The Expression of the Gene for Azurin from *Alcaligenes Denitrificans* in *E. coli*

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Joseph Anthony Bateson

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Abstract

Azurin is a protein which funtions in electron transport and has been found to bind copper when it is expressed in its native bacterial host. In this thesis the azurin from *Alcaligenes denitrificans* was used. This protein is 129 amino acids long with a molecular weight of 14, 600 daltons. The azurin coding gene from *Alcaligenes denitrificans* had previously been cloned into a plasmid which allows an *E. coli* expression system to be used.

Azurin was purified from the *E. coli* hosts using the same procedures as for purifying copper-azurin from the native hosts but was found to remain apparently impure, according to spectrophotometric data. Efforts to increase the production of the protein by using different expression systems and by refining the existing expression system failed to increase the apparent yield of copper-azurin. Efforts to refine the purification procedure also failed to increase the amount of copper-azurin that was purified. Various experiments were performed to demonstrate that azurin was expressed and processed correctly in the *E. coli* host.

Protein was expressed in a copper-rich and copper-sparse environment. Copper-azurin was purified from the copper-rich environment, while very little copper-azurin could be extracted from the copper-sparse environment.

The results described in this thesis suggest that when azurin from *A. denitrificans* is expressed in an *E. coli* host using standard media with no copper added, the predominant form of azurin produced in zinc-azurin. As mutants are going to made of this protein, conditions where the protein would bind only copper were required. The ideal conditions for this are still to be calculated but results from this thesis would suggest that copper concentrations in the region of 0.25 mM lead to 65% incorporation of copper, compared to 17% when no copper is added to the *E. coli* growth medium. *E. coli* cells were shown to grow with no apparent inhibition of growth in 3.0 mM of $CuSO_4$. This concentration of copper in the growth medium may allow the production of a much higher ratio of copper-azurin compared to zinc-azurin than has been achieved so far.

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And if you've taken the time to read this, well then....thank you!

A masters requires labour, patience and at times forces one to think,
And its of the later point that I'd like to acknowledge zinc.
For it confused us all and left us to a person without a clue,
As to why my blue copper protein was anything but blue.

A special thanks to Lois and my housegroup fellowship,
For support, encouragement and all round great friendship.
And finally the biggest thanks of all, and who else should get the last word,
Thank you Jesus Christ, my friend, my brother. Jesus Christ my Lord!

God Bless.

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Chapter 1 : Introduction

Azurin is a metalloprotein which binds copper and functions as an electron carrier in the electron transport chain of several bacteria including members of the genera *Alcaligenes, Pseudomonas, Neisseria, Methylomonas* and *Bordetella.*

1.1 METAL BINDING PROTEINS

Many proteins bind one or more metal ions as part of their structure. These proteins are termed metalloproteins, and include metalloenzymes, which have catalytic activity, respiratory proteins, in which the metalloprotein acts as an oxygen carrier, and electron transfer proteins. There are also several metalloproteins which act as metal-storage proteins and in some instances metal-binding proteins have the function of sequestering toxic metals in a form which is less harmful to the organism (Cotton, 1988; Branden, 1979; Otsuka, 1988). A summary of metals known to be associated with proteins are shown in Table 1.1 (compiled using information from Otsuka, 1988).

There is a great deal of interest as to the nature of metalbinding in proteins. The amino acid residues which act as ligands and the manner of the ligand interactions which result in the unique spectral properties possessed by many of these proteins are of interest. For many years spectral and synthetic analogue studies were the most successful methods for obtaining information about the metal-binding site of a metalloprotein but more recently nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR) and X-ray crystallography have been employed. These structural studies have been coupled with site-directed mutagenesis of the native proteins.

Three dimensional protein structures solved by X-ray crystallography are available for several metalloproteins. Zinc, iron and copper binding proteins have been especially well studied and characterized (Branden, 1979).

Table 1.1

A Summary of Metals Known to Complex With Proteins		
METALS	EXAMPLES	PROTEIN CLASS
cadmium	metallothionein	regulatory protein
calcium	calmodulin	metalloenzyme, homeostasis, muscle regulation, cell control
*chromium	glucose tolerance factor	regulatory protein
cobalt	vitamin B_{12} dependent enzymes	enzyme
copper	azurin, plastocyanin	metalloenzymes, electron transport, oxidation reactions, detoxifying proteins
iron	transferrin, cytochromes	metalloenzyme, electron transport, homeostasis
nickel	jack bean urease	metalloenzyme
manganese	pyruvate carboxylase	metalloenzyme
magnesium	DNA/RNA polymerases	metalloenzyme
molybdomen	bacterial nitrogenases	metalloenzyme
selenium	glutathione reductase	metalloenzyme
tungsten	bacterial dehydrogenases	metalloenzyme
vanadium	nitrogenase enzyme of nitrogen fixing bacteria	metalloenzyme
zinc	DNA/RNA polymerase	metalloenzyme, electron transport

* There is some doubt as to the existence of the glucose tolerance factor (Haylock *et al.*, 1983; Shepherd *et al.*, 1992).

1.2 CLASSES OF COPPER PROTEINS

Copper proteins fulfil important biochemical functions in animals, plants and bacteria, where they function in electron transport systems, some enymes of amino acid metabolism, and other enzymes (Ettinger, 1985).

Some copper-binding proteins, such as azurin, bind only one copper ion, while others bind several copper ions or different metal ions in addition to copper. For example, superoxide dismutase binds two copper ions and two zinc ions (Adman, 1991). Copper binding proteins display a diverse range of spectroscopic properties, sizes and three dimensional structures (Adman, 1991). Spectroscopic properties suggest that three types of binding centre exist for copper. The unpaired electron in Cu(II) produces a magnetic moment that gives rise to paramagnetic spectra. For Cu(II) metalloproteins the nature of these spectra can be used to determine the type of copper centre. Vänngård devised a scheme to classify copper-binding centres into three types, according to their paramagnetic spectra and other spectroscopic properties. These were called type I, type II, and type III centres (Fee, 1975). Some proteins contain more than one type of copper centre. An example of this is the multi-copper oxidase laccase, which possesses type I, type II and type III copper centres (Adman, 1991).

1.3 TYPE I COPPER CENTRES

Type I copper centres possess a unique EPR spectrum, which contains an unusually small hyperfine coupling constant. These centres have large redox potentials of between 0.3 and 0.8 V, which compare with values of 0.16 V for most copper redox reactions (Boas, 1984; Fee, 1975). The unique features of the EPR spectra of the type I copper centres are believed to be due to the N_2S donor set found in these centres (Ainscough *et al.*, 1987).

These copper centres have a maximum absorption in the region of 600 nm, which gives the intense blue colour associated with proteins containing this centre. Several lines of inquiry have been used to elucidate the cause of the 600 nm absorption maxima. These experiments included substitution of copper with cobalt, the synthesis of organic analogues of the copper centre, and infrared absorption, circular dichroism (CD) and magnetic circular dichroism spectroscopy of several proteins which contain a type I copper centre exclusively. The results suggested that the cause of the absorption maxima in the 600 nm region is due to a charge-transfer interaction between the sulphur group of a cysteine and the Cu(II) (McMillin *et al.*, 1974; Solomon *et al.*, 1976; Thompson *et al.*, 1977).

Type I centres possess an extinction coefficient of between 1,000 - 10,000 $M^{-1}cm^{-1}$ at about 600 nm which is approximately 100 fold larger than corresponding extinction

coefficients found in simple complexes of copper with amino acids and small peptides (Boas, 1984).

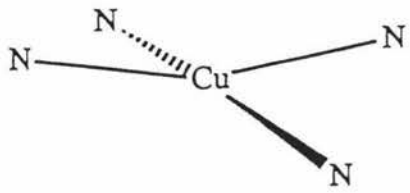
1.4 TYPE II COPPER CENTRES

Type II copper centres possess EPR spectra similar to that exhibited by most small Cu(II) complexes. They have larger hyperfine splitting constants than other copper centres. The copper-binding centre appears to have a square planar geometry with nitrogen and oxygen acting as coordinating ligands (Boas, 1984).

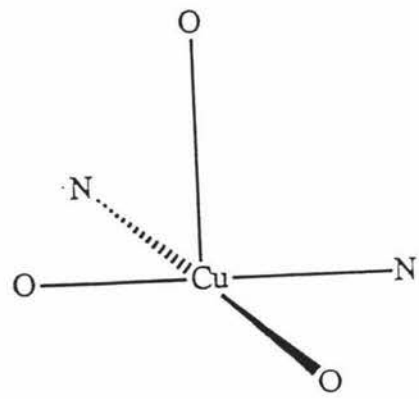
Centres of this type are found in multi-copper oxidases, such as superoxide dismutase and galactose oxidase where they are essential for enzyme activity (Boas, 1984). Laccase, ascorbate oxidase and ceruloplasmin possess all three types of copper centre (Adman, 1991). Examples of type II copper sites, as seen in superoxide dismutase and galactose oxidase copper, are shown in Figure 1.1.

Figure 1.1

Diagrams of the Type II Copper Sites of Superoxided Dismutase and Galactose Oxidase



superoxide dismutase copper centre



galactose oxidase copper centre

There is some contention as to whether the term type II copper centre should be reserved for proteins which possess exclusively this type of copper centre or extended to include those centres which are found in proteins in conjunction with type I centres. It is usual to

include those sites which possess the EPR spectrum typical of this site, irrespective of the presence of other types of copper sites in a protein (Fee, 1975; Adman, 1991).

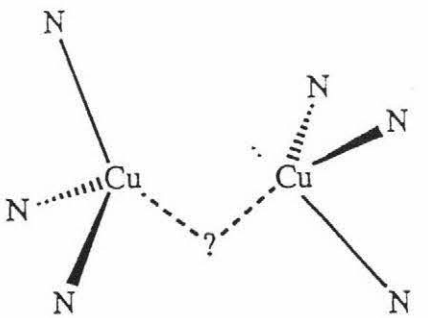
1.5 TYPE III COPPER CENTRES

Type III copper centres are binuclear, binding two copper atoms They also have the ability to associate with oxygen. They are involved in several different biological processes including oxygen transport (e.g. hemocyanin), hydroxylation (e.g. tyrosinase) and the four electron reduction of O_2 to H_2O (e.g. laccase).

When both copper atoms in the type III centre are in the oxidised Cu(II) state, the normal paramagnetic spectrum is absent. Centres in this state have been shown to be non-paramagnetic over a large range of temperatures (Fee, 1975; Boas, 1984).

The type III copper centre has been modelled using the EPR information and the consideration of the possible binding of O_2. This model is consistent with resonance Raman and UV/visible spectra. X-ray crystallography has revealed the structure of the type III copper centre in ascorbate oxidase, where six histidine residues act as ligands for the two copper ions in this centre (Messerschmidt, 1989). The modelled structure for the type III copper centre of hemocyanin is shown in Figure 1.2.

Figure 1.2
Diagram of the Copper Pairs in Hemocyanin



A schematic diagram of the copper pairs in hemocyanin as seen in the crystal structure at 3.2 Å resolution. A question mark (?) indicates a possible endogenous binding ligand (Volbeda & Hol, 1989)

1.6 CHARACTERISTICS OF BLUE COPPER PROTEINS

Small proteins which possess only the type I copper centre are termed blue copper proteins. The most studied of these proteins are azurin, plastocyanin and pseudoazurin. The only function attributed to blue copper proteins to date is electron transfer and consequently these proteins are often referred to as cupredoxins, analogous to the iron containing electron transfer proteins, ferredoxins (Adman, 1985 & 1991).

The UV/visible spectra of blue copper proteins exhibit an intense absorption maximum in the 600 nm region, a weaker band in the 460 nm region and a broad peak, which is less pronounced in plastocyanin and stellocyanin than for azurin, in the 780 nm region (Ainscough *et al.*, 1987). The exact positions of these absorption maxima vary between cupredoxins. Plastocyanins have an absorption maximum in the region of 600 nm, compared with 620 nm for azurins, suggesting a difference in the electron density of the copper atom. Crystal structures revealed that the bond length of Cu-S(Cys) is almost identical for these two proteins. A possible explanation for the difference in the absorption maxima is that a weaker axial interaction with a methionine ligand in azurin results in less charge on the copper ion, which would be expected to decrease the energy of the charge-transfer band and cause a shift in the absorption maximum (Lever, 1984; Ainscough *et al.*, 1987).

The absorption maximum at 780 nm has also been assigned to the sulphur atom of methionine $S(Met)_{(\sigma)}$, although there is a possibility that it is due to a charge transfer reaction from copper to the sulphur group of cysteine (Ainscough *et al.*, 1987). The absorption maximum observed in the 460 nm region is assigned to a charge transfer reaction between copper and a nitrogen of one of the histidines in the copper-binding site (Solomon *et al.*, 1980a).

It has been suggested that the large redox potential possessed by these proteins is due to the electron-rich cysteine thiol group located at the type I copper-binding site (Ainscough *et al*, 1987). The degree of interaction of copper with the sulphur group of Met_{121} and the carbonyl oxygen of Gly_{45}, found in azurins and possibly stellocyanin, appears to tune the redox potential of blue copper proteins, while the interaction with the three strongly bound ligands (1 Cys and 2 His) remains essentially constant (Gray and Malmstrom, 1983; Ainscough *et al.*, 1987). The minor differences seen in the lengths of

these bonds in different blue copper proteins could explain the wide variation in the redox potential (Ainscough *et al.*, 1987).

1.7 CLASSIFICATION OF TYPE I COPPER PROTEINS

Adman has devised a classification system which divides the blue copper proteins into four classes (Adman, 1988). A further four classes have since been added to this list bringing the total to eight (Ryden, 1988; McManus *et al.*, 1992). The cupredoxins are divided into these classes based on their spectroscopy properties, the number of cysteines in the protein, the type of EPR spectrum and the number of histidines at the copper centre (Adman, 1985 & 1991). A summary of the classification of type 1 copper proteins into the eight classes is summarised in Table 1.2. Some parameters of some classes are still to be investigated and published.

Table 1.2

Classification of Type I Copper Proteins						
Example	λ_{max} in 600 nm region	Other λ_{max} in visable region	No. of Cys Residues	EPR Type	No. of His Residues	Reference
azurin	625		3	axial	2	Adman, 1985; Adman *et al.*, 1989
pseudoazurin	595	470	1	axial	2	Adman, 1985; Adman. *et al.*, 1989
plastocyanin	595	780	1	axial	2	Adman, 1985; Adman. *et al.*, 1989
amicyanin						Rydén, 1988
phytocyanin						Rydén, 1988
rusticyanin						Rydén, 1988
auracyanin	596		1		2	McManus *et al.*, 1992
cucumber basic blue	595	480, 790	3	rhombic	2	Adman, 1985

1.8 BINDING SITE OF BLUE COPPER PROTEINS

Structures have been determined for various azurins, pseudoazurins, plastocyanins, cucumber basic blue protein and stellocyanin (for examples refer Adman *et al.*, 1978; Coleman *et al.*, 1978; Guss & Freeman, 1983; Petratos *et al.*, 1987; Guss *et al.*, 1988; Baker, 1988; Fields *et al.*, 1991). Several features are common to all blue copper-binding proteins. The copper atom in these proteins is not usually buried more than 8 Å beneath the surface of the protein where it is held by 4 ligands. Three of these ligands are on a loop between two β-strands while the fourth ligand, a histidine residue located 30 or more residues closer to the N-terminus than the other ligands, is brought adjacent to them by the three dimensional folding of the protein. The three ligands on the loop are arranged in the order Cys N(x) His N(y) Met, where N(x) and N(y) represent a variable number of residues (Adman, 1991). Slight variations to this pattern are observed in the blue copper proteins azurin, where a carboxyl oxygen associated with a glycine residue acts as a weak fifth ligand, and in stellocyanin, which possesses no methionine residues. In stellacyanin the side chain of a glutamine residue, which lies in a loop with a cysteine and histidine ligand, acts as the fourth ligand (Ouzounis & Sanders, 1991, Fields *et al.*, 1991).

In all blue copper proteins the interaction between the two anti parallel ß-strands 4 and 7 appears to be important for the construction of the copper site. A number of other residues, several of which are associatated with β-strands 4 and 7 are generally well conserved (Ouzounis & Sanders, 1991).

The blue copper proteins also possess a pair of generally conserved residues, one next to the cysteine ligand and the other beside the upstream histidine residue. These residues hydrogen bond to each other further stabilising the interaction between the loop containing the copper ligands and the β-sheet containing the fourth ligand. The methionine ligand is always sandwiched between two hydrophobic residues and one or more NH···S hydrogen bonds always exist between main chain amide nitrogens and the cysteine ligand (Adman, 1991).

The upstream histidine is buried more deeply than the downstream histidine and is usually orientated by a hydrogen bond from a residue or main chain atom on a different strand. The downstream histidine has an edge protruding through a more or less extensive hydrophobic face, which is likely to be at least one of the surfaces through

which electron transfer occurs (Adman, 1991). This will be discussed in further detail for azurins later.

Gray & Malmström (1983) proposed that the peptide backbone structure of the blue copper proteins forces the copper-binding site to adopt the conformation observed in these proteins and that variations in the redox potential of different blue proteins can be ascribed to changes in back bonding at the blue copper site induced by protein structure. This is called the rack-induced model. Karlsson *et al.* (1989), investigated this concept by making the $Met_{121}Leu$ ligand mutant of azurin from *P. aeruginosa*. This change resulted in the absorption maxima shifting 5 nm towards a longer wavelength, the absorption coefficient increasing by about 10% compared to the wild type protein and the redox potential increasing by 70 mV. The results are consistent with the rack-induced model for blue copper proteins (Karlsson *et al.*, 1989).

The structure of the apo-azurin from *A. denitrificans* has been solved to 1.8 Å and this structure was compared with the structure of the copper containing azurin from the same species (Shepard *et al.*, 1993). There were only very slight changes, of the order of 0.1 to 0.2 Å, in the atoms at the copper-binding site for these two proteins. This result supports the rack-induced model for copper-binding which suggests the metal enters an existing site which is determined by the structural constraints of the protein molecule.

Nar *et al.* (1992a) has described the crystal structures of several forms of azurin from *P. aeruginosa*. Two species of apo-azurin were observed, one of which was prepared by removing copper from azurin crystals and is very little changed from the structure of holo-enzymes from *P. aeruginosa*. The second apo-azurin was prepared by removing the copper from azurin prior to crystallization and showed that His_{46} and His_{117} had moved 0.6 and 1.6 Å. In apo-azurin prepared in this way His_{117} is located at the surface of the protein where it is believed to provide access for copper to the binding centre.

The copper-binding site of azurin from *A. aeruginosa* has a high affinity for both Cu(I) and Cu(II) which has led to the proposal that the type I copper-binding site is a compromise between the ideal binding sites for reduced and oxidised copper and that a low energy barrier exists between these two forms. Cu(I) binds preferably in a tetrahedral geometry, while Cu(II) prefers a square planar geometry. The type I copper centres possess a distorted tetrahedral geometry (Canters, 1989; Adman, 1991). The crystal structures of holo-azurin from *A. denitrificans* has been solved at high resolution

for both the oxidised are reduced forms (Norris *et al.*, 1983; Baker, 1988; Shepard *et al.*, 1990). These structures showed minimal differences between the two forms suggesting that very little change occurs to the protein structure during electron transfer.

1.9 COMPARISON OF THE BINDING SITES OF PLASTOCYANIN AND AZURIN

The blue copper proteins azurin and plastocyanin both have a β-barrel structure with a single type I copper centre and a hydrophobic patch on the surface of the molecule. Both proteins also display the copper centre in similar position within the tertiary structure (Adman, 1991). However several differences exist between these two proteins and these are listed in Table 1.3.

Table 1.3

Comparison of Parameters of Plastocyanin and Azurin			
Feature	Plastocyanin	Azurin	Reference
Molecular Weight (kDa)	10.8	14.6	Adman, 1985
Number of amino acids	99	128-129	Adman, 1985
NH...S hydrogen bond at the copper-binding site	1	2	Adman, 1991
Distance Between Carbonyl Oxygen and Cu	3.8 Å	3.1 Å	Adman, 1991
Cu-Met-S	2.9 Å	3.1 Å	Adman, 1991
Redox potentials (mV)	340-370	230-330	Adman, 1991

The type I copper centres of azurin and plastocyanin are very similar, yet show two significant differences. The first of these differences involves the carbonyl group preceding the upstream histidine residue. This carbonyl group, associated with a glycine residue, is close to the binding site in azurins where it is typically about 3.1 Å away. This is considerably closer to the copper centre than in plastocyanin, where this carbonyl group is typically 3.8 Å away. This relatively close proximity in azurins allows the carbonyl group to bond to the copper and where it acts as a fifth ligand (Ugurbil and Bersohn, 1977; Baker, 1988; Nar *et al.*, 1991a).

The second difference between the type I copper site of azurins and plastocyanins is the length of the Met(S)···Cu bond which is longer in azurins (3.1 Å) than in plastocyanins,

(2.9 Å). The observation of this weaker interaction has led to speculation that the Met(S)···Cu interaction may not be as important for stabilising the copper atom in azurin as it is in plastocyanin and other blue copper proteins (Baker, 1988; Adman *et al.*, 1989)

1.10 COMPARISON OF TYPE I COPPER-BINDING SITE WITH ZINC-BINDING SITE

More than 160 zinc-binding enzymes have been identified in animals, plants and bacteria. Zinc-binding proteins include aminopeptidases, dehydrogenases, alkaline phosphatase and electron transfer proteins (Yamanaka, 1988; Tsuru, 1988). The presence of zinc in cells is known to govern many metabolic processes and zinc finger proteins, which bind zinc, are known to interact with DNA where they play a role in regulating transcription (Struhl, 1987).

One technique used to study these enzymes is metal substitution. One metal which substitutes for zinc is Cu(II), suggesting that zinc-binding sites can accomodate this atom. The zinc-binding site has been found to be highly asymmetric and the geometry is distorted. Four amino acid side chains have been observed to act as ligands for zinc. In the descending order of frequency these are N-atoms of histidine, the S-atom of cysteine, and the carboxylate groups of glutamate and aspartate. Water has been found to act as a ligand in some zinc-binding proteins (Vallee & Galdes, 1984).

A well studied zinc-binding protein is superoxide dismutase which is an electron transfer protein with two domains. Each domain contains a type II copper centre and a zinc-binding centre. The copper ion is held by three histidine ligands and the zinc by three histidine ligands and one aspartate ligand. The copper and zinc ions are 6 Å apart and share one histidine ligand. The copper-binding site in superoxide dismutase has a distorted trigonal planar arrangement while the zinc-binding site geometry is tetrahedral (Fielden & Rotilio, 1985).

Another example of a zinc binding site is found in carboxypeptidase A. The crystal structure of this protein has been refined to 1.75 Å. Zinc is co-ordinated to two histidine residues and the carboxylate oxygens of two glutamate residues. Water acts as a fifth ligand. When this protein complexes with the dipeptide glycyl-(L)-tyrosine the water ligand is replaced by both the carbonyl oxygen and the anion nitrogen of the dipeptide so the co-ordination number changes from 5 to 6. This phenomenon of changing

co-ordination numbers seems to occur in other zinc-binding proteins as well. Zinc co-ordination in this protein is described as a distorted tetrahedron (Rees *et al.*, 1981).

The relevance of the similarity between zinc and copper-binding sites has become apparent very recently. This has arisen from studies on azurin produced in *E. coli* from recombinant DNA. Azurin produced from *E. coli* often has a diminished A_{620} peak when compared with the same azurin purified from its native organism. This phenomenon has been shown to be due to the production in *E. coli* of azurin in which a high proportion of the protein contains zinc. This form of protein is colourless and therefore it produces no A_{620} peak.

The zinc-binding azurin of *P. aeruginosa* has recently been isolated from *E. coli* (Nar *et al.*, 1992b). The copper centre of azurin is a consequence of the tertiary structure and does not appear to be altered by the binding of copper (Shepard *et al.*, 1993; Nar *et al.*, 1991b). However the binding of zinc, while not causing drastic conformational change, does cause some distortions of the polypeptide backbone and side chains. The largest difference between zinc-azurin and copper-azurin is that the Gly_{45} carbonyl group which is normally considered a weak ligand in azurin has moved from 2.9 Å from the copper to being only 2.3 Å away from the zinc. The movement of this group and the slight adjustment to the backbone atoms connected to it is probably induced by the preference of zinc for tetrahedral co-ordination rather than the trigonal bipyrimidal arrangement that exists in copper-azurin. The Met_{121} has moved significantly away from the zinc, to 3.4 Å. This is too long to form a significant bond with the copper (Nar *et al.*, 1992b).

1.11 AZURINS

Azurin may be classified as a type I, class I copper protein and functions in the electron transport chain of several bacteria. Azurin is believed to transport electrons from cytochrome C to the cytochrome oxidase/nitrite reductase of these organisms (Adman, 1985). A summary of the diversity of azurins and their host bacterial strains is shown in Table 1.4.

Table 1.4

Summary of Azurins That Have Been Identified					
Source	Molecular Weight	Peptide Length	λ_{max}	Sequence Known	Reference
P. aeruginosa	14,600	129	631	yes	Otsuka, 1988; Pascher et al., 1989
P. florescens B-93	14,600	128	635	yes	Otsuka, 1988
P. florescens C-18	14,600	128	625	yes	Ambler & Tobari, 1989
P. florescens D-35	14,600	128	625	yes	Ambler & Tobari, 1989
P. florescens 6009/1	14,600	128	625	yes	Ambler & Tobari, 1989
P. denitrificans	14,600	128	620	yes	Otsuka, 1988
B. bronchiseptica	14,600	129	-	yes	Otsuka, 1988; Arvidsson *et al.*, 1989
A. faecalis	14,600	128	-	yes	Otsuka, 1988
A. denitrificans	14,600	129	619	yes	Otsuka, 1988; Ainscough *et al.*, 1987
P. denitrificans	13,800	124	595	no	Otsuka, 1988
Methylomonas. J	14,600	128	-	yes	Hoitink *et al.*, 1990
Methylomonas. J	14,600	1	-	yes	Hoitink *et al.*, 1990; Nishiyama *et al.*, 1989

Much work has gone into looking at the copper-binding site in azurin. The crystal structure of azurin from *Pseudomonas aeruginosa* has been resolved to 2.7 Å (Adman & Jensen, 1981), while the azurin from *Alcaligenes denitrificans* has been resolved to 1.8 Å (Baker, 1988).

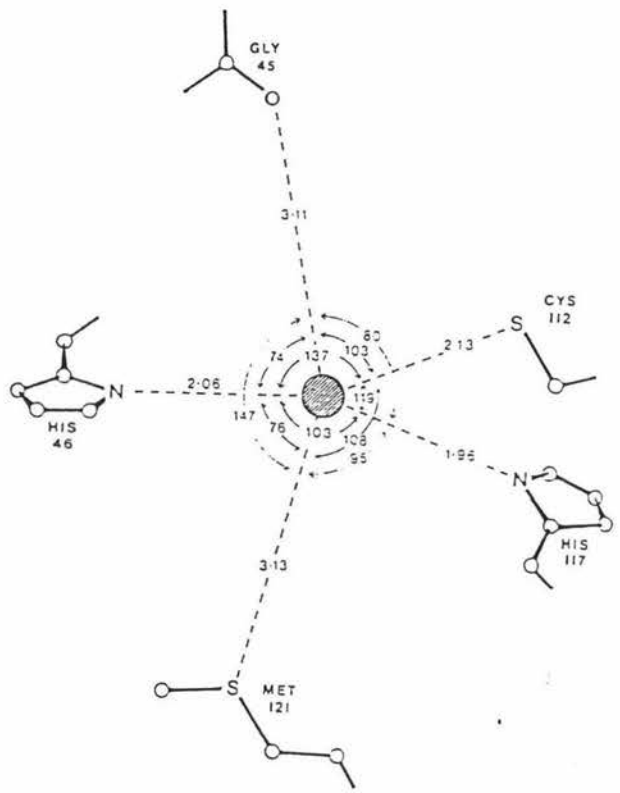
1.12 THE CRYSTAL STRUCTURE OF AZURIN FROM *ALCALIGENES DENITRIFICANS*

The structure of azurin from *A. denitrificans* has been solved and refined to 1.8 Å and is shown in Figure 1.3 (Baker, 1988). This structure revealed that the azurin consists of a β–barrel, or more correctly a β–sandwich network. The protein molecule consists of eight β-sheets that pack together with a filling of non-polar side-chains between them. A small number of polar side-chains are involved in important hydrogen bonds which act to

stablise the structure. The copper-binding site is located in a cavity at one end of the molecule. This cavity is formed between several of the β–strands and their connecting loops. A 28 amino acid residue loop has been identified between residues 53 and 80. This links strands four and five and packs as an extra "flap" on the outside of the β–barrel. This "flap" contains the only α–helix in the molecule.

Figure 1.3

Diagram of the Copper Binding Site of Azurin from *A. denitrificans*



The binding site of azurin from *A. denitrificans* from Baker, (1988).

On the surface of the azurin molecule a hydrophobic patch can be clearly observed. This has been found in all azurins characterised to date and is discussed further in section 1.13. This patch is located adjacent to the copper site at one end of the molecule. The hydrophobic patch contains a number of charged amino acid side-chains which do not make stabilising ion-pairs. The orientation of the copper ligands in this structure appears to be tightly constrained by the surrounding protein structure. For example the

cysteine$_{112}$ residue which acts as a copper ligand is held into position by two NH···S hydrogen bonds.

This crystal structure also revealed the presence of a sulphate (SO_4^{2-}) ion located in a depression between the flap and the main body of the β–barrel. It is bound by a peptide NH of residue 76 and $N^{\sigma 1}$ of His$_{83}$. This is significant as His$_{83}$ or residues in close proximity to it have been implicated by various experiments as a binding site for electron transfer. Azurin is known to react with anionic redox agents such as $Fe(CN)_6^{3-}$ which is used as an oxidising agent when working with azurin as is described in section 2.3.29. The His$_{83}$ residue is external and conserved in all azurins. The nature of the cleft that this residue is located in makes an attractive site for the binding of ions, solvents and other molecules, and its position corresponds roughly to that of an acidic patch located in plastocyanin. All these factors suggest that the His$_{83}$ residue and perhaps the SO_4^{2-} ion are involved in some way in electron transfer.

1.13 THE HYDROPHOBIC PATCHES IN AZURIN

The mechanism by which an electron traverses the 8 Å to the copper atom in the interior of azurin has been the subject of much research. Two hydrophobic patches have been found on the surface of all azurins examined to date. These have been suggested to be involved in the transfer of electrons to the proposed redox partners of azurin which are cytochrome c551 and the cytochrome oxidase/nitrite reductase complex (Adman, 1985; Sykes, 1991). Site-directed mutagenesis experiments have been performed on azurin from *P. aeruginosa* to determine the significance of these two patches.

One of the hydrophobic patches is centred around the residue His$_{35}$. The imidazole ring of this residue contacts the His$_{46}$ copper ligand (Pascher *et al.*, 1989) Pascher *et al.* made a His$_{35}$Lys mutant and demonstrated that the spectroscopic properties of this mutant were essentially identical to those of the wild type. This indicated that His$_{35}$ is unlikely to be involved in the formation of a precursor complex with cytochrome c551 and may therefore not be involved in electron transfer. These findings were supported by Nar *et al.* (1991a) who made the mutants His$_{35}$Gln and His$_{35}$Leu. They showed that the His$_{35}$ of the hydrophobic patch was unlikely to be involved in electron transport between azurin and cytochrome c551 or cytochrome oxidase/nitrite reductase.

The second hydrophobic patch observed in azurin from *P. aeruginosa* is thought to be analogous to that observed in plastocyanin and other blue copper proteins (Adman, 1991). This hydrophobic patch is centred around the downstream histidine copper ligand His_{117}. The $N^{\delta 1}$ of the copper ligand His_{117} lies in the centre of a depressed patch with residues Met_{13}, Met_{44}, Phe_{114}, the conserved residue Pro_{115} and the methyl group of Gly_{116} surrounding it. An outer ring of residues consisting of Leu_{39}, Val_{43}, Met_{64}, Ala_{119}, Leu_{120} and the polar residues Gln_{42} and Tyr_{72} surround these (Baker, 1988).

The $Phe_{114}Ala$ mutant of azurin from *P. aeruginosa* was prepared (Pascher, 1989). Phe_{114} lies adjacent to His_{117}. The red absorption maximum dropped by 7 nm to 621 nm in this mutant, indicating that the copper site had been affected, and the hyperfine splitting constant of the EPR decreased. The redox potential increased by 20-40 mV.

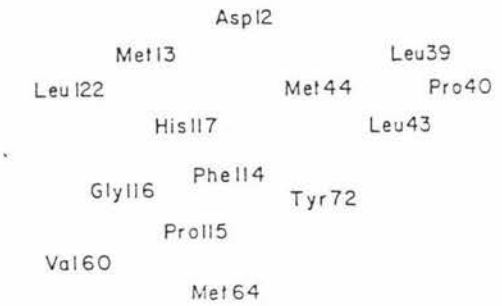
A $His_{117}Gly$ mutant of the azurin from *P. aeruginosa* has also been prepared, with the intention of creating an aperture in the centre of the hydrophobic patch. The ^{1}H-NMR spectrum of this mutant indicates that negligible change was made to the tertiary structure of the protein. Interestingly the UV/visible spectrum revealed a strong absorption at 420 nm and not 628 nm, where a maxima would be expected. This suggested that a type II copper site had been formed and that some Cu(II) was binding to the azurin non-specifically. Copper could be removed from these mutants using 0.2 M NaCl which resulted in a homogeneous EPR spectrum that clearly corresponds to a type II copper centre. When the protein was supplemented with $Cu(NO_3)_2$ and N-methylimidazole an absorption maximum at 630 nm with the corresponding intense blue colour appeared which is characteristic of a type I copper centre. The EPR spectrum of this mutant was similar to that of the wild type indicating that the copper site remains open enough to allow the imidazole group to enter back into the structure (den Blaauwen *et al.*, 1991).

Met_{44}, which also lies in this patch, has been mutated to Lys. This mutant had an absorption maximum at 625 nm, unchanged from the wild type. The pI moved from 5.6 to 6.6 as measured by isoelectric focusing and the redox potential increased by 40-60 mV consistent with an extra positive charge near the copper ion. ^{1}H-NMR indicated that a very slight change had been made in the protein structure and the copper site. Interestingly electron transfer kinetics demonstrated that the $k_{(ex)}$, which was lower in the mutants was pH dependant. This was not the case in the wild type, suggesting that

the Met44 residue and therefore the His_{117} hydrophobic patch is likely to have an important function (van de Kamp *et al.*,1990). A schematic diagram of the hydrophobic patch associated with the His_{117} residue of azurin is shown in Figure 1.4 (Adman, 1985).

Figure 1.4

Schematic diagram of the His_{117} Hydrophobic Patch from Azurin



1.14 COMPARISON OF CHARACTERISTICS AND CRYSTAL STRUCTURES OF AZURIN FROM *A. DENITRIFICANS* AND *P. AERUGINOSA*

While the azurins have been found to possess a conserved eight stranded β-barrel structure and possess similar spectroscopic characteristics, a degree of variability has been noted between azurins. The sequences of the two most studied azurins isolated from *A. denitrificans* and *P. aeruginosa* have been aligned and 38% of the amino acids were observed to be different (Ambler, 1971). Differences in the resonance Raman spectra have also been observed. It is suggested that the differences between azurins could indicate that azurins from different sources may have other functions (Ainscough *et al.*, 1987).

Azurin from *P. aeruginosa* has been shown to undergo a pH-dependant conformational change (Rosen & Pecht, 1976; Werland & Pecht, 1978; Silvestrini *et al.*, 1981). NMR studies suggest that this pH dependance may be linked to the deprotonation of His_{35} with a pK ~7 (Ugurbil & Bersohn, 1977; Ugerbil *et al.*, 1977; Hill & Smith, 1979; Farver & Pecht, 1981). This pH-dependant conformational change does not occur in azurin from *A. denitrificans* (Baker, 1988).

A crucial difference in sequence between these two azurins occurs at position 36, which is a proline residue in the *P. aeruginosa* protein and a valine in the azurin from *A. denitrificans* . This valine forms a hydrogen bond with the carbonyl oxygen of Gly_9. This hydrogen bond is prevented from forming in the *P. aeruginosa* protein by the proline residue. This residue also causes a narrowing of the small cleft where the His_{35} imidazole is positioned, preventing the $N^{\sigma 1}$ from becoming protonated at low pH values. The region around the His_{35} has a role in tuning the redox potential, although it does not appear to be involved in the electron transfer pathway (Nar *et al.*, 1991b).

The differences in the distances between the ligands and the copper atom in azurin from *A. denitrificans* and *P. aeruginosa* are summarised in Table 1.5.

Table 1.5

Summary of Bond Lengths of the Copper-Binding Ligands in Azurins from *A. denitrificans* and *P. aeruginosa*		
Copper to Ligand Bond	Bond length in *A. denitrificans* at pH 5.0	Bond length in *P. aeruginosa* at pH 5.5
S(Cys 112) - Cu	2.14 Å	2.25 Å
N(His 46) - Cu	2.06 Å	2.11 Å
N(His 117) - Cu	1.96 Å	2.03 Å
S(Met 121) - Cu	3.11 Å	3.15 Å
O(Gly 45) - Cu	3.13 Å	2.97 Å

The figures in Table 1.5 for *A. denitrificans* were obtained from Baker (1988) and for *P. aeruginosa* from Nar *et al.* (1991b).

While spectroscopic differences are observed between these two proteins they are more similar to each other than to either plastocyanin or stellacyanin (Solomon *et al.*, 1980b). It is not clear what structural features give rise to the differences in the electronic absorption spectra between the two azurins. The absorption maxima for these two proteins differ considerably as shown in Table 1.6 .

The figures on Table 1.6 for *A. denitrificans* were obtained from Ainscough *et al.* (1987) and for *P. aeruginosa* from Solomon *et al.* (1980b).

Further refinement needs to be done on the crystal structure of azurin from *P. aeruginosa* for a more meaningful comparison to be made of these structures. It is worth noting however that while these proteins do have a very similar structure they may be sufficiently different to have dissimilar modes of action.

Table 1.6

Comparison of λ_{max} in the Region of 600 nm for Azurin from *P. aeruginosa* and *A. denitrificans*	
λ_{max} of *A. denitrificans* azurin	λ_{max} of *P. aeruginosa* azurin
780 nm	779 nm
619 nm	631 nm 567 nm
460 nm	481 nm

1.15 MUTAGENESIS OF THE COPPER CENTRE OF AZURIN AND OTHER TYPE I COPPER CENTRES

The Met_{121} residue of azurin from *P. aeruginosa* has been changed to leucine by *in vitro* mutagenesis. The resulting azurin demonstrated that Met_{121} is not essential for Cu(II) binding by this protein, although the change to leucine at this position may decrease the stability of the protein (Karlsson *et al.*, 1989).

Site-directed mutagenesis experiments have been performed on plastocyanin, pseudoazurin, and on the azurin from *P. aeruginosa* but not as yet on azurin from *A. denitrificans*. These experiments have been designed to determine what is essential for copper-binding and this information could be extrapolated to other proteins with type I copper centres. All the mutants reported have been expressed in *E. coli*.

Two mutants have been made in pseudoazurin of *A. faecalis* S-6. One of these was the Pro_{80}Ala mutant. The Pro_{80} residue lies next to the copper ligand His_{81} of this protein. UV/visible spectra of this mutant revealed no change but the redox potential increased by 139 mV. The ability of this mutant to transfer electrons to nitrite reductase decreased significantly but the apparent K_m of nitrite reductase for pseudoazurin did not change. X-ray diffraction revealed that the pocket left by the removal of the side chain was

occupied by a water molecule, which led to the suggestion that the increase in the redox potential may be caused by the increased accessibility of the solvent to the copper. It appeared that the water molecule moves during the change in the state of copper oxidation (Nishiyama *et al.*, 1992).

Several mutants of plastocyanin from *Populus nigra* var *italica* have been expressed in *E. coli* (Chang*et al.*, 1991). The copper ligand Met_{92} was mutated to all 19 possible amino acid residues. Of these 19 mutants, $Met_{92}Cys$, $Met_{92}His$, $Met_{92}Ala$, and $Met_{92}Gly$ were expressed in *E. coli* although none of the proteins were purified. Western blot analysis showed that azurin was present in the periplasmic space for three of the mutants but not for $Met_{92}Cys$. It was not possible to purify any blue protein from these mutants. It was proposed that the low levels of expression could be due to kinetic difficulties in acquiring the copper or because the thermodynamic binding contant for copper had decreased (Chang *et al.*, 1991).

Mutants of the type I copper centre have been prepared from azurin isolated from *P. aeruginosa*. All 19 possible mutants have been made for the Met_{121} copper ligand and have been expressed in *E. coli*. Five of these have been purified and shown to contain copper. While the intense blue colour was observed in all five mutants some spectroscopic differences were observed in all cases, as shown in Table 1.7 (Chang *et al.*, 1991).

Table 1.7

Spectroscopic Differences Between Met_{121} Mutants of *P. aeruginosa* Azurin		
Mutant	Red Absorption Maximum (nm)	Minor Peak (nm)
wild type	625	445
$Met_{121}Val$	630	459
$Met_{121}Ile$	626	459
$Met_{121}Asn$	622	447
$Met_{121}Asp$	622	445
$Met_{121}His$	612	449

These mutants reveal that the methionine ligand in azurin can be replaced by a large variety of residues without preventing binding of copper. All 19 possibilities of copper ligand His_{46} were also made and the mutant $His_{46}Asp$ was purified. The spectrum of

this mutant showed a drop of 9 nm at the red absorption maximum and an increase of 13 nm at the minor peak (Chang *et al.*, 1991).

Independently, the mutant $Met_{121}Leu$ was prepared and characterised. This protein displayed an intense blue colour confirming the finding of Chang *et al.* that $methionine_{121}$ is not an obligatory ligand for copper-binding in azurin. The absorption maximum increased by 5 nm and the extinction coefficient increased by about 10% compared to the wild-type protein. The λ_{max}:280 nm absorption ratio dropped from 0.56 to 0.33 indicating that while this ligand was not necessary for copper-binding it may stabilise copper-binding. The EPR parameters and in particular the hyperfine splitting were modified, indicating that there is a small change in the copper-ligand interactions. The redox potential of the mutant increased about 70 mV, showing that the strong Cu-S(Met) interaction is not important for the high potential in the blue copper proteins. It was proposed that the introduction of the leucine residue to the copper centre caused a change in the strength of the Cu-S(Cys) interaction which would account for the increase in the absorption maximum and the EPR differences. The result of these experiments suggest that rack-induced bonding is likely (Karlsson *et al.*, 1989).

The $His_{117}Gly$ mutant was discussed in section 1.13.

1.16 The Aims of this Thesis

The original aim of this thesis was to make site-directed mutants of azurin from *A. denitrificans*. Due to difficulties in isolating blue azurin from *E. coli* in good yield, these mutants were not made but attempts were made to overcome the problems encountered when expressing azurin in *E. coli*. This led to an investigation into the possibility of the azurin from *A. denitrificans* binding zinc when expressed in an *E. coli* host, as had been reported for the azurin from *P. aeruginosa* (Nar *et al.*, 1992b). The results obtained from this thesis are consistent with the results reported by Nar *et al.*, (1992b). The possibility of overcoming this problem by expressing azurin in an .host in medium which was supplemented with copper was investigated with a degree of success.