Evidence for Two Distinct Azurins in *Alcaligenes xylosoxidans* (NCIMB 11015): Potential Electron Donors to Nitrite Reductase

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ABSTRACT: We have isolated two type 1 copper-containing proteins ($M_r \sim 13$K) from *Alcaligenes xylosoxidans* (NCIMB 11015) grown under denitrifying conditions. Amino acid sequence analysis of these two proteins shows one to be the previously identified azurin (Ambler, 1971), which we shall call azurin I, and the other to be a related, but previously undescribed, blue copper protein which we show to also be an azurin and propose to call azurin II. Thus, NCIMB 11015 becomes the second system where two distinct azurins are found, the other being *Methylomonas* J (Ambler & Tobari, 1989). On isoelectric focusing, azurin I migrates very similarly to the previously identified azurin from this organism while azurin II migrates similarly to azurin purified from *Alcaligenes denitrificans* NCTC 8582. The sequence of azurin II is 33% different than the azurin I sequence but is only 11% different than the azurin from *Alcaligenes denitrificans* NCTC 8582. Optical spectra for the two proteins are very similar with ϵ_{mM} values of 6.27 and 5.73 mM^{-1} cm^{-1} for azurin I and II, respectively, at λ_{max} ~620 nm. The 291 nm shoulder normally ascribed to the hydrophobic nature of tryptophan 48 is clearly observed in azurin I but is missing in azurin II. Amino acid analysis confirms that this tryptophan is missing in azurin II. Azurin I and azurin II show essentially the same redox potential of 305 ± 10 mV at pH 7.5 and are equally effective electron donors to the purified dissimilatory nitrite reductase of *Alc. xylosoxidans in vitro*. The two azurins crystallized distinctly; azurin I crystallizes as thin needles while azurin II grows as blue elongate rectangular prisms with the tetragonal space group $P4_122$ and unit cell parameters $a = b = 52.65$ Å, $c = 100.63$ Å. The structure of azurin II has been solved and is currently being refined to 1.9 Å resolution.

Denitrification is the anaerobic respiratory pathway by which some bacteria carry out the stepwise reduction of nitrate via nitrite to the gaseous end products N_2O and N_2 (Payne, 1985). A key step in this process is the reduction of nitrite to yield nitric oxide (NO). In the bacterium *Alcaligenes xylosoxidans* (formerly known as *Alcaligenes* sp. NCIMB 11015, *Achromobacter xylosoxidans*, or *Pseudomonas denitrificans*), the enzyme responsible for this reduction is a copper-containing nitrite reductase. Two classes of blue copper proteins, pseudoazurin and azurin, distinguishable by their amino acid sequence and optical spectrum, have so far been implicated in donating electrons to nitrite reductase: pseudoazurin to the green nitrite reductases of *Alc. faecalis* S-6 and *Achr. cycloclastes* (Kakutani *et al.*, 1981; Liu *et al* 1986) and azurin to the blue enzyme of *Ps. aureofaciens* (Zumft *et al.* 1987). However, a "blue copper protein" (probably azurin I) isolated from *Alc. xylosoxidans* was shown not to function as an electron donor to the blue nitrite reductase isolated from *Alc. xylosoxidans*, but cytochrome c_{552} from this organism did function in a coupled assay using yeast lactate dehydrogenase as a primary source of electrons (Miyata & Mori, 1969).

The X-ray crystal structures of two bacterial azurins have been reported, one from *Pseudomonas aeruginosa* (Adman & Jensen, 1981) and the other from *Alc. denitrificans* (Norris *et al.*, 1983; Baker, 1988). The availability of site-directed mutants of both of these azurins has generated new interest in studying these small model electron transfer proteins (Farver, *et al.*, 1993), and led to a number of structural studies of these mutated proteins (Nar *et al.*, 1991; Murphy *et al.*, 1993). An azurin from *Alc. xylosoxidans* has been crystallized (Strahs, 1969) and was in fact the first copper protein to be crystallographically characterised (cell dimensions and space groups were determined). Despite several attempts, crystallization (Norris *et al.*, 1979) and structure determination of this protein have remained problematical; only the tertiary structure at 3.0 Å resolution has appeared from a solution based on anomalous dispersion (Korzun, 1987). However, no atomic details of this structure have emerged.

In this paper, we show that *Alc. xylosoxidans* is capable of synthesizing two biochemically distinct azurins, and that both of these proteins can donate electrons to purified nitrite reductase. The *N*-terminal amino acid sequence of one of these proteins is identical to the azurin purified and sequenced by Ambler (1971) while the other shows a closer similarity

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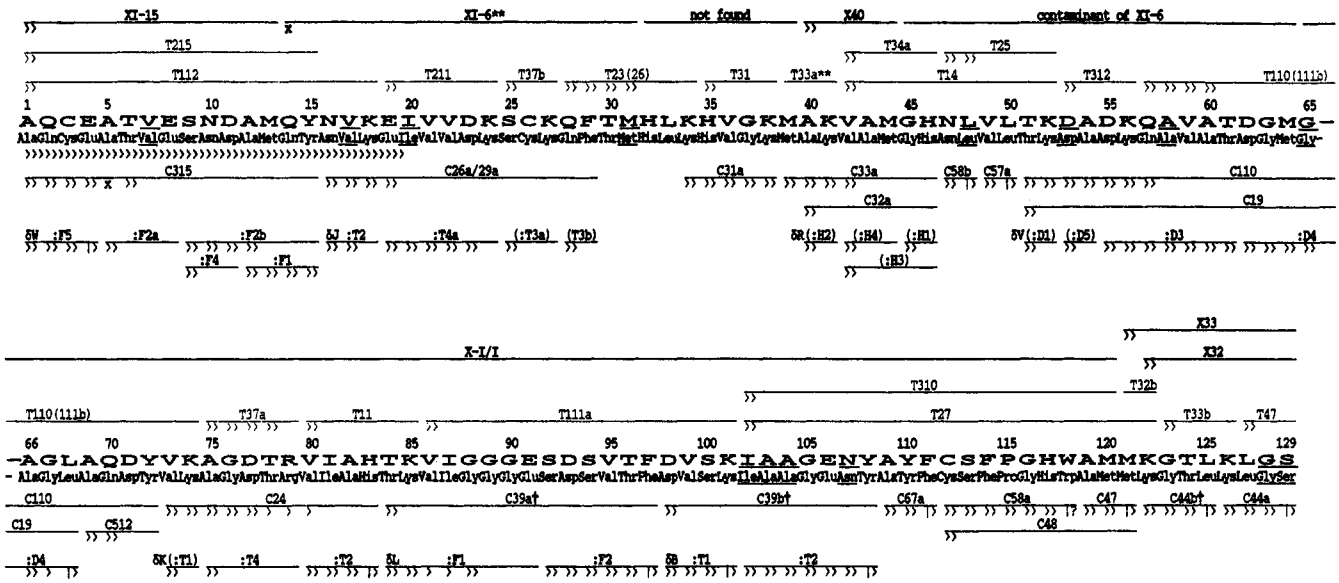

FIGURE 1: Peptides are named with a letter (for method of formation) and numbers and auxiliary letters. The methods of formation used were cleavage with the following: X, CNBr; C, chymotrypsin; T, trypsin; H, thermolysin; F, staphylococcal protease ("Glu-C"); D, pseudomonad protease ("Asp-N"). Automatic sequencer results are shown by >>>>>> below the protein sequence. Under peptide bars: >>, residues determined by dansyl-Edman method; |>, last residue identified as free amino acid. Peptides examined by treatment with carboxypeptidase A are marked †. Peptides that gave substandard quantitative amino acid analyses are marked *, while peptides whose presence was identified by amino acid analysis, although they were still impure are marked **. Small peptides that were only analyzed qualitatively for amino acid composition are named in parentheses. Residues that are different from the sequence of the azurin from *Alcaligenes denitrificans* NCTC 8582 are underlined.

to the azurin from *Alc. denitrificans*. A complete sequence analysis of azurin II is presented. We also present preliminary X-ray crystallographic and other spectrochemical data for both of these azurins.

MATERIALS AND METHODS

Organism and Growth. Alcaligenes xylosoxidans subsp. xylosoxidans 11015 was obtained from the NCIMB culture collection, and grown under denitrifying conditions as described previously (Abraham *et al.*, 1993).

Preparation of Samples for Amino Acid Sequencing. SDS−PAGE (15% gel) was carried out by the method of Laemmli (1970) with the minor modifications described in Bagby *et al.*, (1991). The gel was stained with Coomassie blue quick stain for 1 h and destained until the protein bands could be distinguished. A gel slice was taken containing the protein of interest and incubated in 0.1% sodium acetate, pH 8.5, 0.1% SDS overnight at 37 °C. The buffer containing the protein was then removed and freeze-dried. The resulting residue was resuspended in trifluoroacetic acid (0.1%) and the sample washed with this solution by centrifugation in a ProSpin column (Applied Biosystem) 3 times before *N*-terminal sequence analysis using a gas phase Applied Biosystems 470 sequenator equipment with a 120 on-line analyzer.

Isoelectric Point Determination. The isoelectric points (p*I*) of azurin I and azurin II were determined using a Pharmacia PhastSystem over a pH range of 3−9. The electrophoresis gel was calibrated with a Pharmacia isoelectric focusing calibration kit consisting of 11 proteins of known p*I*.

Molecular Weight Determination. The apparent M_r's of the polypeptides of azurin I and azurin II were determined by SDS−PAGE by comparison of the rates of migration with proteins of known M_r. The M_r of the protein polypeptides was also determined by electrospray mass spectrometry. The proteins were dialyzed against water before analysis and were run with myoglobin as standard on a FISONS VG platform by Dr. John Firmin of the John Innes Centre at Norwich, U.K. The native M_r was measured by gel filtration chromatography on an FPLC Superose-12 column (Pharmacia) equilibrated with 50 mM phosphate buffer, pH 7.0, 50 mM NaCl, and 10% v/v ethylene glycol. The column was calibrated with six standard proteins of known M_r before samples of each azurin were applied.

Metal Analysis. Proteins (1.5 mg) were dialyzed against 25 mM HEPES buffer, pH 7.5, before wet ashing, and the copper and zinc contents were determined by plasma emission spectroscopy by Southern Science Ltd., Brighton, U.K. A similar volume of buffer was digested to provide blanks.

Amino Acid Sequence Determination. An initial automatic sequence analysis had indicated that azurin II was similar but not identical to the azurin from *Alc. denitrificans*. The remainder of the sequence was then determined by standard methods, which have been described elsewhere (Ambler & Wynn, 1973; Ambler *et al.*, 1979, 1984). Samples of 3 μmol of protein were oxidized with performic acid and then digested with trypsin or chymotrypsin, and the peptides were separated and characterized (Figure 1). A smaller quantity was also examined by cyanogen bromide cleavage.

Electron Donation Experiments. Reduced azurins were prepared, in an anaerobic chamber under N_2 containing ≤1 ppm of O_2, by the addition of sufficient 100 mM $S_2O_4^{2-}$ to bleach the blue color. Excess reductant and oxidation products were then removed by gel filtration on a column of Biorad P6DG (0.5 × 8 cm) equilibrated with 25 mM HEPES buffer, pH 7.5. Azurin I was reduced more slowly than azurin II under these conditions, and on subsequent exposure to air, reoxidized more rapidly than azurin II. The reduced proteins were removed from the chamber in a vial

Table 1: Purification of Azurin I and Azurin II from *Alcaligenes xylosoxidans* NCIMB 11015

	azurin I				azurin II			
purification step	ratio, 280/620	vol (mL)	protein concn (mg/mL)	total protein (mg)	ratio, 280/620	vol (mL)	protein concn (mg/mL)	total protein (mg)
CM-cellulose	7.4	480	0.3	144	5.1	240	0.8	192
$(NH_4)_2SO_4$ pptn & dialysis	10.8	46	2.1	92	4.9	25	6.1	152
CM-cellulose	2.8	37	1.5	57	3.5	35	3.4	119
$(NH_4)_2SO_4$ pptn & dialysis	2.8	7	8.0	56				
Sephadex G-50 & dialysis					3.7	25.5	4.5	115

capped with a rubber closure. The assay mixture under N_2 in a 1 cm cuvette contained azurin at the concentrations indicated, 25 mM HEPES buffer, pH 7.5, and 10 mM $NaNO_2$ in a final volume of 0.51 mL. The reaction was initiated by the injection of purified nitrite reductase, 10 μL (0.3 μM final concentration), and the increase in absorbance at 620 nm was monitored using a Shimadzu MPS 2000 spectrophotometer.

Determination of Midpoint Redox Potentials. Redox potentials of both azurin I and azurin II were determined in 25 mM HEPES buffer at pH 7.5. Measurements were made in a 1 cm anaerobic spectrophotometric cell fitted with calomel and Pt electrodes. Titration mixtures contained buffer, potassium ferricyanide (0.5 mM), phenazine methosulfate (0.5 mM), and azurin I (0.16 mM) or azurin II (0.18 mM) in an initial volume of 3.57 mL. Oxidative titrations were performed by the addition of potassium ferricyanide, and reductive titrations by the addition of phenazine methosulfate. The A_{620} at various measured potentials was used to determine the redox state of the azurins.

RESULTS AND DISCUSSION

Isolation of Azurins. During ion exchange chromatography of nitrite reductase on (carboxymethyl)cellulose (CM-cellulose) [step 3 of Abraham *et al.*, (1993)], two bands in addition to nitrite reductase were observed. To obtain better resolution of these species, this step was modified. After the ammonium sulfate fractionation (step 2), 30−100% fraction containing the azurins was extensively dialyzed against water. The CM-cellulose column (9 × 20.5 cm) was equilibrated with 20 mM Tris-HCl buffer, pH 7.0, and then washed with water (1 L) before protein was applied. On the addition of 25 mM NaCl to the elution buffer (5 mM Tris-HCl buffer, pH 7.0), the first blue band (cut 1, azurin I) was eluted. On addition of 50 mM NaCl to the buffer, a second blue band (cut 2, azurin II) was eluted in front of a cytochrome-containing band and the nitrite reductase. Cuts 1 and 2 were concentrated separately by precipitation with 100% ammonium sulfate (w/v); the resulting precipitates were resuspended in water and then dialyzed extensively against water before applying each separately to a second CM-cellulose column (2.5 × 12 cm), which had been equilibrated with 50 mM Tris buffer, pH 7.4, and then washed with water. In each case, the blue protein fraction was eluted with 20 mM Tris-HCl buffer, pH 7.4, in front of a contaminating cytochrome fraction. The absorbance spectrum of cut 1 showed that this protein was no longer contaminated with cytochrome, whereas cut 2 required an additional gel filtration step on a Sephadex G-50 column (2.5 × 50 cm) equilibrated with 50 mM Tris buffer, pH 7.4, containing 25 mM $MgCl_2$ to remove cytochrome. These procedures, after which both proteins gave a single band on SDS−PAGE, are summarized in Table 1.

The relative yield of these two low M_r blue proteins was 2:1, with cut 2 predominating. There are precedents for the synthesis of two azurins in a single bacterial species depending on the growth conditions. For example, methylotrophs, when grown on methylamine or methanol, synthesize amicyanin (the electron acceptor for methylamine dehydrogenase) and, depending on the species, also synthesize azurin or pseudoazurin (Tobari, 1984; Lawton & Anthony, 1985). *Methylomonas J* synthesizes two distinct azurins, only one of which is able to function as the primary acceptor to methylamine dehydrogenase (Ambler & Tobari, 1989). The levels of azurin/pseudoazurin were enhanced when additional copper was added to the growth medium. In the present case, the crude extracts of *Alc. xylosoxidans* have copper added to them to activate nitrite reductase (Abraham *et al.*, 1993). However, we also observe both azurin I and azurin II when NiR is purified from extracts which have not been activated by Cu. The failure of others, who did not activate their crude extracts to observe more than a single species of azurin in *Alc. xylosoxidans* grown under denitrifying conditions (Suzuki & Iwasaki, 1962), is thus apparently not due to one of these species being an apoprotein when Cu is not added. Sequence determination experiments done in 1965 (Ambler, unpublished results) on material that was a gift from Dr. Iwasaki can now be interpreted as showing that his preparations contained both azurin I and azurin II, as peptides corresponding to the C-terminal CNBr fragments of the two proteins were identified. The protein subsequently prepared in Edinburgh (Ambler, 1973) behaved as pure azurin I, and no extraneous peptides were seen in it (Ambler, 1971). The culture was deposited by one of us (R.P.A.) to NCIMB in 1973. In the present case, the culture was obtained directly from NCIMB. The observations of traces of the second sequence in the pre-1965 material supplied by Dr. Iwasaki and the evidence presented here strongly suggest that the culture that reached NCIMB in 1973 was the same as that grown in Japan in the 1960's.

Spectrochemical Properties of the Azurins. The visible absorption spectra of the purified azurins from *Alc. xylosoxidans* show that both proteins have a single absorption maximum at ~620 nm (619 and 622 nm, for azurin I and azurin II, respectively), which is characteristic of the azurin class of small blue copper proteins. Pseudoazurins have additional absorption bands near 450 and 750 nm. The extinction coefficients at ~620 nm (619 and 622 nm) are 6.27 and 5.73 mM^{-1} cm^{-1} for azurin I and azurin II, respectively; these are within the range normally found for other azurins and significantly lower than the value of 10.5 mM^{-1} cm^{-1} reported for the blue protein isolated from *Alc. xylosoxidans* by Suzuki and Iwasaki (1962). The extinction coefficients for the two proteins at 280 nm are 17.76 and 21.18 mM^{-1} cm^{-1}, respectively.

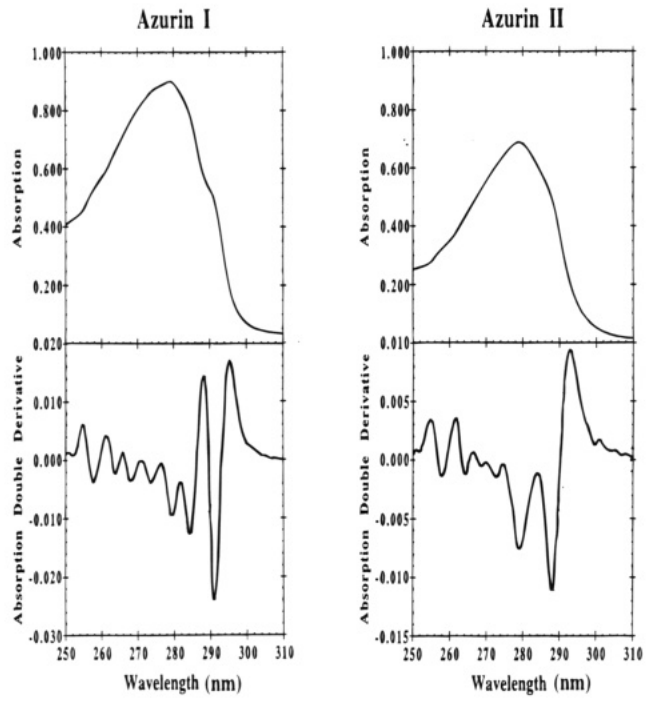

FIGURE 2: The UV spectra of the purified azurins. Azurin I has a distinct shoulder at ~290 nm. This difference is highlighted in the derivative spectra given as insets.

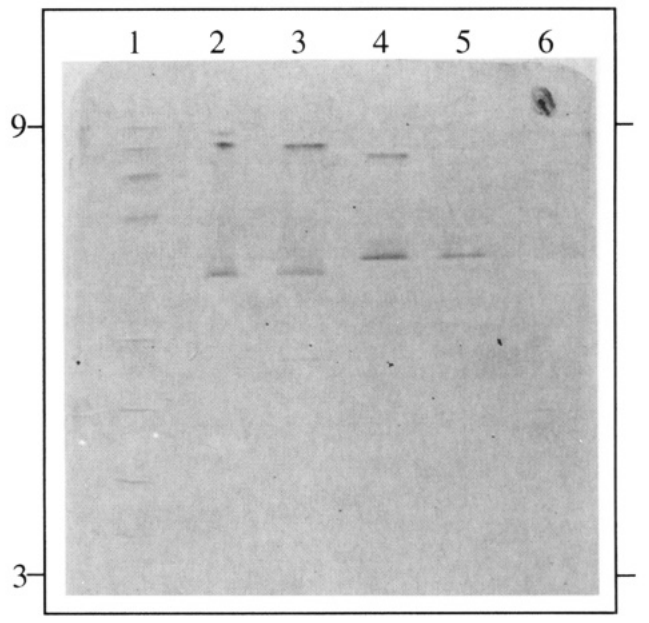

FIGURE 3: Isoelectric focusing gels corresponding to azurin I (lane 2) and azurin II (lane 4). For comparison, isoelectric runs of azurin from *Alc. dentrificans* (lane 5) and azurin from *Alc. xylosoxidans* (lane 3), as prepared by Ambler for original sequence determination, are also shown.

A comparison of the UV absorption spectra of the two proteins (Figure 2) shows that azurin I has a shoulder at 291 nm which is absent in azurin II. This shoulder has long been assigned to arise from the hydrophobic environment of tryptophan 48 in azurins (Finazzi-Agro *et al.*, 1970). Its disappearance has been associated with the denaturing of the protein. The differences in the UV spectra of the two azurins indicate differences in the environment of aromatic amino acid residues that these two proteins contain or a difference in the nature of residue 48. The derivative UV spectra of the two azurins were used as a convenient method to distinguish between them during purification.

Both azurin I and azurin II ran as two bands of similar intensity on isoelectric focusing. Figure 3 shows the bands corresponding to azurin I and azurin II. Also shown are the isoelectric runs of azurin from *Alc. denitrificans* and azurin from *Alc xylosoxidans*, as prepared by Ambler for original sequence determination (Ambler, 1971). Azurin I runs identically to originally sequenced azurin from this organism (p*I* 8.8 and 7.1) while azurin II (p*I* 8.6 and 7.3) runs similarly to azurin from *Alc. denitrificans* (p*I* 7.3, sometimes this also shows the second band at p*I* 8.6). The reason for two bands is not clear, but it may be due to a different overall charge of the molecule due to the presence of either an apoprotein or a Zn-substituted protein. The amino acid sequence shows that it is not due to contamination. Indeed, the electromass spectrometric measurements also showed that the proteins are very clean.

The molecular weights of the two azurins were determined by a variety of methods. SDS−PAGE and gel filtration gave average M_r's of 13.5 kD and 12.5 kD for azurin I and azurin II, respectively. The electrospray mass spectrometric measurements gave M_r's of 13 941 and 13 772 D for azurin I and azurin II, which compared well with amino acid sequence analysis, 13 940 D for azurin I and 13 759 D for azurin II.

Metal analyses of the two proteins showed the presence of both Cu and Zn. The preparations of azurin I described here contained 0.73 Cu atom/mol and 0.27 Zn atom/mol, and those of azurin II contained 0.78 Cu atom/mol and 0.24 Zn atom/mol. These values are consistent with full occupancy of the metal-binding site in both proteins with either Cu or Zn.

EPR spectra of the oxidized proteins at pH 7.4 showed characteristics typical of type 1 copper centers and were very similar except for a small difference in the $A_{||}$ values which were 55 G for azurin I and 57 G in azurin II. The midpoint potentials for both azurins are the same within experimental error at 305 ± 10 mV, which is significantly different from that of 230 mV reported by Susuki and Iwasaki (1962) for the azurin from this organism.

N-Terminal Sequence and Structural Implications. The results of *N*-terminal amino acid analysis for both azurins were compared with that previously determined by Ambler (1971) for azurin I from this organism and the sequences of pseudoazurin from *Alc. faecalis* S-6 and azurin from *Alc. denitrificans*. It was evident that azurin I is identical in its *N*-terminal amino acid sequence to that of the protein sequenced by Ambler (1971). However, the protein we have designated azurin II is clearly a different protein. Azurin II is not a pseudoazurin because it contains a cysteine residue at position 3, which is highly conserved among the azurins and is involved in the formation of the disulfide bridge characteristic of the azurin family. Azurin II shows a strong homology (see below) to the amino acid sequence found in the azurin isolated from *Alc. denitrificans* (Ryden & Lundgren, 1976).

Amino Acid Sequence of Azurin II and Structural Implications. In view of the close similarity of the *N*-terminus of azurin II with the *N*-terminus of azurin from *Alc. denitrificans* and the substantial difference with azurin I, it was decided to undertake a full sequence analysis. As expected, the complete sequence of azurin II shows a close identity (89%) to azurin from *Alc. denitrificans*. Figure 4 compares the amino acid sequence of azurin II with azurin I and azurins

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Alc. xylosoxidans AzI   AECSVDIAGNDQMQFDKKEITVSKSCKQFTVNLKHPGKLAKNVMGHN
Ps. aeruginosa          AECSVDIQGNDQMQFNTNAITVDKSCKQFTVNLSHPGNLPKNVMGHN
Alc. xylosoxidans AzII  AQCEATVESNDAMQYNVKEIVVDKSCKQFTMHLKHVGKMAKVAMGHN
Alc. denitrificans      AQCEATIESNDAMQYNLKEMVVDKSCKQFTVHLKHVGKMAKVAMGHN
                        *.§   . .** **.. . . * **§****..* * *...*  **#*

Alc. xylosoxidans AzI   WVLTKQADMQGAVNDGMAAGLDNNYVKKDDARVIAHTKVIGGGETDS
Ps. aeruginosa          WVLSTAADMQGVVTDGMASGLDKDYLKPDDSRVIAHTKLIGSGEKDS
Alc. xylosoxidans AzII  LVLTKDADKQAVATDGMGAGLAQDYVKAGDTRVIAHTKVIGGGESDS
Alc. denitrificans      WVLTKEADKQGVATDGMNAGLAQDYVKAGDTRVIAHTKVIGGGESDS
                         **. ** *.   *** .** ..*.* .*.******.**.** **

Alc. xylosoxidans AzI   VTFDVSKLAAGEDYAYFCSFPGHFALMKGVLKLVD
Ps. aeruginosa          VTFDVSKLKEGEQYMFFCTFPGHSALMKGTLTL-K
Alc. xylosoxidans AzII  VTFDVSKIAAGENYAYFCSFPGHWAMMKGTLKLGS
Alc. denitrificans      VTFDVSKLTPGEAYAYFCSFPGHWAMMKGTLKLSN
                        *******.  ** * .*#.***# *.#** * *
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FIGURE 4: Comparison of the amino acid sequence of azurin II with azurin I is made with azurins from *Alc. denitrificans and ps. aeruginosa*. Azurin's I and II show 68.2% sequence identity. The following coding is used: residues which are common to these four azurins are marked by *, similar residues are marked by ●, cysteines forming the disulfide are marked as §, and ligands to Cu are marked by #.

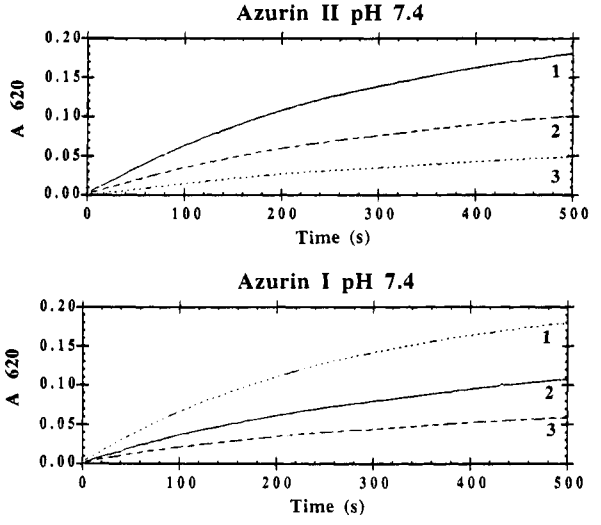

FIGURE 5: Progress curves measured at 620 nm for azurin I and azurin II in the presence of catalytic amounts of nitrite reductase.

from *Alc. denitrificans and Pseudomonas aeruginosa*. Azurin II reveals a number of interesting substitutions; e.g., W-48 is replaced by leucine and hence the absence of the ~291 nm shoulder. Azurins have a tryptophan at only positions 48 and 118, with a single tryptophan at position 48 being the most common situation. Two of the *Alcaligenes* species (NCTC 8582 and two *Alc. faecalis* strains) have W-118 with NCTC having both W-48 and W-118. The other notable changes with respect to NCTC's sequence are at residues 129, 102−104, 57, and 7.

The amino acid sequence of azurin II differs from that of azurin I in 43 out of the 129 residues in the proteins. Only one of the generally conserved positions is changed in azurin II, with Ile-102 occurring rather than leucine. However, the protein only differs from the azurin from *Alc. denitrificans* NCTC 8582 in 14 positions, which is close similarity for electron transport proteins from different bacterial species (Meyer *et al.*, 1986). The taxonomic positions of strains NCICB 11015 and NCTC 8582 are discussed below.

Electron Donation to Nitrite Reductase. The ability of azurins I and II to function as electron donors to nitrite reductase was compared spectrophotometrically using reduced, but reductant-free, proteins. Their rates of nitrite-dependent oxidation were determined from the increase in absorbance at 620 nm in the presence of catalytic amounts of nitrite reductase (Figure 5). Both proteins functioned effectively as electron donors to nitrite reductase, the progress curves were biphasic, with an initial linear region followed by an extended nonlinear phase. In the concentration range 37−140 μM, the initial rate of oxidation was linear with the concentrations of both azurin I and azurin II. Our results are in disagreement with a previous study which reported that a small blue copper protein isolated from this organism did not donate to nitrite reductase, and that cytochrome *c*-552 was the physiological electron donor (Suzuki & Iwasaki, 1962, Miyata & Mori, 1969). However, we note that the nitrite reductases used in the earlier studies were not fully activated and lacked type 2 Cu (Abrahams *et al.*, 1993; Grossmann *et al.*, 1993).

The ability of purified small blue copper proteins to donate electrons to the respective purified copper-containing nitrite reductase has been examined for a variety of denitrifying bacteria. Copper-containing nitrite reductases are colored because of the type 1 copper centers that they contain, and are blue or green depending on the ratio of the absorbance bands at ~460 and ~596 nm, presumably arising from detailed differences in the geometry of the ligation at the type 1 Cu site. In *Achr. cycloclastes* (Liu *et al.*, 1986) and *Alc. faecalis* (Kakutani *et al.*, 1981), pseudoazurins have been shown to donate electrons to green copper-containing nitrite reductases, whereas in *Ps. aureofaciens* it is azurin which donates electrons to a blue copper-containing nitrite reductase (Zumft *et al.*, 1987). It has also been reported that the pseudoazurin from *Achr. cycloclastes* does not donate electrons to the green copper-containing nitrite reductase from *Bacillus halodenitrificans*, for which no natural electron donor has yet been identified from the organism (Denariez *et al.*, 1991). Thus, it would appear that small blue copper proteins show, at least, some specificity in their interaction with purified nitrite reductases.

Preliminary Crystallographic Data for Azurin I and Azurin II. Crystals of the two azurins were grown using the hanging-drop technique. In a typical experiment, 3 μL of buffered protein solution (10 mg ml^{-1}) was mixed with 3 μL of reservoir buffer over 1 mL reservoirs. Crystals of an azurin from *Alc. xylosoxidans* have been obtained before (Strahs, 1969; Korszun, 1987) with very similar cell dimensions as we have determined for azurin II. Crystals of azurin I, buffered with 0.1 M sodium acetate, pH 5.6, originally formed stacks of very thin plates, but recently, high-quality needles (0.015 × 0.7 mm) have appeared. These grew over a reservoir of 560 μL of saturated ammonium sulfate (aqueous) and 440 μL of 0.1 M sodium acetate, pH 5.6, in ca. 12 weeks. Azurin II crystals were grown from 0.1 M KH_2PO_4, pH 6.0, over a reservoir containing 620 μL of saturated ammonium sulfate (aqueous) and 380 μL of 0.1 M KH_2PO_4, pH 6.0. Single crystals were obtained within 7 days; these form elongate rectangular prisms. The largest crystals were 0.1 × 0.1 × 0.5 mm.

Diffraction data were taken from crystals of azurin II using a Rigaku rotating-anode source and a Rigaku R-Axis IIc oscillation camera (Sato *et al.*, 1992) at the Department of Chemistry, University of Manchester. CuKα X-rays were used at a crystal to image plate distance of 170 mm. Oscillation data were collected over 84° to ~2.8 Å resolution. The data were auto-indexed and reduced using the dedicated software (Higashi, 1990; Sato *et al.*, 1992). The unit cell parameters were refined as $a = b = 52.65$ Å, $c = 100.63$ Å, and the space group was considered to be $P4_122$ or $P4_322$ based upon the systematic absences. The data reduction gave

a merging *R* factor of 13%.

A high-resolution diffraction data set to 1.9 Å was collected using the Weissenberg camera (Sakabe, 1991) at beamline 6A-2 at the Photon Factory, using an X-ray wavelength of 0.970 Å with a crystal to image plate radius of 429.7 mm. The data were reduced using Weis (Higashi, 1989) and then scaled and merged using the CCP4 suite (CCP4, 1994), giving a merging *R* factor of 4.0% to 1.9 Å for 43 645 reflections with 7212 unique reflections. These data have a completeness of 62% to 1.9 Å. The structure determination of azurin II by molecular replacement using azurin of *Alc. denitrificans* as a search model (Baker, 1988) has provided a consistent solution with the *AMoRe* (Navaza, 1994) package in the space group $P4_122$. The quality of this initial electron density map has been sufficiently good to recognize the amino acid substitutions at positions 7, 20, 48, 103, and 104 prior to the information from amino acid sequence analysis became available. Further details of this work will be published elsewhere (Dodd *et al.*, 1995).

Alcaligenes Taxonomy. The genus *Alcaligenes* was named by Castellani and Chalmers (1919), but became a repository for undistinguished Gram-negative saprophytes, some of which also appear named *Achromobacter*. In the last 10 years, the techniques of nucleic acid hybridization have been brought to bear on the group (De Ley *et al.*, 1986), many organisms have been rejected from the genus, and the accepted strains have been clustered.

The organism from which the two azurins have been purified was isolated as a denitrifying organism from soil in Japan (Iwasaki & Mori, 1955). The organism was tentatively identified as *Pseudomonas denitrificans*, but further examination showed that it should be put in the *Alcaligenes/Achromobacter* group, and it was deposited in the culture collection as *Alcaligenes* sp. NCIMB 11015. De Ley *et al.*, (1986) put it in a group they called *Alc. xylosoxidans* subsp. *xylosoxidans*, close to strain NCTC 8582 (Figure 2), which they now call *Alc. xylosoxidans* subsp. *denitrificans*. They associate *Alc. xylosoxidans* in a family of which the other known members are the *Bordetella* species (the causative organisms of whooping cough), and the type strain of *Alcaligenes faecalis*. All these organisms produce azurins (Sutherland & Wilkinson, 1963), though the sequences of the azurins (Ambler, 1968) are not clustered together to the exclusion of those from denitrifying pseudomonads. Some organisms called *Alcaligenes faecalis* (e.g., strain S-6; Hormel *et al.*, 1976) are almost certainly misnamed, as they produce a pseudoazurin rather than a true azurin.

CONCLUSION

We show here that *Alc. xylosoxidans* is capable of producing two biochemically distinct azurins, both of which can donate electrons to the purified Cu-containing blue dissimilatory nitrite reductase from this organism. Contamination of bacterial cultures is always a possible explanation of unexpected results, but we believe that the evidence we have presented here makes it unlikely that our finding of two distinct azurins in NCIMB 11015 is caused by it being a mixed culture. The finding of peptides corresponding to the *C*-terminal CNBr fragments of two azurins in the original material supplied to one of us (R.P.A) by Dr. Iwasaki further makes the possibility of contamination very unlikely. The azurin II crystallizes in the same space group with strikingly similar cell dimensions to those observed by Strahs (1969) and Korszun (1987). Azurin I crystallizes with distinct morphology from significantly different crystallization conditions to azurin II. The *N*-terminal sequence of azurin I is identical to that reported by Ambler (1971) for an azurin from this organism. Azurin II is not a pseudoazurin due to the presence of Cys-3 and the lack of 450 and 750 nm bands, and its sequence shows high homology to azurin from *Alc. denitrificans*. The question why the organism produces two electron donor proteins with similar redox potentials is interesting and can only be speculated upon at this stage. The close similarity of azurin II to the sole known azurin from NCTC 8582 does raise the question whether the latter organism also has the other azurin. Furthermore, the close taxonomical relationship between *Alc. xylosoxidans* 11015 and *Alc. denitrificans* 8582 would suggest a similar relationship between the other proteins of the two organisms.

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