *Hinc*II and *Sma*I, which facilitate the cloning experiments necessary for site-directed mutagenesis.

Upon transformation of *E. coli* JM101 cells with pCH5 and growth in the presence of isopropyl- β -thiogalactopyranoside large amounts of azurin were produced and transported into the periplasm. This was verified from SDS-polyacrylamide gel electrophoresis and concomitant Western blotting of a

sample from the periplasm (data not shown).

Isolation and Purification of wt Azurin—The isolation of wt azurin was performed as described under "Experimental Procedures." After the first cation-exchange column, the purity of azurin amounted to 60% as calculated from the ratio A_{620}/A_{280} , which amounts to 0.30 for pure azurin from *A. denitrificans* (Norris *et al.*, 1979). Analysis of this azurin by IEF electrophoresis showed one strong band, at pI 8.6, under oxidizing conditions and two bands under reducing conditions, at pI 8.6 and 7.7. It was not possible to obtain a single reduced form of azurin by reconstitution with copper and using a large excess of reductant. It is known that *P. aeruginosa* azurin as obtained after heterologous expression of the *azu* gene in *E. coli* contains two forms of azurin: apoazurin and a nonreconstitutable form of azurin called azurin* (Van de Kamp *et al.*, 1990b). Presumably *A. denitrificans* azurin also consists of these two forms, with azurin* exhibiting the same isoelectric point as oxidized holozurin (pI 8.6). Fortunately, the two forms could be separated by anion-exchange chromatography under reducing conditions. IEF electrophoresis of the azurin obtained after DEAE column chromatography showed only one band at pI 8.6 when the protein was oxidized and one band at pI 7.7 when the protein was reduced. The purity of the azurin was checked by SDS-polyacrylamide gel electrophoresis and by measuring the A_{620}/A_{280} ratio. After the last cation-exchange step azurin was obtained with a purity better than 95%. Yields of wild type azurin amounted to 12.5 mg per 5 g of cell paste.

The ratio holozurin/total azurin obtained after the first chromatography step appeared to depend upon the growth time after induction: for an overnight culture this ratio was 0.5, for a culture which had grown for 4 h after induction it was 0.75. Several spectroscopic (UV-visible, electron spin resonance, and NMR) and electrophoretic experiments were performed in order to confirm that wt azurin isolated from *E. coli* is identical to azurin isolated from *A. denitrificans* (Groeneveld *et al.*, 1987; Groeneveld and Canters, 1988) (data not shown). No differences could be detected.

Construction of the N47L Mutant; Isolation and Purification of N47L Azurin—For the construction of the N47L mutant, the 270-bp-long *Hinc*II-*Xma*I fragment (the *Xma*I site is identical to the *Sma*I site) of pCH5 was ligated into the polylinker of M13 mp18 and transformed to *E. coli* JM101. The single-stranded DNA of this clone was subjected to oligo-directed site-specific mutagenesis with the use of the selection method of Kunkel (1985). An 18-nucleotide-long oligodeoxyribonucleotide (ATGGGCCACCTCTGGGTG; leucine codon is underlined) was used to substitute the Asn⁴⁷ codon AAC for the leucine codon CTC. The mutation was confirmed by single-stranded DNA sequencing. To check that no unwanted mutations had occurred in the course of the mutagenesis procedure the whole DNA fragment was sequenced. Subsequently, the *Hinc*II-*Xma*I fragment of pCH5 was exchanged for the fragment containing the mutation. The resulting plasmid, called pCH8, was screened for the mutation by hybridization with the oligonucleotide that was used for the mutation according to Sambrook *et al.* (1989).

Growth of *E. coli* JM101 cells transformed with pCH8 resulted in the synthesis of azurin in comparable amounts to the wt protein as detected on SDS-polyacrylamide gel electrophoresis (data not shown). The procedure for isolation and purification of N47L azurin was identical to the wt azurin. However, 60% of the initial yield (as determined after the first column) was lost in the subsequent chromatography steps. 3.2 mg of pure N47L was obtained per liter of culture.

Stability of N47L Azurin—The N47L azurin showed a

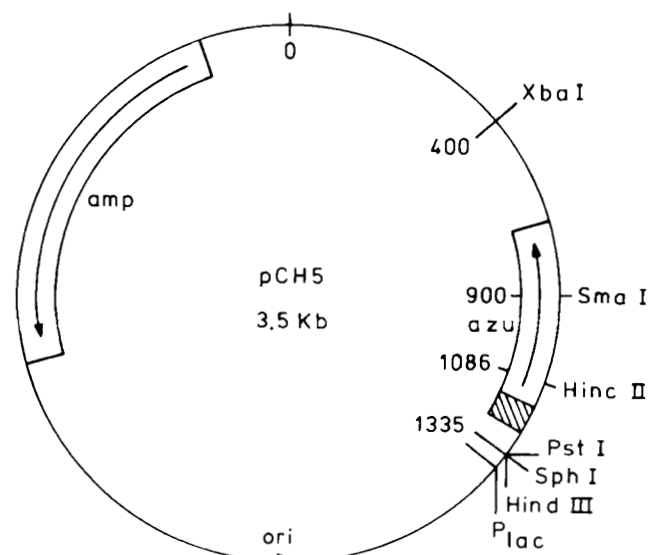


FIG. 2. Map of pCH5. Unique relevant restriction sites and the *lac* promoter P_{lac} are indicated. The reading frame for β -lactamase (*amp*) and *azu* are indicated by open boxes. The signal sequence of the *azu* gene is represented by a hatched box.

drastically decreased stability in comparison to wt azurin. Reduced N47L azurin started to denature when the pH was lowered beneath 7 at 4 °C. Oxidized pure N47L azurin shows a slight tendency to aggregate during sample handling and concentration. This sometimes made it a laborious task to obtain clear-cut spectroscopic results (see below).

In order to obtain an idea of the stability of N47L compared to wt azurin, the optical density of solutions of the oxidized proteins at 287 nm were measured as a function of the temperature (see "Experimental Procedures"). For wt azurin the onset of a transition in the structure was seen at 73 °C as a sudden rise in the absorbance. This transition was not reversible as the colorless protein could not be reconstituted or reoxidized to blue azurin. For N47L azurin the onset of a transition was seen at 63 °C. In this case the irreversible transition resulted in an opaque white protein solution.

Structural Characterization of the N47L Mutant—Superposition of the electronic absorption spectra of wt and N47L azurin showed that the absorption envelopes are nearly identical. This holds for the intensities as well as for the positions of the maxima. The only difference is an upward shift of 1 nm in the λ_{\max} of the main sulfur to copper charge transfer band (Table I).

The EPR spectra (Fig. 3 and Table I) of wt and of N47L azurin also are similar with the exception of the appearance of an additional weak signal at low and high field in the g_{\perp} area for N47L azurin. This additional signal is due to an extra copper containing species that could not be removed totally. Incubation of the sample in 0.2 M NaCl, which in the case of wt azurin results in removal of adventitiously bound copper, had no effect on the spectrum. Reduction of the sample with dithionite led to the disappearance of the "blue copper" signal, while the extra signal remained. Its features derive from paramagnetic copper, possibly coordinated by four oxygen

ligands ($g_{\parallel} = 2.419$, $g_{\perp} = 2.057$ en $A_{\parallel} = 179 \times 10^{-4} \text{ cm}^{-1}$) (Addison, 1985). A similar spectrum has been observed for wt azurin at pH 2 (Ainscough *et al.*, 1987). The signal probably originates from partially denatured azurin in which the copper is coordinated by carboxylic or carbonyl groups and possibly water. This explanation is corroborated by IEF experiments performed after the spectroscopic experiments, which show an impurity band at the position of denatured holozurin. In order to check for possible structural differences between N47L and wt azurin, ¹H NMR spectra of both proteins in the reduced form were recorded (Fig. 4). Comparison of the two spectra revealed that no big structural changes have occurred due to the mutation. In fact the two spectra bear a surprisingly large similarity. The positions of a number of ligand resonances could be assigned by comparing spectra of reduced and slightly oxidized samples. In the presence of 8 μM oxidized azurin five resonances broadened significantly and clearly originated from ligand protons. Their assignments based on a comparison with wt data (Groeneveld *et al.*, 1988) are shown in Fig. 4.

Midpoint Potential—The midpoint potentials of wt and N47L were determined by static titration of reduced azurin with potassium ferricyanide (Goldberg and Pecht, 1976). For wt azurin a value of 286 (± 4) mV was found, and for N47L a value of 396 (± 1) mV (Table II) was found. Azurins as a class have midpoint potentials in the range of 270–370 mV (Adman, 1985), which indicates that the increase in midpoint potential for N47L is rather drastic. The values of the midpoint potential were checked by cyclic voltammetry. Porous glassy carbon as well as gold electrodes modified with pyridine-4-carboxaldehyde thiosemicarbazone (Hill *et al.*, 1985) gave good quasi-reversible reactions with wt azurin. The same experiments were performed for N47L, but the response of this protein was less reversible: the distance between the peak potentials was much larger than 59 mV, and the peak positions were less well resolved. Extracted parameters are shown in Table II.

We also determined the midpoint potential of N47L azurin as a function of temperature to determine the thermodynamic parameters of the reduction of the protein. Measurements were done at five temperatures between 10 and 40 °C by titration with potassium ferricyanide (Goldberg and Pecht, 1976). The results (Table III) show that the enthalpy change as well as the entropy change have increased considerably.

Redox Activity—The redox activity of N47L was probed by determining the self-exchange rate with NMR spectroscopy. The self-exchange rate (k) can be derived directly from the line broadening induced by addition of a small amount of oxidized azurin to a reduced azurin solution (Canters *et al.*, 1984; Groeneveld and Canters, 1988) according to the following relation:

$$\Delta T_2^{-1} = k[Az_{ox}]$$

ΔT_2^{-1} stands for the observed line broadening. Canters *et al.* (1984) and Groeneveld *et al.* (1988) have shown that the ligand resonances in azurins obey the conditions for this equation to be valid. Measurements on the resonance designated by peak 2 in Fig. 4 were performed with varying amounts of oxidized azurin between 0 and 50 μM . From these experiments a value of $0.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 25 °C for the self exchange rate for N47L azurin was extracted. This value lies in the same range as the electron self exchange rates of wt azurin and *P. aeruginosa* azurin (0.9×10^6 and $0.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively (Groeneveld and Canters, 1988; Groeneveld *et al.*, 1988)).

TABLE I
Parameters derived from UV-Vis and EPR spectra
for wt and N47L azurin

	λ_{\max}	$\epsilon_{\lambda_{\max}}$	$A_{\lambda_{\max}}/A_{280}$	g_{\parallel}	g_{\perp}	A_{\parallel}
	nm	$\text{M}^{-1} \text{ cm}^{-1}$				10^{-4} cm^{-1}
wt	619	5100	0.30	2.254	2.058	60
N47L	620	5100	0.30	2.246	2.057	60

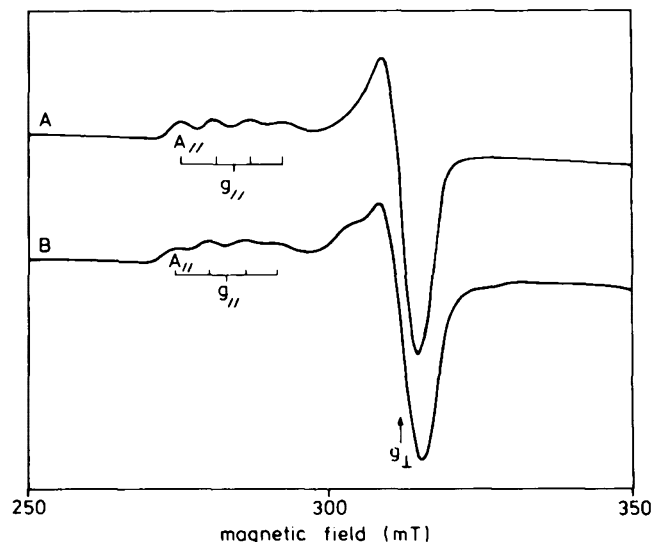


FIG. 3. X-band EPR spectra of 1 mM samples of wt (A) and N47L (B) azurin in 20 mM MOPS buffer, pH 7.0, with 40% glycerol at 77 K. EPR parameters are given in Table I.

FIG. 4. Resolution enhanced 300 MHz ¹H NMR spectra of 2 mM reduced wild type (a) and N47L azurin (b) in 20 mM phosphate buffer, pH 8.7, at 298 K. Positions of ligand resonances are indicated by 1–5. 1–4 are assigned to Cε1 or Cδ2 protons of His⁴⁶ or His¹¹⁷: 5.34, 6.78, 6.92, and 7.06 ppm for N47L azurin and 5.5, 6.78, 6.95, and 7.03 ppm for wt azurin (Groeneveld and Canters, 1988); 5 represents the CεH₃ of Met¹²¹: 0.56 ppm for N47L azurin and 0.53 ppm for wt azurin (Groeneveld and Canters, 1988).

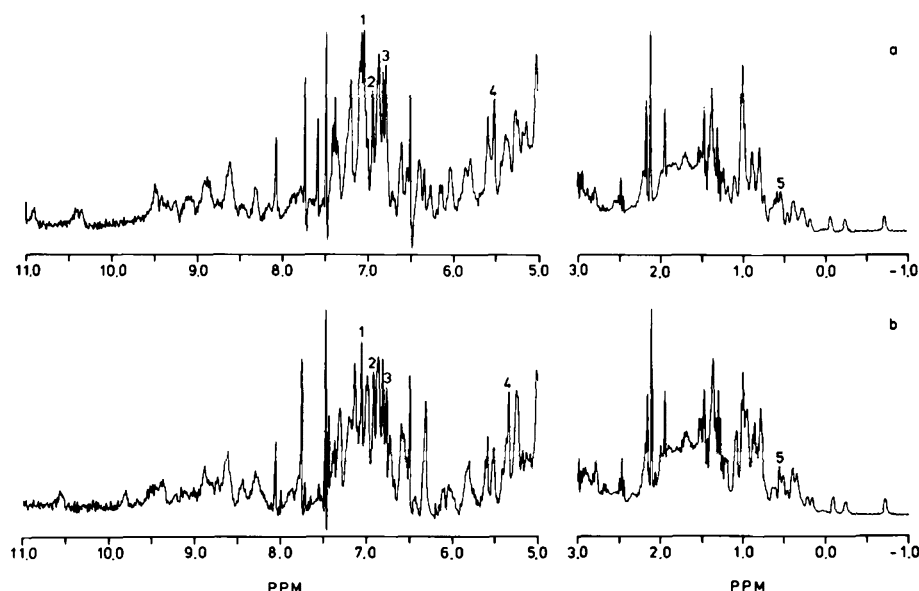


TABLE II

Midpoint potentials of wt and N47L azurin

In mV at pH 7.0 and 298 K, errors are given in parentheses.

	$E^{\circ a}$	$E^{\circ b}$	$\Delta E_p^{b,d}$	$E^{\circ c}$	$\Delta E_p^{c,d}$
wt	286 (4)	268 (2)	117	268 (2)	86
N47L	396 (1)	366 (8)	136	384 (8)	188

^a Calculated from titration of reduced azurin with potassium ferricyanide.

^b From cyclic voltammetry with glassy carbon electrode.

^c From cyclic voltammetry with gold electrode modified with pyridine-4-carboxaldehyde thiosemicarbazone.

^d Distance between peak potentials.

TABLE III

Thermodynamic parameters for the reduction of wild type and N47L azurin

All values with reference to the normal hydrogen electrode at pH 7.0 and 25 °C.

	$\Delta E/\Delta T$	ΔS°	ΔH°
	mV K ⁻¹	J K ⁻¹ mol ⁻¹	kJ mol ⁻¹
wt ^a	-0.33	-97.0	-55.6
N47L	-1.45	-205	-99

^a From Ainscough *et al.* (1987).

DISCUSSION

The present study shows that the *azu* gene of *A. denitrificans* can be expressed to a high level in *E. coli* under control of the *lac* promoter. To achieve this, the upstream sequence of the *azu* gene, which contains inverted repeat sequences, was deleted up to 50 nucleotides in front of the Shine-Dalgarno sequence. Azurin as isolated from *E. coli* cells is obtained in two forms. One form is apoazurin which can be reconstituted with copper. The other form, azurin*, is not reconstitutable and is likely to be zinc azurin, by analogy with heterologously expressed *P. aeruginosa* azurin from *E. coli*.² Fortunately, the holoazurin could be purified to homogeneity by making use of the different isoelectric points of zinc azurin and reduced copper azurin. Because the holoazurin/azurin* ratio appeared to depend on the growth time after induction, it is possible that zinc is slowly incorporated in azurin during the time the protein resides in the periplasm. It is known that

zinc in bacteria is more abundant than copper (Williams, 1983).

¹H NMR spectroscopy revealed that the overall structure of reduced N47L azurin is similar to the wt structure. In addition, when compared to the wt data, the positions of the ligand resonances have not shifted significantly, which indicates that the copper site structures are very similar in the reduced wt and mutant protein. The same holds for the structure of the oxidized copper site: the electron spin resonance spectra as well as the UV-visible spectra look very similar. It is known that azurins as a class exhibit a certain spread in their EPR parameters, especially in the g_{\parallel} values (Addison, 1985). Also the slight conformational change of the copper site of *P. aeruginosa* azurin brought about by lowering the pH from 9 to 5 results in noticeable changes in the EPR parameters (Groeneveld *et al.*, 1987). This spread and variation is much larger than the difference in EPR parameters observed here between the wt and N47L azurin. The conclusion seems justified, therefore, that the equilibrium structure of the azurin is not appreciably affected by a change in the hydrogen bonding pattern around residue 47.

This conclusion is reinforced by the results of the experiments on the redox activity of the wt and mutant proteins: the electron transfer rate of the N47L azurin appears to be as high as that of the wt azurin. Since it may be expected that the electron transfer rate is very sensitive to changes in the geometry of the copper site and the near by lying so-called hydrophobic patch (Baker, 1988), the constancy of the electron self-exchange rate confirms that the protein structure around the copper site is not influenced to a great extent by the N47L mutation. In view of the very strongly conserved character of Asn⁴⁷ in blue copper proteins this conclusion was totally unexpected.

However, there appear to be two features of the azurin that are clearly affected by the mutation: stability and midpoint potential. Although this was not investigated in quantitative detail, the decreased stability of the mutant manifested itself clearly in a reduced resistance to pH and temperature variations and to variations in redox state. It also was apparent from the low temperature at which denaturation of the mutant set in as compared to wild type. This decreased stability of N47L azurin is understandable. The azurin structure consists of eight β -strands which are connected by various shorter and longer loops. Asn⁴⁷ occurs at a crucial position in this structure

² H. Nar *et al.*, unpublished results.

where it assists in holding the two ligand containing loops (Fig. 1) together by hydrogen bonds (Cys¹¹² S γ ..HN 47, Asn⁴⁷ O δ 1..HN 113, Asn⁴⁷ N δ 2..OG Ser¹¹³ (Baker, 1988)). Replacement of this Asn by a Leu naturally weakens the thermal stability of the protein, as two hydrogen bonds are lost by the substitution. Alber *et al.* (1987) have shown that hydrogen bonds at the surface as well as in the interior of the protein play a role in the stabilization of the folded structure. The temperature at which the structural transition occurs in wt azurin is in good agreement with the results of differential scanning calorimetry experiments on *P. aeruginosa* azurin. The latter more elaborate experiments were performed under the same conditions considering protein concentration, pH, and buffer and revealed a value of 80 °C for the T_m of *P. aeruginosa* azurin (Engeseth and McMillin, 1986). The authors interpreted this irreversible transition as melting of the β -strands with concomitant loss of the blue copper site.

Equally interesting is the effect the mutation has on the midpoint potential. The redox titrations show that the midpoint potential of the N47L azurin has increased by 110 mV with respect to the wt. The cyclic voltammetry experiments confirmed these results although the electrochemistry of the mutant protein exhibited not as good a reversibility as the wt protein. For this reason the redox titration data are considered the more reliable and they have been used in the further analysis. The loss of reversibility in the electrochemistry of the N47L is possibly caused by a fouling up of the electrode surface by denatured protein which might promote radial diffusion at the expense of lateral diffusion.

It is interesting to notice that the change in midpoint potential is not brought about by the introduction or deletion of a single point charge, but by replacement of a neutral polar residue for a neutral nonpolar residue. Point charge effects on midpoint potentials as well as on pK_a values of titratable groups have been reasonably well accounted for by existing theories and simulation algorithms in a number of cases (Cutler *et al.*, 1989; Bashford *et al.*, 1986). One way to estimate the contribution from the partial charges on the Asn⁴⁷ side chain to the midpoint potential is to use simply Coulomb's law. We have used partial charges of +0.38, -0.38, and 0 for the C γ , O δ 1, and N δ 2 of Asn⁴⁷ (Hermans *et al.*, 1984; Van Gunsteren and Berendsen, 1987) and values of 5.59 and 6.41 Å for the distance of the copper to the Asn⁴⁷ O δ 1 and C γ , respectively. One then finds that the electrostatic interaction of the Asn⁴⁷ side chain with the extra charge that enters the copper upon reduction accounts for a contribution to the midpoint potential of from 110 to 30 mV depending on the choice of the effective dielectric constant in the range of from 1 to 4 (Harvey, 1989; Gilson and Honig, 1986). Although the crystallographic distances have a certain margin of uncertainty (± 0.1 Å) and the above calculation consequently cannot be considered very precise, the result shows that the presence of the dipole on the Asn side chain may explain at least partially the observed difference in midpoint potential between wt and N47L azurin. It remains to be seen how the calculated effect will be affected when the dynamics of the protein are taken into account.

The above calculation does not provide insight into the entropy effects seen on reduction. When using Coulomb's law the contribution of the entropy to the Gibbs free energy of reduction is hidden in the temperature dependence of the dielectric constant ϵ . Since ϵ is used here as an adjustable parameter ("effective dielectric constant") and its temperature dependence is unknown the entropic effect cannot be estimated from the temperature dependence of ϵ . Qualitative insight into the origin of the large difference in reduction

entropy between wt and N47L azurin may be obtained by looking at the electrostatic interactions again. In the oxidized form the metal center plus its ligands has a net charge of +1 (Cu(II) plus a negatively charged cysteinate, 2 neutral histidines and a neutral methionine side chain as ligands) and will have an electrostatic attractive interaction with the side chain of Asn⁴⁷. This will restrict the mobility of the β -strand containing Asn⁴⁷ and the ligand His⁴⁶ with respect to the loop that contains the other three ligands. Reduction of the copper abolishes this electrostatic interaction and may increase the internal degrees of freedom of the protein, giving rise to a positive contribution to the entropy of reduction. This effect is absent for N47L azurin and leads to a more negative entropy of reduction as compared to wt azurin, in accordance with observation. A quantitative account of the differences in reduction entropy has to await the results of a molecular dynamics simulation. The present case may qualify for such an analysis since the mutation has been realized in the interior of the protein, and solvent effects will play a less dominant role than in cases where a surface residue has been mutated.

It is worthwhile to point out that a similar effect on the midpoint potential has been observed for myoglobin where the replacement of a leucine for an asparagine caused a drop in midpoint potential of 82 mV (Varadarajan *et al.*, 1989). There the observed change in midpoint potential could be ascribed to an enthalpy effect. Equally interesting is that the effect reported in the present study, which is due to a mutation in the second coordination sphere of the copper, is larger than the effect (70 mV) that a mutation in the first coordination sphere of the copper has on the midpoint potential of *P. aeruginosa* azurin (Karlsson *et al.*, 1989). In that case, the ligand Met¹²¹ had been replaced by a leucine. The effects reported here will become amenable for theoretical analysis by means of molecular dynamics simulations once the three-dimensional structure of the N47L mutant has been reported.

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