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

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry at Massey University

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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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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).

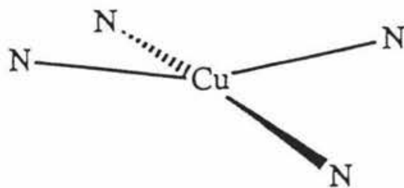
1.4 TYPE II COPPER CENTRES

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).

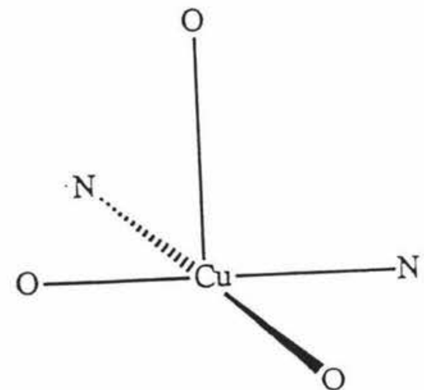
Centres of this type are found in multi-copper oxidases, such as superoxide dismutase and galactose oxidase where they are essential for enzyme activity (Boas, 1984). Laccase, ascorbate oxidase and ceruloplasmin possess all three types of copper centre (Adman, 1991). Examples of type II copper sites,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

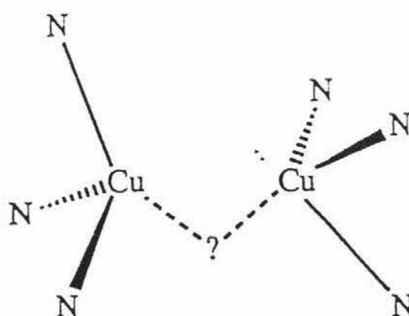
Type III copper centres are binuclear, binding two copper atoms They also have the ability to associate with oxygen. They are involved in several different biological processes including oxygen transport (e.g. hemocyanin), hydroxylation (e.g. tyrosinase) and the four electron reduction of O_2 to H_2O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

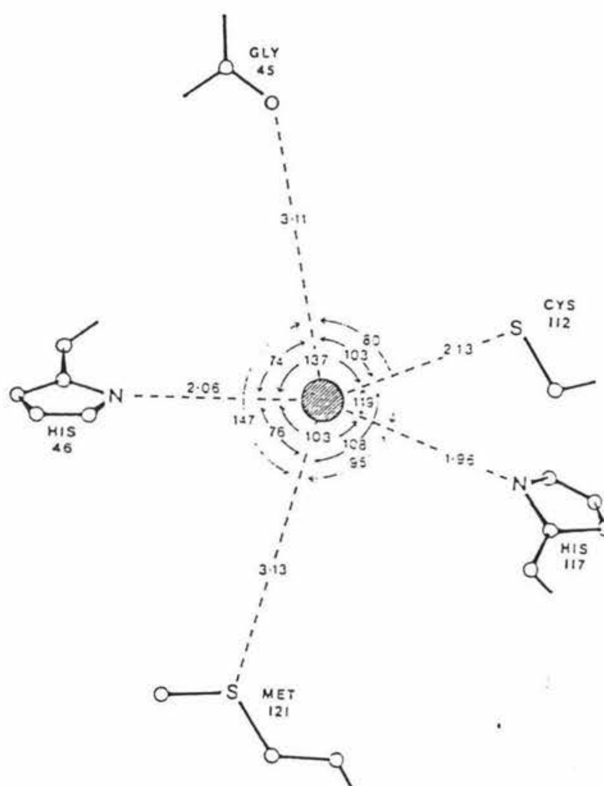
1.12 THE CRYSTAL STRUCTURE OF AZURIN FROM *ALCALIGENES DENITRIFICANS*

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.

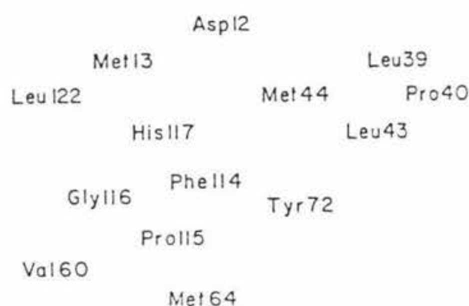
A His_{117}Gly mutant of the azurin from *P. aeruginosa* has also been prepared, with the intention of creating an aperture in the centre of the hydrophobic patch. The ^{1}H-NMR spectrum of this mutant indicates that negligible change was made to the tertiary structure of the protein. Interestingly the 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 Met_{44} 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.

Chapter 2 : Materials and Methods

2.1 CHEMICALS

All chemicals used were of the highest grade available. They were obtained from various pharmaceutical companies.

2.2 SOLUTIONS

2.2.1 General Buffers

High Salt Buffer	(pH8.0)	
	12.0 g	NaCl
	12.1 g	Tris
	0.27 g	EDTA
	Made up to 1 litre with Milli Q water.	
TE buffer	(pH8.0)	
	0.27 g	EDTA
	1.21 g	Tris
	Made up to 1 litre with Milli Q water.	

2.2.2 Solutions Used for the Extraction and Purification of Azurin

Osmotic Shock Solution	(pH8.0)	
	0.27 g	EDTA
	3.63 g	Tris
	Made up to 1 litre with Milli Q water.	
Acetate Buffer	(pH4.1)	
	42 mL	glacial acetic acid
	Made up to 15 litres with Milli Q water and pH adjusted with ammonia.	

40 mM or 50mM Copper Sulphate

6.38 g or 7.98 g	copper sulphate (hydrated)

Made up to 1 litre with Milli Q water

2.2.3 Cell Growth Media

All cell growth media were autoclaved before use.

SOC Media

0.019 g	KCl
0.20 g	$MgCl_2$
0.25 g	$MgSO_4$
0.36 g	glucose
0.5 g	yeast extract
0.6 g	NaCl
2.0 g	tryptone

Made up to 1 litre with Milli Q water.

Luria Broth (pH7.5)

5 g	NaCl
5 g	yeast extract
10 g	tryptone

Made up to 1 litre with Milli Q water.

Luria Broth Agar Plates

400 mL	Luria broth
6 g	agar

2YT Broth (pH7.5)

10 g	NaCl
10 g	yeast extract
16 g	tryptone

Made up to 1 litre with Milli Q water.

2.2.4 Solutions of DNA Sequencing

Acrylamide for Sequencing Gels

	288 g	urea
	342 g	acrylamide
	1.8 g	bis-acrylamide
	Made up to 500 mL with Milli Q water	
	5 g	amberlite
	stirred for 30 minutes at room temperature and filtered.	
	60 mL 10 X TBE	
	Made up to 600 mL with Milli Q water.	
TBE buffer	(pH8.0)	
	108 g	Tris
	55 g	boric acid
	40 mL	0.05M EDTA
	Made up to 1 litre with Milli Q water.	

2.2.5 Solutions for Large or Small Scale Plasmid Preparations

All solutions for large or small scale plasmid preparations were autoclaved before use.

Solution i	(pH8.0)	
	2.7 g	EDTA
	3.0 g	Tris
	9 g	glucose
	Made up to 1 litre with Milli Q water.	
Solution ii		
	2 mL	10 M NaOH
	Make up to 95 mL with Milli Q water.	
	5 mL	20% SDS
Solution iii	(pH4.8)	
	11.5 mL	glacial acetic acid

	60 mL	5 M potassium acetate
	Made up to 100 mL with Milli Q water.	
STET	(pH8.0)	
	0.157 g	Tris
	1.86 g	EDTA
	8 g	sucrose
	pH adjusted to 8.0 with NaOH	
	0.5 g	Triton X-100
STE	(pH7.8)	
	0.27 g	EDTA
	1.21 g	Tris
	4 g	NaCl
50 mM Calcium chloride		
	7.35 g	calcium chlorid-2 hydrat
	Made up to 1 litre with Milli Q water.	
3.5 M Ammonium acetate in 20% PEG solution (pH7.0)		
	27 g	ammonium acetate
	20 g	PEG 8000
	Dissolved in 80 mL of Milli Q water and the pH was adjusted to 7.2 Made up to 100 mL with Milli Q water.	
TAE buffer	(pH8.0)	
	242 g	Tris
	57.1 mL	glacial acetic acid
	100 mL	0.5 M EDTA
	Made up to 1 litre with Milli Q water.	

2.2.6 Solutions for SDS Gel-Electrophoresis

10% SDS		
	50 g	SDS
	Made up to 500 mL with Milli Q water.	

Acrylamide for SDS-polyacrylamide Gel Electrophoresis Resolving Gel

32 g	acrylamide
0.2 g	bisacrylamide

Made up to 100 mL with Milli Q water.

Acrylamide for SDS-polyacrylamide Gel Electrophoresis Stacking Gel

4 g	acrylamide
0.105 g	bisacrylamide

Made up to 50 mL with Milli Q water.

SDS-polyacrylamide Stacking Gel Buffer (pH8.7)

1.8 g	Tris
4 mL	10% SDS

Made up to 100 mL with Milli Q water

SDS-polyacrylamide Stacking Gel Buffer (pH6.8)

3 g	Tris

Made up to 50 mL with Milli Q water

Tank Buffer

40 mL	10% SDS
12.1 g	Tris
57.6 g	glycine

Made up to 4 L with Milli Q water.

Coomassie Blue Stain

0.625 g	Coomassie brilliant blue R-250
225 mL	methanol
25 mL	glacial acetic acid

Made up to 500 mL with Milli Q water.

Destain 1

100 mL	methanol

	900 mL	glacial acetic acid
	Made up to 2 litres with Milli Q water.	
Destain 2		
	100 mL	methanol
	100 mL	acetic acid
	Made up to 2 litre with Milli Q water.	
Quench Solution		
	4 mL	10% SDS
	1.0 mL	2-mercaptoethanol
	2.0 mL	glycerol
	2.5 mL	stacking gel buffer

2.3 METHODS

2.3.1 Preparation of Azurin Coding Fragment by Gel Electrophoresis

The 3.5 kbp expression plasmid pCH5, described in detail in Section 3.1.1, was digested with the restriction enzymes XbaI and HindIII. These enzymes cleave the 0.9 kbp azurin coding sequence from the vector sequence of pCH5. DNA was extracted from the reaction mixture using phenol/chloroform as described in Section 2.3.5 and was precipitated with ethanol as described in Section 2.3.6. When necessary, the cohesive ends resulting from this digestion were filled using T4 DNA polymerase to produce blunt ends as described in Section 2.3.8. Gel electrophoresis was used to separate the 0.9 kbp and 2.6 kbp fragments. The 0.9 kbp fragment was excised from the gel using a sterile razor blade. Agarose was removed from the DNA using Geneclean® II, from Bio101, Inc, USA, as described in Section 2.3.9.

2.3.2 Small-Scale Plasmid Preparation

Small-scale plasmid preparations were performed as described in Sambrook *et al.* (1989).

2.3.3 Medium Scale Plasmid Preparation

A 20 mL culture of *E. coli* XL-1 or JM101 cells was grown overnight in LB with appropiate antibiotic selection. Cells were harvested by centrifugation for 10 minutes at 5000*g* in a SS34 rotor and the supernatant was discarded. The cell pellet was resuspended in 1 mL of solution

i. This was incubated at room temperature for 5 minutes and divided evenly between 4 X 1.5 mL microcentrifuge tubes. To each microcentrifuge tube, 0.5 mL of solution ii was added. This solution was mixed by inversion and allowed to stand for 10 minutes on ice. To each tube, 0.375 mL of solution iii was added and the tubes were gently vortexed for a few seconds before being incubated at room temperature for 5 minutes. The microcentrifuge tubes were centrifuged at high speed for 5 minutes, the supernatants were evenly divided between 8 microcentrifuge tubes and the DNA was precipitated with 5 mL/tube of isopropanol as described in Section 2.3.6. The DNA pellet was resuspended in either water or TE buffer and pooled into a total volume of 600 µL. To this, 6 µL of DNase-free RNase (10 mg/mL) was added and the solution was incubated at 37°C for 30 minutes. Phenol/chloroform was added to the DNA solution and after mixing and centrifugation the DNA in the aqueous layer was precipitated with ethanol as described in Sections 2.5.5 and 2.5.6. The final DNA pellet was resuspended in 100 µL of TE.

2.3.4 Large-Scale Plasmid Preparation

Large scale plasmid preparations were carried out as described in Sambrook *et al.* (1989).

2.3.5 Extraction of DNA Using Phenol/Chloroform

An equal volume of phenol was added to a DNA solution suspended in TE buffer. This was vortexed rapidly for 1 minute and the aqueous (TE) and alcohol (solvent) phases were separated by centrifugation for 5 minutes at 13,000 rpm. The aqueous layer was transferred to an equal volume of 1 part phenol to 1 part 24:1 chloroform:isoamyl alcohol. This was vortexed as before and centrifuged for 3 minutes. Again the aqueous phase was removed then added to an equal volume of 24:1 chloroform:isoamyl alcohol. This was vortexed for one minute and the phases were separated by either centrifugation for 1 minute or allowing the solution to stand for several minutes until the phases were seen to separate . The aqueous phase which should contain the DNA was removed.

2.3.6 DNA Precipitation

DNA was precipitated by of two methods. Either an equal volume of isopropanol or two and a half volumes of 95% ethanol was added to the DNA solution. The solution was incubated at -70°C for 30 minutes before the DNA was pelleted out of solution by centrifugation at 13,000 RPM for 10-15 minutes.

2.3.7 Agarose Gels

DNA fragments were run on 1% agarose gels. This gel was made up and run in TAE buffer. Gels were stained with ethidium bromide solution.

2.3.8 End Filling

Cohesive (sticky) ends created by restriction enzymes were removed where necessary using Klenow fragment. A reaction mixture containing up to 1 μg of DNA suspended in 10 μL of TE buffer was prepared. To this, 2 μL of BRL react buffer 2, 1 μL (1 unit) of Klenow fragment and 1 μL of 2 mM (dNTP's) were added. The reaction mixture was brought to 20 μL with Milli Q water. This reaction was incubated at 30°C for 15 minutes and the Klenow fraction was then inactivated by heating the reaction at 75°C for 10 minutes. Unincorporated nucleotides were removed from the reaction mixture using Geneclean® II obtained from Stratech Scientific Ltd. This was done according to the manufacturers instructions.

2.3.9 Extraction of DNA from Low Melting Point Agarose Gels

DNA was extracted from low melting agarose gel using Geneclean® obtained from Stratech Scientific Ltd. All procedures were carried in accordance with the manufacturers instructions.

2.3.10 Cloning DNA Fragments

DNA fragments and vectors were prepared and cloned using standard protocols as described in Ausubel *et al.*, (1989).

2.3.11 Phosphorylation

Phosphatase treatment of vectors was performed using bovine alkaline phosphatase from Boehringer Mannheim, Germany, according to the manufacturers instructions, differing only in the length of time allowed for the reaction. A 15 minute reaction time was found to be optimal (as described in Sections 3.5.1 and 3.5.2) and this was implemented for most phosphatasing reactions.

2.3.12 Plasmid Transformation Using $CaCl_2$ Preparation of Competent Cells

Plasmid transformation using 50 mM $CaCl_2$ was performed as described in Sambrook *et al.* (1989).

2.3.13 Preparation of Cells for Electroporation

E. coli strains JM101, RR1 and XL1 were prepared for electroporation by the protocol described in Dower *et al.* (1988).

2.3.14 Microdialysis for Electroporation

A DNA sample suspended in 20-40 μL of TE buffer was carefully pipetted onto a Millipore 0.025 μm pore size membrane and this was dialysed against sterile 10% (v/v) glycerol for 30 minutes.

2.3.15 Electroporation

A Bio Rad Gene Pulser (model 16523098) was used for electroporation. Bio Rad 0.2 cm cuvettes were used. If these were recycled cuvettes they were thoroughly rinsed in 95% ethanol and placed at approximately 37°C for 30 minutes to dry. Dried cuvettes were then placed on ice for 5 minutes. Between 2 and 4 μL of DNA was placed in the cuvettes with 40 μL of *E. coli* cells and the solution was incubated on ice for 2 minutes. Alternatively, the DNA sample was pre-incubated with the *E. coli* cells in microcentrifuge tubes for several minutes before being placed in the cuvettes. The external metal plates of the electroporation cuvettes were dried and the cuvette placed in the slide. The following parameters were used:

capacitance	25 μF (max)
voltage	5 kV (max)
resistance	400-800 ohms

Immediately following electroporation 0.5 mL of SOC media was added and cells were resuspended using a Pastuer pipette before being transferred to a sterile microcentrifuge tube and incubated at 37°C with aeration for 30 minutes. Cells were plated on LB^{amp} plates and incubated at 37°C overnight.

2.3.16 Oligonucleotides and Primers

Oligonucleotides for mutagenesis were obtained from the Separation Science Unit, Massey University. Primers for sequencing were either obtained from Bio Rad or specific primers from Oligos Etc. Inc., Wilsonville, USA.

2.3.17 Isolation of Oligonucleotides by Reverse-phase Chromatography on a Silica Gel

Oligonucleotides were cleaned using Sep-Pak 18 following methods described in Sambrook *et al.* (1989).

2.3.18 SDS-polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gels (12%) were prepared as follows:

Separating gel volumes

7.5 µL	TEMED
75 µL	10% ammonium persulphate
150 µL	10% SDS
3.5 mL	water
3.75 mL	Tris separating buffer
7.5 mL	30% acrylamide solution

Ammonium persulphate was added last to initiate gel polymerisation.

Stacking gel volumes

2 µL	TEMED
25 µL	10% ammonium persulphate
50 µL	10% SDS
0.67 mL	30%
1.25 mL	Trizma Stacking gel buffer
3.0 mL	water

Gel (8 X 10 cm) were run using apparatus purchased from Hoefer Scientific Instruments Ltd (Mighty Small - model SE250). SDS-polyacrylamide gels were run in tank buffer. A current of 5-10 mA was applied until the dye front indicated that stacking had occurred, which usually took about 30 minutes. The current was then increased to between 10-20 mA until the dye reached the bottom of the gel (approximately 2 hours). These gels were stained for 1-2 hours with Coomassie brilliant blue R-250. Destaining was carried out using destain 1 for several minutes before destaining overnight with destain 2. Further destaining was performed using either destain 1 or destain 2.

2.3.19 Silver Staining of SDS-Polyacrylamide Gels

SDS-PAGE gels were run as described in Section 2.3.18 and were stored in 1:1 methanol:water for at least 1 hour. To make the staining solution 0.8 g of silver nitrite, dissolved in 4 mL of Milli Q water was added dropwise to a solution of 21 mL of 0.36 NaOH, which contained 2 mL of 14.8 M ammonium hydroxide. This solution was used immediately to stain the SDS-PAGE gel for 15 minutes. The stain solution was removed by washing the gel with water for 5 minutes, with regular changes of water. The gel was developed in 500 mL of water which contained 2.5 mL of 1% citric acid and 0.25 mL of 38% formaldehyde. As soon as the gel went dark (10 to 15 minutes) it was removed from the developing solution and washed with water before being returned to approximately 200 mL of 1:1 methanol:water solution which contained a few mL of acetic acid.

2.3.20 Spectrophotometric Detection of Azurin

Copper-containing azurin can be detected in relatively low concentrations because of its distinctive spectroscopic properties. A HP8452 spectroscophotometer/computer was used for spectroscopic analyses and scans were recorded between 260 nm and 700 nm. Azurin is expected to produces two distinct peaks in this.region, the first is at 280 nm which is common to all proteins which contain the amino acid residue tryptophan. The second absorption maxima is in the region of 620 nm and is characteristic of the type I or blue copper proteins. It is a result of the association of copper in the type I copper binding site and is only apparent when oxidised copper occupies this copper binding site. When pure azurin from *A. denitrificans* should produce a A_{620nm}:A_{280nm} ratio of 0.3 (Ainscough *et al.*, 1987).

2.3.21 Expression of Azurin in pCH5

Azurin was produced in JM101, RR1 and XL1 *E. coli* strains which had been transformed with pCH5. These strains were cultured overnight with ampicillin selection. A 1 mL sample from each of these cultures was transferred to 4 X 500 mL Luria broths for large-scale azurin production and 50 ml cultures for small-scale production. These were grown with ampicillin selection at 37°C with aeration to an A^{600} of approximately 1.0. IPTG was added to a concentration of 6 mg/L and the cultures were incubated for a further four hours. For large-scale azurin preparations, cells were removed from solution by centrifugation for 10 minutes at 5000*g* at 4°C in GSA centrifuge bottles. For small-scale azurin preparations, cells were pelleted by centrifugation at 5000*g* for 10 minutes in 50 mL Falcon tubes. Following centrifugation the supernatant was decanted and discarded.

2.3.22 Extraction of Azurin from Cells

Protein was removed from cells using osmotic shock. Cells were resuspended in 30 mM Tris/1 mM EDTA using 0.1 volumes of the original culture volume. The suspension was shaken for 15 minutes at room temperature and the cells were pelleted by centrifugation at 5000*g* for 10 minutes at 4°C. The supernatant, containing the azurin, was decanted from the cell pellet. The cell pellet was discarded and the supernatant of identical fractions were pooled.

2.3.23 Addition of Copper

Following the extraction of azurin from the *E. coli* expression host using osmotic shock, copper sulphate and $K_3Fe(CN)_6$ were added to a concentration of 2.5 mM and 0.1 mM respectively. Alternatively, azurin was expressed in copper rich LB media. This was prepared by adding copper sulphate to the LB media at the time of inoculation of pCH5 containing cells. A concentration of 25 mM resulted in azurin binding the copper without causing any apparent inhibition of cell growth as described in Section 3.6.6.

2.3.24 Initial Purification of Azurin by pH Change

Acetic acid (1 M) was added to the osmotic shock fractions to lower the pH to 4.1. The solution was transferred to a fresh GSA bottle and centrifuged for 15 minutes at 7500*g* at

4°C. The supernatant was drained off the pellet into a clean flask. The pellet, which consisted of proteins which were insoluble at pH 4.1, was discarded.

2.3.25 Preparation of CM Cellulose for Azurin Purification

Pre-swollen CM52 was stirred into 1.5 volumes of 0.5 M ammonium acetate buffer, pH 4.1. Fines were removed according to the manufacturers instructions and the resin was equilibrated on a Buchner funnel until the pH and conductivity of the eluate were the same as the washing buffer (0.05 M ammonium acetate buffer, pH 4.1). Before loading into a column (20 X 2 cm) the equilibrated CM52 resin was poured into a Buchner flask and degased. Once loaded the column was kept at 4°C. When not in use a 0.02% azide solution was washed through the column, to prevent bacterial growth.

2.3.26 Application of Azurin onto CM Sephadex or CM Cellulose Column

After the osmotic shock fractions had been brought to the correct copper sulphate concentration the pH and conductivity was adjusted so that it was the same as the ammonium acetate buffer. This was achieved by diluting the azurin-containing solution with Milli Q water. The conductivity was measured on a Radiometer CDM 2e conductivity meter. The azurin-containing solution was then loaded onto the column. At pH 4.1 the azurin should bind to the column so the eluate was discarded after being examined on SDS-PAGE gels. The binding of copper-azurin would be expected to produce a blue band at the top of the resin.

2.3.27 Elution of Azurin from CM Sephadex or CM Cellulose Columns

Before eluting the azurin from the column, other proteins were eluted by increasing the pH of the ammonium acetate buffer. Elution was performed using a stepwise system rather than a gradient system. The pH of the elution buffer was raised to 4.5 and then to 4.8.

After washing at pH4.8 the pH was raised to 5.1 to elute azurin from the column. Fractions were collected using a Pharmacia fraction collector. Theoretically azurin can be determined using spectrophotometric methods which identify the protein by the appearance of a wavelength maximum in region of 620 nm. On several occasions however, no such

maximum could be seen, although polyacrylamide gels indicated the presence a protein corresponding to the size of azurin.

Once the azurin had been eluted from the column, the column was washed with 2 M NaCl. This removed all remaining proteins. The column was then re-equilibrated with acetate buffer pH 4.1.

2.3.28 Preparation of DEAE Cellulose Column for Azurin Purification

Pre-swollen DEAE 52 was mixed with an excess of 0.5 M ethanolamine buffer (pH 9.2). The fines were removed and 5 mM ethanolamine buffer (pH 9.2) was added. The resin was mixed with the buffer and left to stand for approximately 40 minutes. The remaining fines were removed. This process was repeated several times until no fines were apparent.

2.3.29 Application of Azurin to DEAE Cellulose Column

Before chromatography, the azurin was equilibrated in 40 mM diethylamine buffer, pH9.0, by dialysis. At pH9.0 azurin, which has a pI of 5.5, would be expected to bind to the column as it should be negatively charged. However the azurin appeared to pass through the matrix as it could be detected in fractions immediately after the void volume of eluate had passed through the column.

2.3.30 Gel Filtration

A AcA 54 Ultragel from IBF Biotechnics was used for gel filtration. Acetic acid buffer (50 mM, pH 5.1) was used.

2.3.31 Concentration of Protein Containing Solutions

Protein was concentrated using Amicon ultrafiltration stirred cell concentraters and Amicon YM10 membranes.

2.3.32 Atomic Absorption

Atomic absorption spectroscopy of azurin samples was performed by Assoc.Prof. Roger Reeves using a GBC 950AA atomic absorption unit.

2.3.33 Protein Sequencing

Azurin was sequenced using an applied Biosystems Model 470A Protein Sequencer with an on-line 120 PTH-analyser.

2.4 CALCULATIONS

2.4.1 Calculation for Amount and Purity of DNA Present in a TE Buffer or Water Solution

amount of DNA present in a solution
= A_{260} X dilution factor X 50 (µg of DNA/ 1 absorption unit)

DNA which is not contaminated by protein has $A_{260/A280}$ ratio of 1.8. A lower ratio indicates a degree of contamination.

2.4.2 Calculation for Quantity of Azurin Present in a Solution

The number of moles of azurin present in a solution
= (A_{620}/molar extinction coefficient x length of light path) x (# of mL's of solution/1000 mL)
= (A_{620}/5100 M^{-1} cm^{-1} x 1 cm) x # of mL's of solution/1000 mL

The grams of azurin present in a solution
= number of moles of azurin x molecular weight
= number of moles of azurin x 14,600

2.4.3 Calculation of Purity of Azurin in a Solution

The A_{620}:A_{280} ratio for pure azurin from *Alcalagenes denitrificans* is 0.3 (Ainscough *et al.*, 1987).

Purity is calculated as shown below;

% purity = (A_{620}:A_{280})/ 0.3 x 100

Chapter 3 : RESULTS

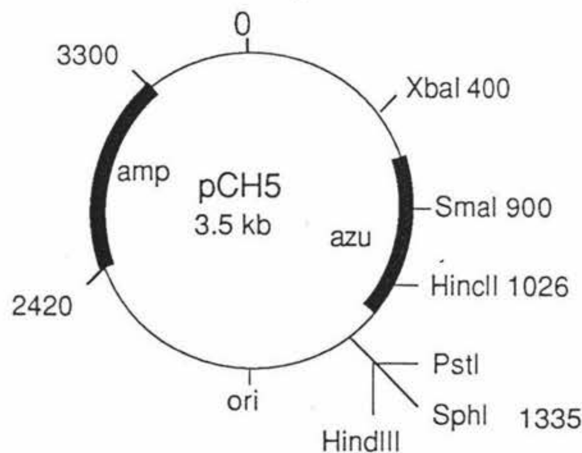
3.1 INTRODUCTORY EXPERIMENTS

3.1.1 Expression Plasmid pCH5

The plasmid pCH5, which carries an azurin coding sequence, was obtained from Hoitink and Canters, Leiden, The Netherlands (Hoitink pers. com). This plasmid was derived through a series of steps. The first step was the cloning of the 1.8 kbp ClaI fragment, which contained the coding sequence for azurin from the genomic DNA of *Alcaligenes denitrificans*, into the AccI site of pUC19. This clone was called pCH1 (Yanisch-Perron *et al.*, 1979). Modifications to the pCH1 expression vector were performed by Canters and others to increase the yield of the azurin gene product. These modifications included the introduction of a Pst1 site 50 nucleotides upstream of the start codon for azurin transcription and a stop codon, introduced in frame with the open reading frame for LacZ. The introduction of this stop codon prevented the formation of a fusion product between azurin and the LacZ gene product. This modified azurin coding sequence was cleaved from the vector using Pst1 and Xba1 which removed a 0.9 kbp fragment. This 0.9 kbp azurin coding fragment was cloned into pUC19. A SmaI restriction site from the pUC19 polylinker was removed from this construct by cleaving the plasmid with BamHI and EcoRI. The resulting construct was called pCH5 and is shown in Figure 3.1 (Hoitink pers. com). *E. coli* cells transformed with this plasmid can be selected for ampicillin resistance.

Figure 3.1

Map of Azurin Expression Vector pCH5

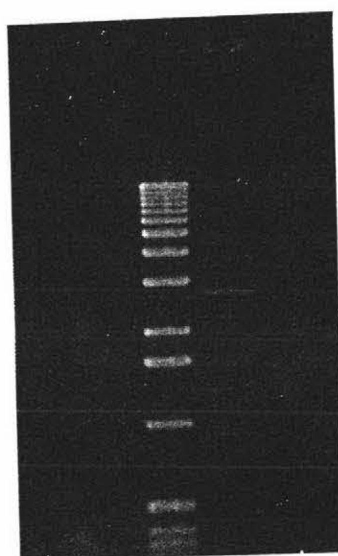


3.1.2 Detection of the 0.9 kbp DNA Fragment Coding for Azurin

The presence of the azurin coding sequence fragment was verified by cleaving pCH5 DNA obtained from Canters with XbaI and HindIII. These enzymes cleave either side of polylinker releasing a 0.9 kbp fragment which contains the azurin coding sequence. The expected 0.9 kbp fragment was observed as shown in Figure 3.2.

Figure 3.2

Verification of the Presence of the 0.9 kbp Fragment in pCH5



pCH5 DNA (1.0 μg) was digested with XbaI and HindIII. The digested DNA was analysed by electrophoresis through a 1.0% agarose gel. Lane 1 has a BRL DNA standard ladder. Lane 2 reveals partially digested pCH5 DNA, revealing the 0.9 kbp azurin coding insert, the 2.6 kbp vector fragment and a 3.5 kbp band corresponding to linearised pCH5.

3.1.3 Transformation of the Azurin Expression Plasmid pCH5 into *E. coli* (XL-1) Cells

The expression plasmid pCH5 was transformed into *E. coli* (XL1) cells made competent by treatment with $CaCl_2$, as described in Section 2.3.12. Transformants were selected by growth on ampicillin plates. Small scale plasmid preparations were performed on 5 mL cultures from several transformed colonies as described in Section 2.3.2. The resulting DNA was enzymatically digested with HindIII and XbaI and analysis of these digests using gel electrophoresis revealed the presence of a 0.9 kbp azurin coding fragment and a 2.6 kbp vector fragment for all the colonies.

3.1.4 Large-Scale Preparation of the Azurin Expression Vector pCH5

A standard large-scale plasmid preparation of pCH5 DNA was performed to provide a single consistent source of plasmid DNA. One litre of medium was inoculated with 0.1 mL of overnight culture and plasmid DNA was purified as described in Section 2.3.4. The quantity and purity of this DNA was determined by measuring the absorbance of a diluted sample at 260 and 280 nm and these values are indicted below. Quantity and purity of DNA were calculated as described in Section 2.4.1 and revealed that 95 μg of DNA, with some protein contamination, was produced.

absorbance value at 260 nm	0.105
absorbance value at 280 nm	0.092
ratio of $A_{260}:A_{280}$	1.14

3.2 AZURIN EXPRESSION

3.2.1 Plasmid Transformation for Azurin Expression

DNA from the large-scale plasmid preparation was transformed into *E. coli* XL-1 cells made competent using the $CaCl_2$ method as described in Section 2.3.13. Fresh transformations were performed for each large-scale azurin preparation as it had been reported that the plasmid is unstable in *E. coli* cells (Hoitink pers. com).

3.2.2 Large Scale Azurin Preparations Using *E. coli* XL-1 Strain

E. coli XL-1 cells, transformed with pCH5, were grown in 2 litres of LB broth and expression of azurin was induced with IPTG as described in Section 2.3.21. Azurin was extracted using osmotic shock treatment as described in Section 2.3.22 and purified as described in Sections 2.3.24, 2.3.25 and 2.3.27. Azurin was quantified during purification by the measurement of the A_{620} of appropriate fractions as described in Section 2.4.3. The method of purification and the amount and purity of azurin produced are shown in Table 3.1.

Table 3.1

Summary of Azurin Production Using the pCH5-*E.coli* (XL-1) Expression System				
Preparation Number	Weight of cells	columns used	amount of azurin/2 L	purity of azurin
1	12.4	CM cellulose	-	-
2	14.1	CM cellulose	-	-
3	14.0	CM cellulose, DEAE cellulose, gel filtration	0.5 mg	56 %

Three separate attempts were made to express azurin from *A. denitrificans* using the pCH5-*E. coli* (XL-1) expression system. The first two attempts did not yield an absorbance maximum at 620 nm, suggesting that azurin was not being expressed from the plasmid. The third preparation gave a measurable absorption reading at 620 nm, though no distinctive peak was observed. The amount of azurin produced was calculated as described in Section 2.4.2, to be 0.5 mg. The purity of this azurin fraction was calculated, as described in Section 2.4.3, to be approximately 50%, when compared to the purity achieved when this protein was purified from *A. denitrificans*.

In the early stages of purification the concentration of azurin was too low to be detected by measurement of a maximum at 620 nm, so yields were not calculated until after the protein had been passed through the CM cellulose column. As the azurin becomes more concentrated its presence should be detectable by eye due to the intense blue colour of the protein, which corresponds to the 620 nm absorbance maximum. During the third attempt of protein purification a blue colour was observed although this was of a much lower intensity than the results of Hoitink *et al.* (1990) suggested. A sample of the azurin- containing fraction which had been isolated from the CM cellulose column from the third azurin preparation was analysed using polyacrylamide gel electrophoresis and the result is shown in Figure 3.3.

The result of the PAGE analysis shown in Figure 3.3 suggests that the protein solution has a high degree of purity, in contrast to the 56% purity calculated by spectral analysis. This apparent discrepancy could at least in part be caused by variable intensity of staining between the two protein bands. Alternatively it could be due to the incomplete saturation of the protein with copper, as the 620 nm peak is totally related to binding of copper to the protein.

Figure 3.3

Polyacrylamide Gel Analysis of Azurin Purified from the pCH5-*E coli* (XL-1) Expression System

A sample of the partially purified protein eluted the CM cellulose column from the third preparation of azurin using the *E. coli* (XL-1)/ pCH5 expression system, was examined by polyacrylamide gel electrophoresis, followed by silver staining of the gel. Lane 5 shows a set of molecular weight markers while lane 5 shows that the main protein present in these fractions has a molecular mass corresponding to azurin (14.6 kDa) and a minor band of a contaminating protein of approximately 36 kDa.

On the basis of the absorbance value at 620 nm, where oxidised copper binding azurin has an absorption maximum, the amount of azurin obtained was estimated to be 0.5 mg from 2 litres of culture as described in Section 2.4.3. This was a considerably lower yield than obtained by Hoitink *et al.* (1990), where a yield of 12.5 mg of azurin per litre of cell culture was reported. The only difference between the protocol used here and that used by Hoitink *et al.* (1990) was the use in this study of *E. coli* XL-1 cells instead of *E. coli* JM101.

3.2.3 Large Scale Azurin Preparation Using *E. coli* JM101 Strain

As the yield of protein from the pCH5-*E. coli* (XL-1) expression system used in this study gave considerably lower yields than those reported by Hoitink *et al.* (1990) who used the pCH5-*E. coli* (JM101) expression system, a preparation was made using an *E. coli* (JM101) host. The results from the first two preparations are summarized Table 3.2.

Polyacrylamide gel electrophoresis indicated that there was little contamination of the azurin with the majority of protein revealed by silver staining of the gel migrating as a single band with a mobility corresponding to the molecular weight of azurin and was essentially identical to the silver stained gel shown in Figure 3.2.

Table 3.2

Summary of Azurin Production Using the pCH5-*E. coli* (JM101) Expression System				
Preparation Number	Weight of cells	columns used	amount of azurin/2 L	purity of azurin
1	18.9	CM cellulose	0.5 mg	69%
2	-	CM cellulose	5.5 mg	37%

Two attempts were made to express azurin from *A. denitrificans* using the pCH5-*E. coli* (JM101) expression system. The yield and purity of azurin was calculated using the 620 nm value and the $A_{620:280}$ ratio and the calculation described in Sections 2.4.2 and 2.4.3. Both the purity and yield were lower than reported by Hoitink *et al.* (1990) who reported 12.5 mg of azurin per 1 litre of cell culture.

3.2.4 Protein Sequencing

A sample of the purified protein was analysed by N-terminal protein sequencing as described in Section 2.3.33. The sequence of the first eight amino acids was obtained and proved to be identical to the first eight amino acids of *A. denitrificans* azurin as reported by Hoitink *et al.* (1990) and shown in Table 3.3. This indicates that the azurin from *A. denitrificans* had been processed correctly by the *E. coli* host cells. Sequence analysis revealed no evidence of secondary sequences, suggesting that the protein was of a relatively high purity, in contrast to the results indicated by the value of the $A_{620:280}$ ratio and supporting the result obtained by gel electrophoresis.

Table 3.3

Comparison of Azurin Sequence from *A. denitrificans* as Determined in this Study by N-terminal Protein Sequencing and by DNA Sequencing as Reported by Hoitink *et al.*, (1990)	
Sequence Determined for Protein Purified in this Study	ala - glu - cys - gln - ala - thr - iso - glu
Sequence Reported by Hoitink *et al.* (1990)	ala - glu - cys - gln - ala - thr - iso - glu

The protein sequence of the N-terminus of azurin from *A. denitrificans* was determined by direct sequencing of the protein produced in *E. coli*. The sequence reported by Hoitink *et al.*, (1990) was obtained by deduction from the DNA sequence of the gene, assuming that the 19 amino acid pre-sequence was correctly processed. This result shows that the N-terminal signal peptide for translocation across the *A. denitrificans* cell

membrane has been correctly processed in the *E. coli* host used to express azurin in this work.

3.3 CLONING OF THE AZURIN GENE INTO pHN1403

3.3.1 Large Scale Plasmid Preparation of pHN1403

As the pCH5-*E. coli* (XL-1) or pCH5-*E. coli* (JM101) expression system appeared to produce low yields of azurin an alternative expression system that become available in the laboratory through Dr.H.H.Nicholson was used. This expression system used the plasmid pHN1403, a pUC119 derivative (Muchmore *et al.*, 1989). It had been used successfully for the large-scale expression of T4 lysozyme (Poteete *et al.*, 1991). To use this vector the azurin coding sequence from pCH5 had to be cloned into the vector via a third plasmid as the polylinkers of the pCH5 and pHN1403 plasmids were in the opposite orientations.

The plasmid pHN1403 was prepared by standard large-scale plasmid preparation procedures as described in Section 2.2.4 gave a yield of 260 µg of DNA with an $A_{260:280}$ ratio of 1.6.

3.3.2 Cloning of the Azurin Insert into the Expression Vector pHN1403

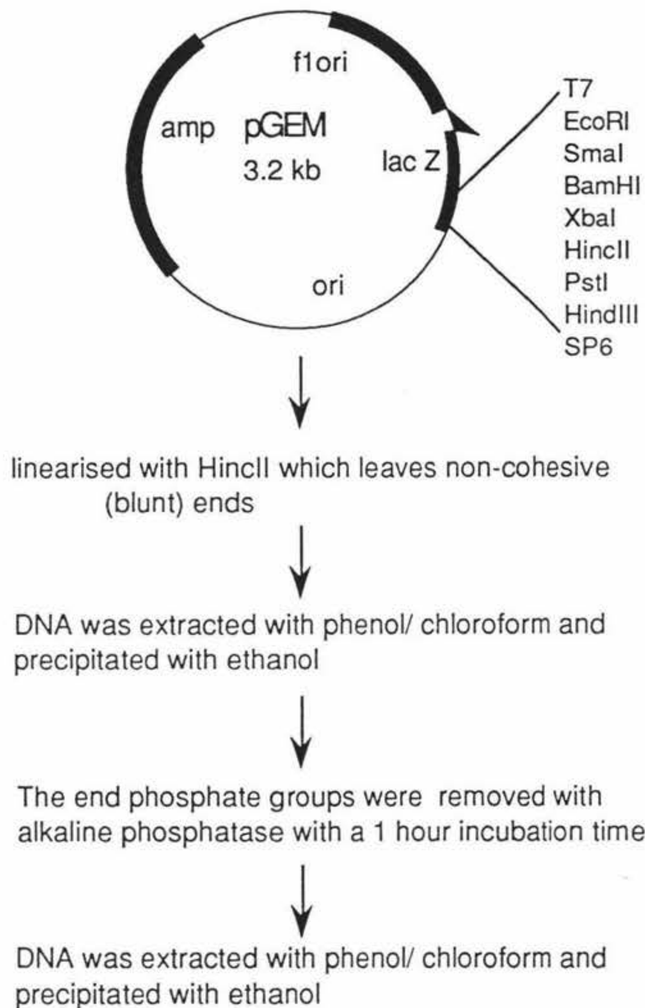
Direct cloning of the 0.9 kbp azurin-coding fragment from pCH5 into pHN1403 was almost impossible as the fragment could not be cloned in the correct orientation to allow gene expression as polylinkers of the two expression vectors are in opposite orientations to each other with respect to their promotor systems. This could be overcome by cleaving the 0.9 kbp azurin coding fragment which had been cloned into the polylinker of pCH5 and cloning this fragment by blunt end ligation into the intermediate vector, which would allow the fragment orientation to be reversed. The plasmid pGEM3Zf(+) was readily available and suitable for this purpose.

3.3.3 Preparation of pGEM Vector

The vector pGEM3Zf(+) was prepared for the insertion of the 0.9 kbp azurin coding fragment as shown in Figure 3.4.

Figure 3.4

Preparation of pGEM3Zf(+) for Blunt End Insertion of 0.9 kbp Fragment Containing the Azurin Coding Sequence



The vector pGEM3Zf(+) (from Promega), was completely digested with the restriction enzyme HincII which cleaves once in the polylinker and produces non-cohesive (blunt) ends. DNA was extracted using phenol/chloroform, precipitated with ethanol and the end group phosphates were removed with alkaline phosphatase as described in Section 2.3.11, with an incubation time of one hour. DNA was again extracted using phenol/chloroform and precipitated with ethanol.

3.3.4 Preparation of Azurin Fragment by Gel Excision

The azurin coding sequence was obtained by two methods, the first of which was by gel excision, performed as described in Section 2.3.1.

3.3.5 Preparation of Azurin Fragment by Complete Digestion of Vector

The second procedure used for preparing the 0.9 kbp fragment containing the azurin coding sequence involved complete digestion of the pCH5 vector as shown in Figure 3.5.

3.3.6 Ligations of the 0.9 kbp Fragment from pCH5 into pGEM

Attempts were made to clone the 0.9 kbp azurin-coding fragment into pGEM. The ligations reactions for this are summarized in Table 3.4. The 0.9 kbp fragments used in these reactions were produced by two different methods as described in Sections 3.3.4 and 3.3.5.

Table 3.4

Ligation Reactions for Coning the 0.9 kbp Azurin-Coding Fragment into the Expression Vector pHN1403								
	1	2	3	4	5	6	7	8
azurin insert	4	4	-	-	1	-	-	-
pGEM	2	1	-	-	-	1	-	-
pHN1403	-	-	-	-	-	-	1	-
diluted pHN1403	-	-	-	-	-	-	-	1
ligase	1	1	-	-	-	-	-	-
5 X ligase buffer	4	4	4	4	4	4	4	4
water	9	10	15	15	15	15	15	15
ligation 1	-	-	1		-	-	-	-
ligation 2	-	-	-	1	-	-	-	-
total	20	20	20	20	20	20	20	20

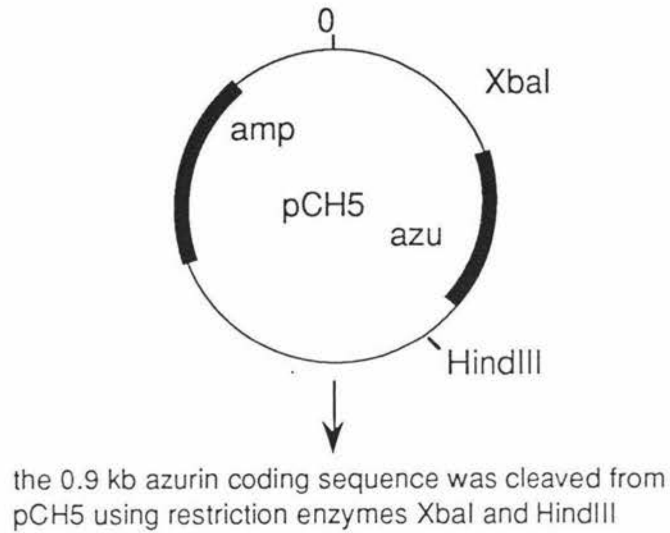
These ligation reactions were incubated at 16°C overnight. Ligations 1 and ligations 2 act as dilutions of the original ligations and were prepared by removing 1 μL from the first two ligation reactions and adding water and ligase buffer to a volume of 20 μL. The concentration of pGEM used was 250 ng/μL (0.25 pmoles/μL). The concentration of 0.9 kbp insert ends was approximately 500 ng/μL (1.8 pmoles/μL). All volumes recorded in the table are given in μL.

Legend for Figure 3.5

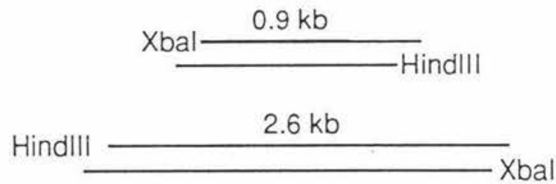
The expression vector pCH5 was incubated with the restriction enzymes XbaI and HindIII, which cleave the 0.9 kbp fragment from the vector. The restriction enzyme PvuI was added to the restriction digest. This cleaves the 2.6 kbp pCH5 vector fragment into two fragments without cleaving the 0.9 kbp azurin coding fragment. DNA was extracted with phenol/chloroform and precipitated with ethanol.

Figure 3.5

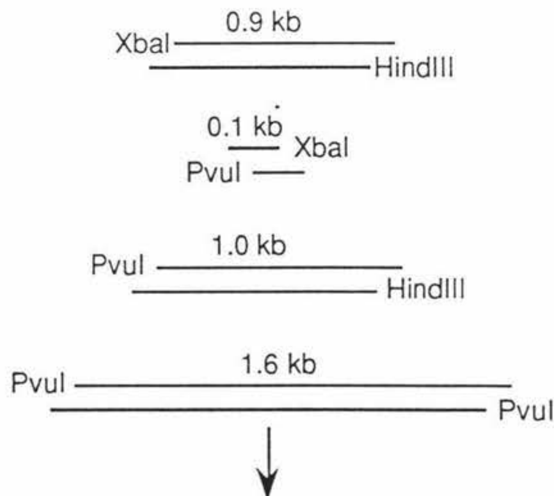
Preparation of the 0.9 kbp Azurin Coding Sequence From pCH5



the 0.9 kb azurin coding sequence was cleaved from pCH5 using restriction enzymes XbaI and HindIII



PvuI restriction enzyme was added to the digest and buffer conditions changed for this reaction



DNA was extracted with phenol/chloroform and precipitated with ethanol

When the incubations were complete, the ligations reactions were microdialysed for 30 minutes as described in Section 2.3.15. Four (4) µL of each ligation reaction was transformed into 20 µL of *E. coli* JM101 cells by electroporation as described in Section 2.3.16 and these cells were plated on LB agar with ampicillin selection. Standard small-scale plasmid preparations were performed, as described in Section 2.3.2, for the resulting colonies. The resulting DNA was digested with HincII and EcoRI which would removed a 0.9 kbp fragment from the 3.2 kbp pGEM vector. The resulting colonies were analysed but gave inconsistent and often unexplainable results. Many colonies appeared to contain the pGEM vector which did not contain the 0.9 kbp azurin coding fragment. Over 80 colonies were analysed and the pGEM-0.9 kbp azurin coding fragment construct was not observed.

3.4 CLONING DIRECTLY INTO pHN1403 FROM pCH5

3.4.1 Partial Digests of pHN1403

The strategies described in Section 3.3 did not result in the isolation of a clone with pGEM containing the 0.9 kbp azurin coding fragment. A strategy for cloning the azurin sequences directly into pHN1403, eliminating the need for blunt end ligation into pGEM was designed. The first stage in this cloning procedure involved the preparation of the pHN1403 expression vector which had to be partially digested with PstI. A partial digest of pHN1403 with PstI was found to be obtainable by using 0.1 units of enzyme per digest reaction and incubating this reaction at 37°C for 60 minutes. The conditions for partial digestions of pHN1403 with PstI is shown in Figure 3.6 and the procedure of preparation of pHN1403 for partial digestion is shown in Figure 3.7.

3.4.2 Preparation of the Azurin Insert for Cloning in Partially Digested pHN1403

The 0.9 kbp fragment which carried the azurin coding sequence was prepared such that it had 1 XbaI and 1 PstI cohesive end. The preparation of this insert is shown in Figure 3.8

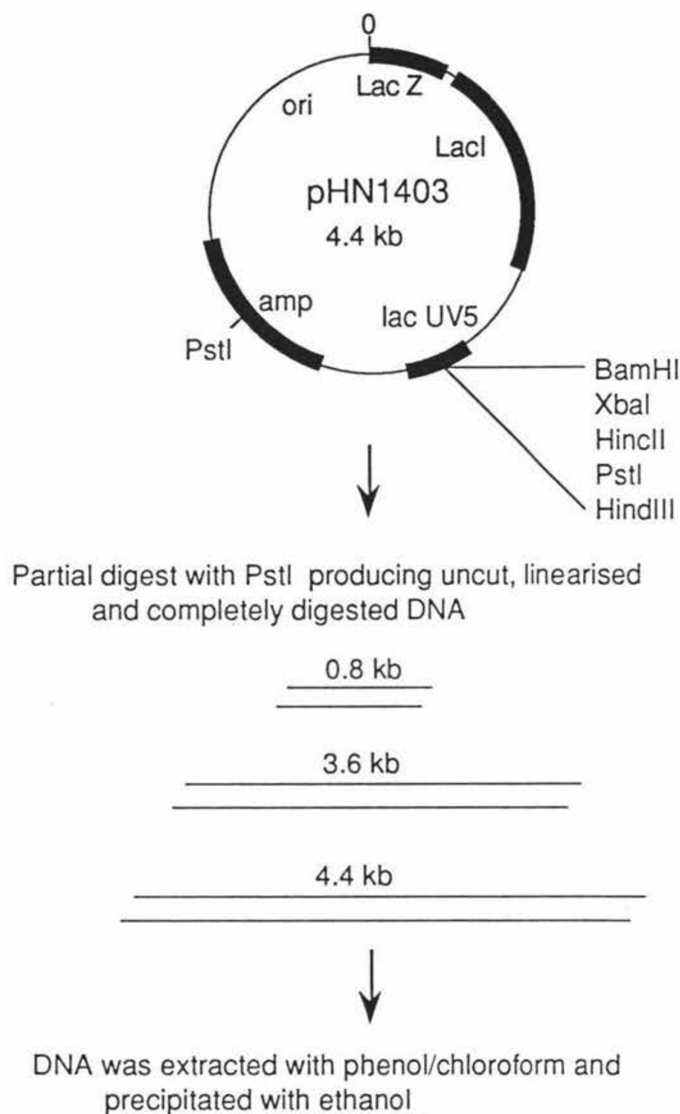
Figure 3.6

Partial Digest Condition for pHN1403 with PstI

The correct conditions for a partial digest of pHN1403 with PstI were assertained by incubating DNA with 0.1 units of enzyme per digest reaction, incubation times of 5, 20 and 60 minutes at 37°C were used as shown in Lanes 3, 2 and 1 respectively. Examination of these restriction digests on a 1% agarose gel indicated that when the DNA was digested for 5 or 20 minutes the vector remained largely undigested, while after 60 minutes full length linearised pHN1403 and the products of complete digestion were both observed. Lane 4 contains a series of standard molecular weight markers.

Figure 3.7

Preparation of pHN1403 Expression Vector for Cloning of 0.9 kbp Azurin Coding Fragment From pCH5



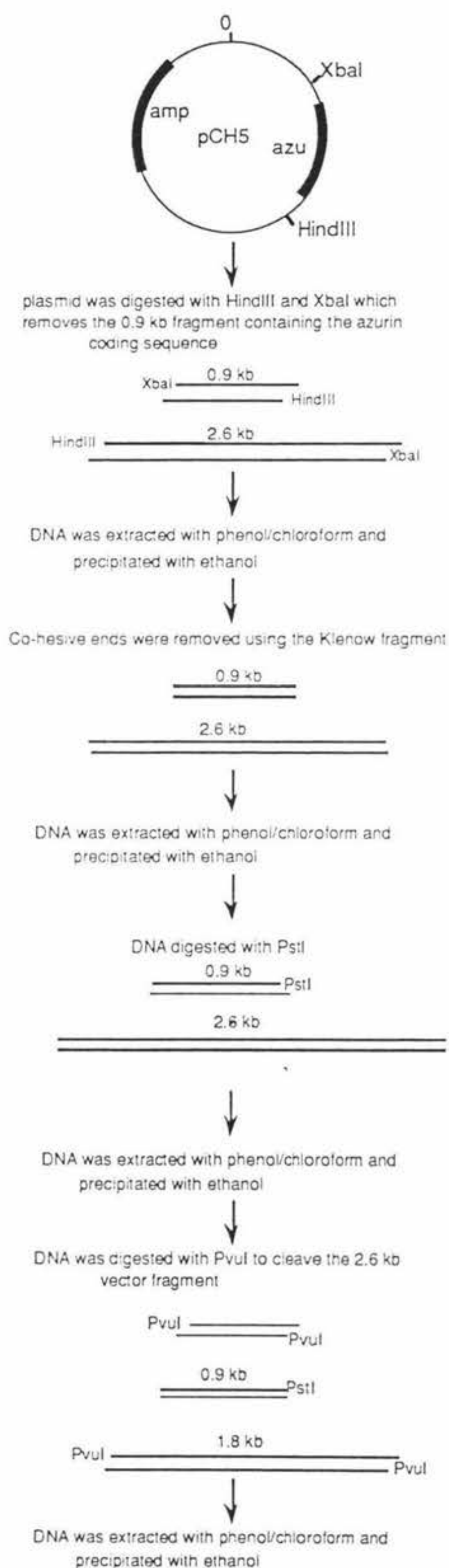
The pHN1403 expression vector was prepared for direct cloning of the 0.9 kbp azurin coding fragment from pCH5 by preparing a partially digested vector. In theory some pHN1403 would be cleaved exclusively at the PstI site in the polylinker. Other pHN1403 molecules would either be cleaved uniquely at the alternative PstI sites, cleaved at two or three site or remain completely undigested.

Legend for Figure 3.8

The 0.9 kbp fragment, which contained the azurin coding sequence was cleaved from pCH5 using HindIII and XbaI. The cohesive ends left by these enzymes were removed by the addition of the Klenow fragment of DNA polymeraseI DNA was further digested with PstI which cleaves the 0.9 kbp fragment close to the HindIII. The 0.9 kbp fragment therefore contains one blunt and one PstI cohesive end.

Figure 3.8

Preparation of the 0.9 kbp Azurin Coding Fragment for Cloning into Partially Digested pHN1403



3.4.3 The Ligation of the 0.9 kbp Fragment into Partially Digested pHN1403

To clone the azurin gene into pHN1403 a series of identical ligations were using the reaction mixture in Table 3.5.

Table 3.5

Ligase Reactions for Cloning the 0.9 kbp Azurin Coding Fragment Directly into pHN1403		
ligase	1 μL	1 unit
ligase buffer	2 μL	
pHN1403 (PstI digest)	2 μL	20 ng
0.9 kbp azurin coding fragment	4 μL	
water	13 μL	

These ligase reactions used the expression vector pHN1403 which had been partially digested with PstI and the 0.9 kbp azurin coding fragment which possessed one XbaI cohesive end and one PstI cohesive end which was prepared as described in Figure 8

3.4.4 Transformation and Examination of the Ligation Reactions

A 2 μL fraction of each ligation described in Section 3.4.3 was transformed into *E. coli* JM101 cells by electroporation and selected for by growth on LB^{amp} plates. Twelve colonies grew and standard small scale preparations were performed as described in Section 2.3.2. The resulting DNA was enzymatically digested with HincII, which should cleave in the pHN1403 vector fragment and the 0.9 kbp insert fragment once each, producing a 0.6 kbp and a 3.5 kbp fragment. Digests were analysed by gel electrophoresis as described in Section 2.3.8. The expected cleavage pattern was not observed indicating that the 0.9 kbp azurin coding fragment had not been cloned into the pHN1403. The colonies that grew appeared to contain only pCH5 suggesting that some of this plasmid had remained undigested when the 0.9 kbp fragment containing the azurin coding sequence had been prepared. This fragment was never been separated from the pCH5 vector by gel excision. Instead the vector had been supposedly completely digested with PvuI as shown in Figure 3.5.

3.4.5 Ligation of Azurin Insert from pCH5 into pGEM

The 0.9 kbp azurin coding fragment containing the azurin coding sequence was cleaved from 5 μg of pCH5 using XbaI and HindIII. PvuI was added. This cleaves the vector

fragment into two but does not cleave the 0.9 kbp azurin coding fragment. Ten (10 μg) of calf thymus tRNA was added to prevent loss of DNA during ethanol precipitation The volume at this stage was 15 μL. Following phenol/chloroform extraction and ethanol precipitation the pellet was resuspended in 20 μL of annealing buffer. The fragments were end filled using by adding 20 μL of 2 X synthesis buffer and 1 μL (1 unit) of the Klenow fragment. Following overnight incubation at 37°C, the Klenow fragment was heat inactivated at 65°C. DNA was extracted with phenol/chloroform and precipitated with ethanol. The resulting DNA pellet was resuspended in 10 μL of TE and digested overnight with PvuI to cleave the pCH5 vector fragment. DNA was extracted with phenol/chloroform and precipitated with ethanol.

Simultaneously a HincII restriction digest was performed on pGEM and the 5' phosphates were removed by incubating DNA for 15 minutes with bovine alkaline phosphatase. Ligation reactions were performed as shown in Table 3.6.

Table 3.6

Ligation Reactions Performed to Clone the 0.9 kbp Azurin Coding Fragment into pGEM

	1	2	3	4	5	6
ligase	1	1	-	-	1	-
pGEM	1	1	-	-	1	1
1 mM DTT	1	1	-	-	1	1
0.9 kbp azurin coding fragment	1	2	-	-	-	1
ligase buffer	2	2	-	-	2	2
water	4	3	9	9	5	5
ligation reaction 1	-	-	1	-	-	-
ligation reaction 2	-	-	-	1		-
total	10	10	10	10	10	10

Ligation reactions were performed to clone the 0.9 kbp azurin coding fragment from pCH5 into pGEM. This fragment was linearised by HincII which uniquely in pGEM polylinker and leaves blunt ends. Ligations 1 and ligations 2 act as dilutions of the original ligations and were prepared by removing 1 μL from the first two ligation reactions and adding water and ligase buffer to a volume of 20 μL. All ligations were incubated at 16°C overnight.

At the completion of the incubation time each ligation was microdialysed and a fraction of each was electroporated into 40 μL of *E. coli* JM101 cells and plated onto LB^{amp} plates. Only a few colonies appeared on the negative control plates while over 50 appeared on the experimental plates. Standard small scale plasmid preparations as were performed on

twenty four of the resulting colonies as described in Section 2.3.2. The DNA from these preparations were examined to identify whether the azurin coding fragment had been cloned into the pHN1403 expression vector. Clones which contained the 0.9 kbp azurin coding fragment cloned in both orientations were isolated and further enzymatic digestions were performed to confirm that they were the correct clones. The pGEM plasmid which had the 0.9 kbp azurin coding fragment cloned into in the correct orientation would be called pT201, while the complementary plasmid would be called pT202.

DNA was digested with the restriction enzyme HincII, which cleaves once in the polylinker and once in the azurin insert. If the azurin gene has been cloned in the correct orientation a 0.6 kbp fragment would be cleaved from the 4.1 kbp vector, leaving a 3.5 kbp fragment. If the insert has been inserted in the opposite orientation then fragments of 0.3 kbp and 3.8 kbp would be produced. DNA was also digested with SmaI which cleaves once in the middle of the azurin coding sequence and once at one end of the pHN1043 polylinker. A similar sized fragment of approximately 0.5 kbp would be cleaved from the vector independent of which orientation the 0.9 kbp azurin coding fragment had been cloned into the plasmid. A BamHI/ HindIII digest removes the 0.9 kbp azurin coding fragment independently of orientation this fragment is cloned in. These digests were examined on a PAGE gel as shown in Figure 3.9.

These results appear to indicate that pT201 is the pHN1403 vector with the 0.9 kbp azurin coding fragment from pCH5 cloned into the polylinker region in the correct orientation to allow protein expression. Conversely pT202 is pHN1403 with the 0.9 kbp azurin coding fragment from pCH5 cloned in the polylinker region in the incorrect orientation to allow protein expression.

3.4.6 Cloning the Azurin Gene into pHN1403

Standard small scale plasmid preparations were performed for pT201 and pT202 as described in Section 2.3.2. The 0.9 kbp fragment which contained the azurin coding sequence was cloned from pT201 into pHN1403 a shown in Figure 3.10

Figure 3.9

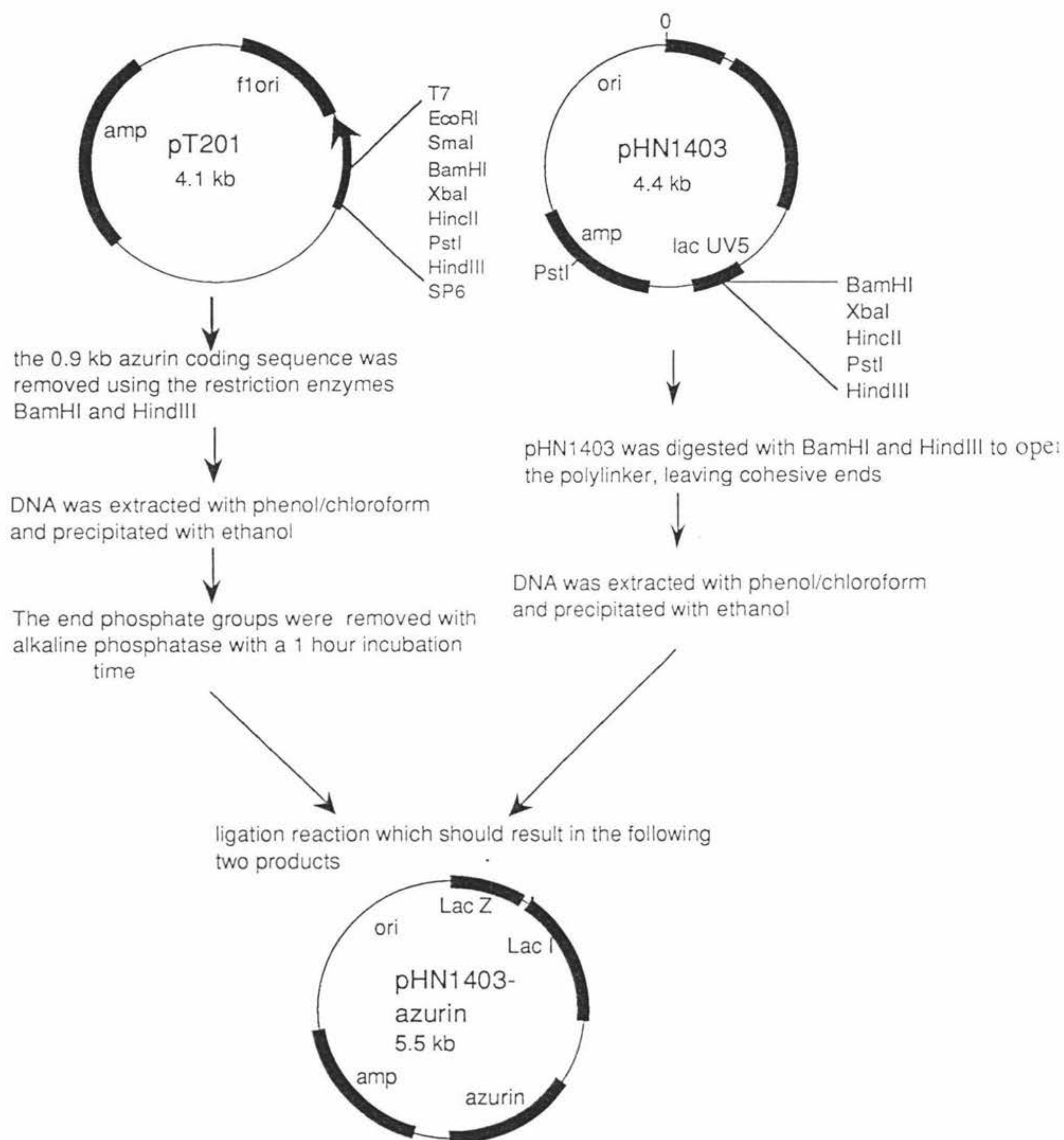
Agarose Analysis of pGEM With the 0.9 kbp Azurin-Coding Fragment Cloned into the Polylinker Region

Lane 1, 5 and 9 show BRL standard markers. Lanes 2 and 6 are SmaI digests of pT201 and pT202 respectively. The 0.5 kbp band expected in this digest is not apparent. Lanes 3 and 7 show the HincII digest of pT201 and pT202 respectively, which shows a 0.6 kbp in the pT201 plasmid and a 0.3 kbp fragment in the pT202 vector, though the later is not visible on the photograph of the PAGE gel. Lanes 4 and 8 are BamHI/ HindIII digests of pT201 and pT202 respectively and show that a 0.9 kbp fragment has been cleaved from this vector.

Legend for Figure 3.10
The 0.9 kbp azurin coding fragment was cleaved from pT201 using BamHI and HindIII. The vector fragment was further digested with PvuI which cleaves the vector into two fragments but does not cleave the 0.9 kbp azurin coding fragment. The pHN1403 expression vector was prepared by digesting this vector with BamHI and Hind III, which cleave in the polylinker providing cohesive ends complementary to those of the 0.9 kbp azurin coding fragment. This allows the azurin coding fragment to be incorporated into the pGEM vector unidirectionally. DNA was extracted with phenol/chloroform, which removed small pieces of DNA cleaved from the polylinker of the pHN1403, leaving only linearised vector

Figure 3.10

Cloning the 0.9 kbp Azurin Coding Fragment From pT201 to the Expression Vector pHN1403



3.4.7 Ligations of Azurin Insert into pHN1403

Ligations of the 0.9 kbp fragment which contains the azurin coding sequence and the pHN1403 vector, which was prepared as shown in Figure 3.10, were performed as indicated in Table 3.7.

Table 3.7

Ligation Reactions for Cloning the 0.9 kbp Azurin Coding Fragment From pT201 into Expression Vector pHN1403						
	1	2	3	4	5	6
pHN1403	1	0.5	-	-	1	-
ligase	1	1	-	-	1	1
5 X ligase buffer	2		-	-	2	2
pT201	5	4	-	-	-	2
ligation reaction 1	-	-	1	-	-	-
ligation reaction 2	-	-	-	1	-	-
water	11	12.5	19	19	6	5
total	20	20	20	20	10	10

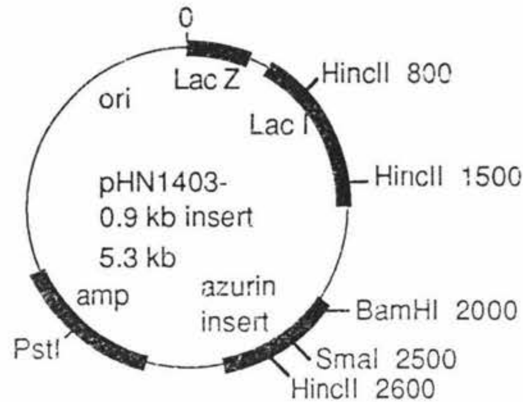
These ligation reactions were incubated at 16°C for 3 hours and were then moved to 37°C for a further 30 minutes. Ligations reactions 3 and 4 act as dilutions of the original ligations and were prepared by removing 1 µL from the first two ligation reactions and adding water and ligase buffer to a volume of 20 µL.

Following the completion of the incubations the ligation reactions were microdialysed as described in Section 2.3.14. Unfortunately ligations 2, 3 and 4 were lost during microdialysis. Ligation 1 and both controls were electroporated into *E. coli* JM101 cells. The electroporated cells were plated as 10 µL and 100 µL aliquots on to LB^{amp} plates and were incubated at 37°C overnight. A large number colonies grew on the experimental plate. Twelve colonies were selected from the experimental plate and standard small scale plasmid preparations were performed as described in 2.3.2. The resulting DNA was digested with HindIII and BamHI and this DNA was analysed on a 1% agarose gel. No photograph of this gel is available.

The resulting construct had a molecular size of 5.3 kbp. This construct is shown in Figure 3.11.

Figure 3.11

Plasmid Map of pHN1403-azu



The pHN1403 with the 0.9 kbp fragment which contained the azurin coding sequence. The numbers refer to the base number from the origin to the nearest 100.

3.4.8 Medium Scale Plasmid Preparation of pHN1403-Azu

A medium scale plasmid preparation was performed for one of these successful clones of pHN1403-Azu as described in Section 2.3.3. Calculations based on the $A^{260:280}$ ratio for the DNA samples were performed as described in Section 2.4.1 and revealed that the yield was approximately 300 µg and was slightly contaminated with protein. DNA was resuspended in 50 µL of TE and was digested with HincII, BamHI and SmaI. The results of these digests confirm that the 0.9 kbp fragment containing the azurin coding sequence had been cloned into the pHN1403 vector. These results are shown in Figure 12.

Figure 3.12

Agarose Analysis of pHN1403-Azu

The DNA from a medium scale plasmid preparation of pHN1403-Azu was digested with the restriction enzymes HincII, BamHI and SmaI. Lane 1-4 contain DNA concentration standards of 2.5, 5, 10 and 20 ng respectively. These indicate that approximately 10 ng of DNA was digested here. A summary of restriction digest reactions of pHN1403-Azu are shown in Table 3.8 Lane 5 ia a SmaI digest, lane 6 is a BamHI. HindIII digest and lane 7 is a HincII digest.

Table 3.8

Fragment Sizes of pHN1403-Azu After Restriction Enzyme Digest		
Restriction Enzyme	Expected Fragment Size	Fragment Size Observed
HincII (Lane 7)	0.6 kbp	0.6 kbp
	1.1 kbp	1.1 kbp
	2.6 kbp	2.6 kbp
BamHI/HindIII (Lane 6)	0.9 kbp	0.9 kbp
	4.4 kbp	4.4 kbp
SmaI (Lane 5)	5.3 kbp	5.3 kbp

DNA from the medium scale plasmid preparation of pHN1403-0.9 kbp azurin coding insert was digested with HincII, SmaI and BamHI/HindIII. Analysis on 1% agarose gels revealed the expected pattern of fragment sizes for these digests as shown on Figure 13.

3.4.9 Small Scale Azurin Preparations Using pHN1403-Azu

To ensure that the pHN1403-azurin clone was capable of producing azurin, cultures were grown on a small scale and treated with IPTG to induce expression from the *lac* promoter present in the pHN1403 vector, adjacent to the azurin coding sequence.

The pHN1403-azurin plasmid was introduced into *E. coli* strains RRI and JM101 by electroporation as described in Section 2.3.15. Colonies were selected and 5 mL cultures were grown overnight. From these overnight cultures aliquots of 0.5 mL were used to inoculate fresh 5 mL cultures which were grown to different cell densities prior to the addition of IPTG to induce azurin production from the *lac* promoter as indicated in Table 3.9.

On reaching the required cell density, 5 µL of 24 mg/mL (0.5 M) IPTG was added to each culture. All cultures were incubated for a further 3 hours after the induction of the last culture, so that those that were induced at lower cell densities were induced for a longer time period. The cells were harvested and resuspended in 0.5 mL of osmotic shock solution, transferred to microcentrifuge tubes and shaken at room temperature for 5 minutes. Cells were harvested by centrifugation and the supernatant containing the azurin retained. Azurin production was assessed by analysis of the supernatant by PAGE.

Table 3.9

Optical Densities (600 nm) at Time of Protein Induction		
Time (min) Prior to Addition of IPTG (120 µg)	Optical Density of JM101 (600 nm)	Optical Density of RR1 (600 nm)
40	0.287	0.286
100	0.533	0.522
150	0.816	0.763
200	1.06	0.961
225	1.08	1.13

The actual cell densities when azurin expression was induced in 5 mL cultures of JM101 and RR1 cells which contained the pHN1403-Azu expression plasmid.

In order to detect any azurin not released by the osmotic shock procedure the cell pellet was lysed as described in Section 2.3.4 for the large scale plasmid preparations. Any azurin remaining in the cells would be collected in the supernatant fraction.

3.4.10 Detection of Azurin Using Polyacrylamide Gel Electrophoresis

The production of azurin was analysed from the osmotic shock fraction and the total cell extract fraction, using 12% polyacrylamide gels as described in Section 2.3.18. There was no protein band corresponding to the molecular weight of azurin on these gels

indicating that this system was not expressing azurin. It was concluded that these clones were not producing azurin. There appears to be no obvious reason as to why this expression system failed. One possibility is that the leader sequence between the promotor and the azurin interferes with *lac* promotor. This possibility was not explored. A photograph of the polyacrylamide gel was not taken.

3.5 PHOSPHATASING EXPERIMENTS

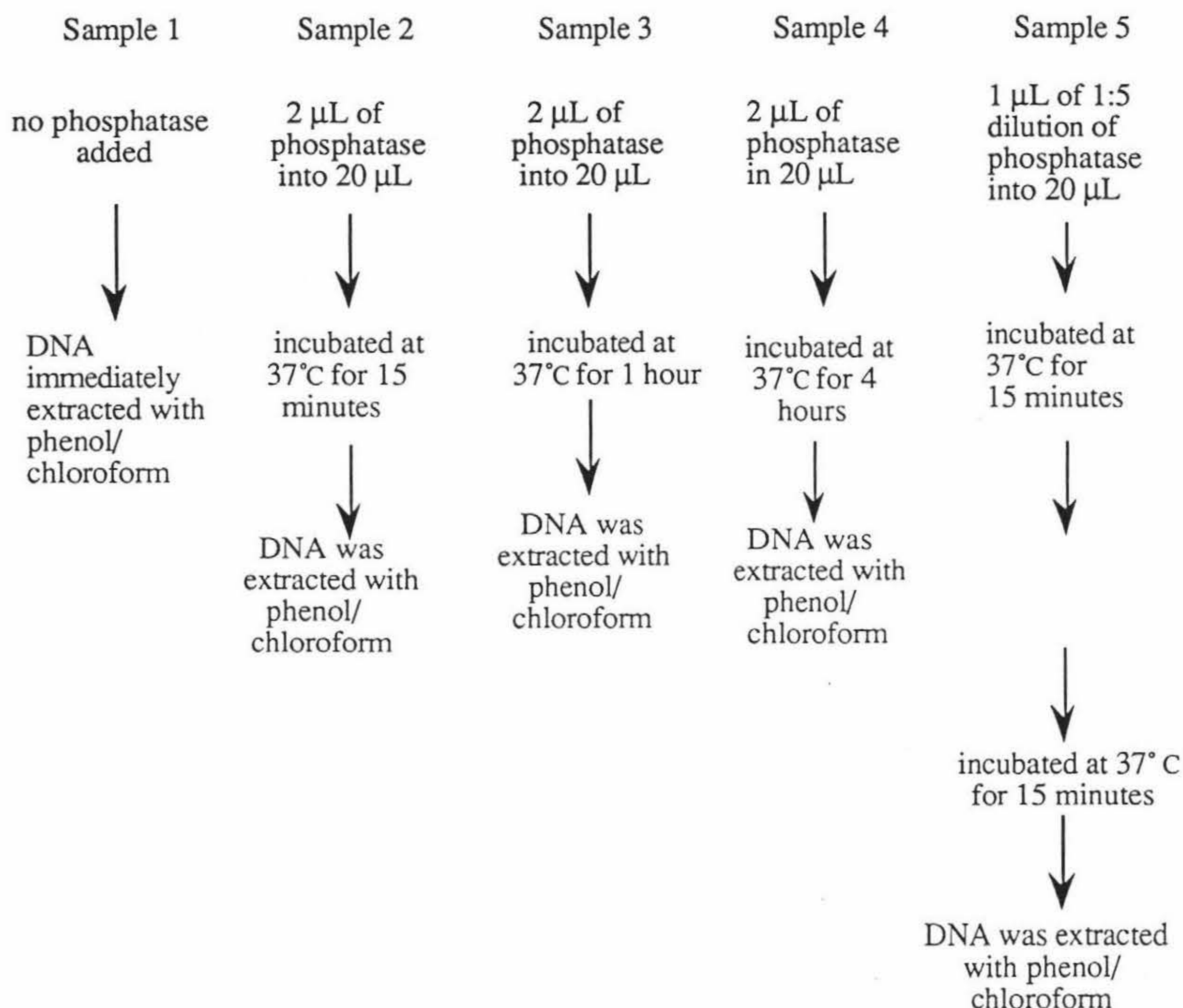
3.5.1 Different Incubation Times of pGEM With Phosphatase

Bovine alkaline phosphatase enzyme, obtained from Boehringer Mannhiem, was used to remove the free 5'-phosphates when HincII restriction digests had been performed as part of a cloning procedure. The removal of these free phosphate groups prevents the vector from religating.

In the preceding experiments several negative controls had revealed colony growth. Negative controls consisted of vector, which for this experiment was pGEM, which had been linearised by a restriction digest with a unique cleaving enzyme. This linearised vector was subsequently treated with bovine alkaline phosphatase enzyme, microdialysed as described in Section 2.3.15 and electroporated into non-ampicillin resistant cells as described in Section 2.3.16. Colony growth from the cells containing linearised vector indicated that the cells contained an ampicillin resistant plasmid which in the case of negative plates indicated that either undigested or religated plasmid was been electroporated into the cells. It had been noted that the amount of growth on the negative plates varied depending on the incubation time of the bovine alkaline phosphatase enzyme and the plasmid. An experiment to find the optimum incubation time for this enzyme was performed and is outlined in Figure 13.

Figure 3.13

The Optimum Time for Incubation with Alkaline Phosphatase for pGEM

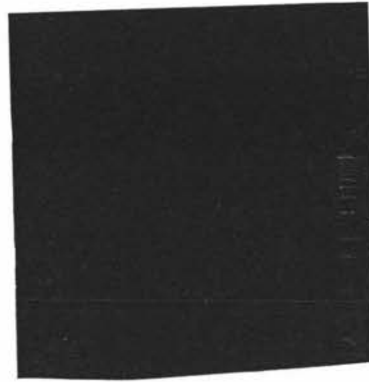


To determine the optimum time of incubation with bovine alkaline phosphatase for pGEM 5μg (20 μL) of pGEM was digested completely with HincII and aliquoted in to five fractions of 4 μL. From this point the fractions were incubated for different lengths of time before the dephosphorylation reaction was stopped by the extraction of DNA

To ensure that DNA was completely digested after being treated with the restriction enzyme HincII a fraction of each was analysed on a 1% agarose gel as shown in Figure 15. This analysis was performed after treatment with Bovine alkaline phosphatase.

Figure 3.14

DNA Prepared by Digestion with the Restriction Enzyme HincII



Following treatment with bovine alkaline phosphatase 1 µL fractions of each ligation were analysed using gel electrophoresis. This indicated complete digestion of pGEM had occurred with HincII, proving that a difference in the number of colonies resulting from cells which contained linearised pGEM was due to the religation of the linearised pGEM rather than a variation in the degree of digestion of the plasmid when transformed into the cells. Lane 1-5 correspond to DNA treated with Bovine alkaline phosphatase for 30 + 30 minutes, 4 hours, 1 hour, 15 minutes and 0 minutes respectively. Lane 6 is a standard DNA ladder.

3.5.2 Ligations of Phosphatased Vectors

Ligation reactions were performed on the linearised vectors to ascertain their ability to religate after being incubated with bovine alkaline phosphatase for various lengths of time. All reactions were incubated at 37°C overnight and microdialysed as described in Section 2.3.15. Five (5) µL of each ligation reaction was electroporated into JM101 cells by electroporation as described in Section 2.3.16. Cells were plated onto LB^{amp} plates and the resulting colonies were counted as shown in Table 10.

The results of this experiment suggest that the optimum incubation time for bovine alkaline phosphatase enzyme is either 15 minutes or two incubations of 30 minutes, with a 1:10 dilution of the enzyme. When DNA is incubated for longer periods of time with this enzyme the effectiveness of the reaction is lowered. This could be due to either a second activity of the phosphatase enzyme or the action of a contaminating protein, which digests DNA from the free ends. This digestion would result in the presence of a free

phosphate group on the terminal nucleotide. It is unlikely that the phosphates are not removed or that they are removed and subsequently replaced.

Table 3.10

Number of Colonies Obtained From Cell Transformed with DNA Treated with Alkaline Phosphatase for Different Periods of Time			
Incubation Time With Alkaline Phosphatase	Number of Colonies on Plate 1	Number of Colonies on Plate 2	Average Number of Colonies on Plate
0 minutes	120	90	105
15 minutes	3	0	1.5
1 hour	4	6	5
4 hours	32	55	43.5
30 minutes 30 minutes	2	2	2

3.6 IMPROVEMENTS TO THE OF PURIFICATION PROCEDURE OF AZURIN FROM *E. COLI*

Since the pCH5/*E. coli* expression system apparently gave low yields of azurin and the alternative vector pHN1403 had not proved to be effective in expressing azurin in *E. coli*, attention was turned to refining the expression and purification methods for azurin in an effort to increase the yield of copper binding azurin produced. A series of experiments testing each step of the expression and purification were devised and performed using the pCH5-*E. coli* expression system.

3.6.1 Effect of Azurin Production in Different Cell Lines and the Effect of Growing Cells to Different Densities before Induction

E. coli strains, JM101 and RR1 were transformed with pCH5 by electroporation as described in 2.3.16. Six 5 mL cultures of each transformed cell strain were grown overnight with ampicillin selection and 1 mL of these cultures were used to inoculate 100 mL of LB^{amp}. These cultures were incubated at 37°C with aeration until reaching the required density indicated in Table 3.11.

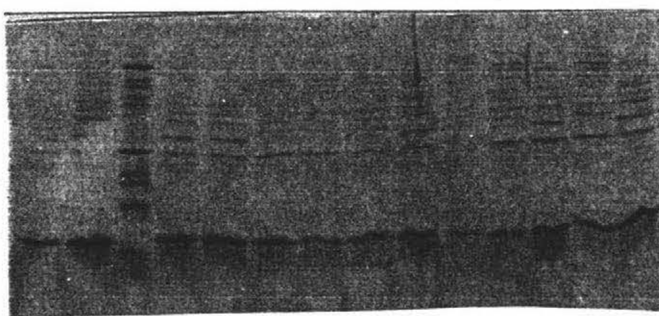
Table 3.11

Optical Density of JM101 and RR1 Cell Cultures at Point of Induction of Azurin Expression		
Required Density at (600 nm)	Actual Density of JM101	Actual Density of RR1
0.7	0.725	0.710
0.8	0.843	0.810
1.0	1.030	1.002
1.2	1.215	1.197
1.4	1.460	1.405
max	1.900	1.630

Azurin expression was induced by the addition of IPTG to a concentration of 100 μM. Cultures were incubated at 37°C for 4 hours and the cells were harvested by centrifugation in 50 mL Nunc tubes, for 15 minutes at 5000 g. Cell pellets were resuspended in 1/10th volume (10 mL) of osmotic shock reagent. After shaking these suspensions for 20 minutes cells were removed by centrifugation and the supernatant, which should contain any azurin produced, was analysed by SDS-PAGE as described in Section 2.3.18. The SDS-PAGE results are shown in Figure 15.

Figure 3.15

SDS-PAGE Analysis of Protein Extracted from the Periplasmic Space of Cells Where Expression Had Been Induced at Various Optical Densities



Equal volumes of each of the samples of protein extracted from the periplasmic space of the *E. coli* RR1 and JM101 cells induced at different cell densities were used for SDS-PAGE analysis. Lane 3 and 10 are BRL molecular weight markers. Lane 1 and 2 fraction from JM101 and RR1 cells respectively, induced at $A^{600} = 0.7$. Lanes 4-8 are the fractions from JM101 and RR1 cells respectively, grown to $A^{600} = 0.8$, 1.0 and 1.2 respectively (JM101 and RR1 cell fractions were loaded alternately). Lane 11-14 are the fractions from JM101 and RR1 cells respectively, grown to $A^{600} = 1.4$ and maximum density.

These results suggest that there is no significant difference between the two cell lines regards their ability to produce azurin. These results also suggest that growing cells to higher cell densities before the addition of IPTG to induce azurin expression makes no significant difference to the final yield of azurin produced.

As previous results in Section 3.2.3 had indicated that azurin was the major protein released into the periplasmic space of the host cells, changes to the absorbance of the periplasmic fraction at 280 nm should reflect a corresponding change in the levels of azurin present. This was used as a second assay for estimating the production of azurin, to confirm the results of SDS-PAGE analysis. The values of absorbance at 280 nm for the periplasmic fractions from each of the JM101 and RR1 cell cultures were recorded in Table 3.12.

Table 3.12

Absorbance Values at 280 nm of Periplasmic Protein Fractions of Cells Where Azurin Expression Had Been Induced at Various Optical Densities		
Density of Culture Before Azurin Expression Induced	Effective 280 nm Absorbance for JM101	Effective 280 nm Absorbance for RR1
0.7	0.119	0.103
0.8	0.133	0.113
1.0	0.138	0.141
1.2	0.140	0.139
1.4	0.130	0.135
max	0.151	0.254

Protein samples extracted from the periplasmic space of JM101 and RR1 cells which had been induced at various cell densities were diluted 15 fold. If the volume of the sample was greater than 10 mL, a correction was made to the A^{280} value.

The results shown in Table 3.12 and the results of SDS-PAGE analysis both indicate that there was no significant difference between azurin yields obtained from RR1 and JM101 cells. The results obtained by measuring the absorbance at 280 nm and by SDS-PAGE analysis also suggest that the cell density at the point when azurin expression was induced made no significant difference to the azurin yield. The results using A^{280} results suggest that the yield of azurin was significantly increased when cells were grown to maximum density. The SDS-PAGE analysis does not appear to indicate this difference. One explanation for the high A^{280} value for this sample may be that some cells rupture when cultures are grown to maximum densities releasing other proteins into solution, which would cause an increase in A^{280} value. These proteins would contaminate the

periplasmic fraction but may be present in small enough concentrations so as not to be observable on a SDS-PAGE.

3.6.2 Inducing Azurin Production for Different Lengths of Time

To determine whether the yield of azurin could be increased by increasing the induction time with IPTG, a variety of induction times were used on small scale azurin preparations.

Overnight cultures of JM101 cells which contained the pCH5 plasmid were grown with ampicillin selection. Azurin production was performed as described in 2.3.21 for a small scale azurin preparation with the length of incubation time of the cell culture with IPTG being the only variable. The incubation time with IPTG which was usually 4 hours was varied between 0 and 6 hours in these experiments. Six cultures were induced for 0, 2, 3, 4, 5 and 6 hours with IPTG were grown to densities as indicated in Table 3.13

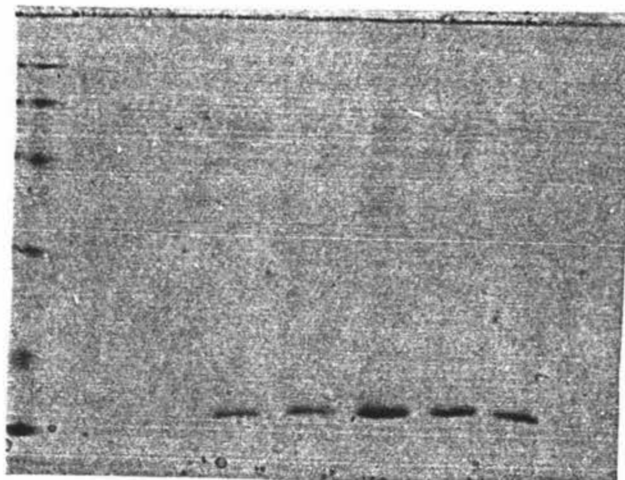
Table 3.13

Actual Optical Densities of Cell Cultures at the Point Where Protein Expression was Induced	
Time of Induction (hours)	actual density
0	1.015
2	0.988
3	1.023
4	0.827
5	0.883
6	1.014

Cells were harvested and azurin was removed by osmotic shock as described in Section 2.3.22. The yield of azurin was estimated by SDS-PAGE analysis as shown in Figure 3.17 and by measuring the absorbance at 280 nm as shown in Table 3.14.

Figure 3.16

SDS-PAGE Analysis of the Periplasmic Fraction of Cell Where Protein Expression Had Been Induced Various Lengths of Time



Equal volumes of each of the samples of protein extracted from the periplasmic space of the *E. coli* JM101 cells induced for various lengths of time were used for SDS-PAGE analysis. Lane 1 is a set of molecular weight markers. Lane 2-5 are periplasmic extracts after cells had been induced for 2, 3, 4 and 6 hours respectively.

The highest absorbance value at 280 nm were obtained with induction times of 4 hours or longer and this trend of increased azurin production with longer incubation times up to 4 hours is in agreement with results observed from SDS-PAGE analysis. The agreement between the protein gel and the absorbance values at 280 nm suggests that the absorbance values at 280 nm can be used to give an indication of the changes in azurin production.

Table 3.14

Estimation of Protein Content in Osmotic Shock Supernatant for Cultures of *E. coli*/pCH5 Induced for Various Lengths of Time			
time induced	volume	280 nm	effective value at 280 nm
0	10.9	0.110 0.106	5.89
2	10.0	0.121 0.127	6.20
3	10.0	0.130 0.131	6.53
4	10.0	0.136 0.147	7.08
5	10.4	0.132 0.140	7.07
6	10.2	0.146 0.154	7.65

Protein samples were extracted from the periplasmic space of cells which had been induced for various lengths of time after addition of IPTG. These were diluted 15 fold and these dilutions were included in the calculation for the effective 280 nm. If the volume of the sample was greater than 10 mL, a correction was made to the A^{280} value.

3.6.3 Azurin Production in JM101 Cells at Different Temperatures and With and Without Copper in Media

Some proteins are not stable at 37°C. While this not been shown for azurin the possibility of increasing the azurin yield by inducing azurin production at a lower temperature was investigated.

Azurin could potentially be stabilised after its synthesis by the addition of copper to the media. Some groups working with azurin have found that the yield increases when the host organism is grown in the presence of copper (Chang *et al.*, 1991). In this experiment copper was added simultaneously with the induction of azurin production with IPTG. In the standard azurin preparations copper was not added to the preparation until after the osmotic shock stage of the purification.

Twelve, 5 mL cultures of JM101 cells transformed with pCH5 were grown overnight with ampicillin selection. One (1) mL fractions from each culture were inoculated into

Legend for Figure 3.17

Equal volumes of the samples of protein extracted from the periplasmic space of the *E. coli* JM101 cells induced at different temperature and grown with and without copper were used for SDS-PAGE analysis. Lane 1 is a set of molecular weight markers. Lanes 2, 4, 6 and 8 show the fractions were azurin production was induced at 37°C with 0.00, 0.10, 0.15 and 0.20 mM $CuSO_4$ respectively. Lanes 3, 5, 7 and 9 show the fractions where azurin production was induced at 30°C with 0.00, 0.10, 0.15 and 0.20 mM $CuSO_4$ respectively.

flasks containing 100 mL of LB^{amp}. Cells were incubated at 37°C with aeration until they reached a density of 1.0 at 600 nm. At this point IPTG was added to the cultures to induce azurin production and copper was supplemented as shown in Table 3.15

Table 3.15

Copper Supplemented to the Cell Growth Media	
amount of 200 μM $CuSO_4$ (μL)	final concentration of copper (mM)
0.00	0.00
25.0	0.05
50.0	0.10
75.0	0.15
100.0	0.20

Following these additions, half of the samples were transferred to 30°C, while their duplicates were incubated at 37°C. All cultures were incubated for a further four hours. Cells were harvested and azurin was extracted from the periplasmic space as described in Section 2.3.22. The amount of azurin present in the periplasmic space was measured by SDS-PAGE analysis shown in Figure 3.18 and the A^{280} values as shown in Table 3.16

Figure 3.17

SDS-PAGE Analysis of Protein Extracted from the Periplasmic Space of JM101 Cells After Induction at 30°C and 37°C and in the Presence and Absence of Copper in the Growth Media

Table 3.16

Absorbance at 280 nm of Protein Sample Extracted from the Periplasmic Space of JM101 Cells After Induction at 30°C and 37°C and in the Presence and Absence of Copper in the Growth Media		
Copper Concentration (mM)	Effective Value at 280 nm 30°C	Effective Value at 280 nm 37°C
0.00	0.161	0.170
0.05	0.155	0.192
0.10	0.166	0.206
0.15	0.154	0.194
0.20	0.194	0.163

The A_{280} values were recorded for protein samples extracted from the periplasmic space of JM101 cells which had been induced at 30°C and 37°C and where cells had been grown in the presence and absence of copper. Samples were diluted 15 fold. This dilution factor was not included in the calculation for the effective 280 nm as the same dilution was used for all samples.

These results suggest that the presence of copper in the LB media up to and including 0.20 mM made no difference to the amount of azurin produced. Supplimentation of copper to the media at levels up to 0.20 mM did not appear to affect cell growth. SDS-PAGE analysis suggested that the amount of protein produced at 30°C was lower than when the cells are incubated at 37°C and this observation is supported by all the A_{280} results with one exception.

3.6.4 Effect on Azurin of Freezing Azurin Containing Cells

The first day of a large scale azurin preparation takes up to 15 hours. Cells are first grown to a density of 1.0 at 600 nm, before azurin expression is induced with IPTG and cells are incubated for a further 4 hours Azurin is extracted from the periplasmic space with osmotic shock and the pH of the azurin containing fraction is reduced to 4.1. Proteins which are insoluble at this pH are removed by centrifugation and the azurin containing solution is loaded onto the Sephadex or cellulose column.

This work could be divided over two days if freezing the harvested cells could be shown to have no effect on the preceding steps of azurin purification. The first day of azurin production would then involve growing cells, inducing azurin expression, harvesting and freezing cells. The second day would involve thawing cells, extracting azurin, removing proteins which are insoluble at pH 4.1 and loading the azurin containing solution onto the

Sephadex or cellulose ion exchange columns. An experiment was performed to investigate the effect of freezing azurin containing cells regards the quantity and purity of protein extracted from the periplasmic space.

Four 5 mL cultures, were inoculated with JM101 cells containing. These cultures were grown with ampicillin selection at 37°C with aeration and 1 mL of each culture was inoculated into 100 mL of LB^{amp}. Cells were incubated at 37°C with aeration until reaching a density of 1.0 at 600 nm. IPTG was added to a concentration of 0.5 mM to induce azurin expression. Cells were incubated for a further four hours. The four 100 mL cultures (A, B, C and D) were divided into two and cells were harvested by centrifugation. One fraction of A, B, C and D was frozen at -20°C and the duplicate fraction was resuspended in 1/10 volume (10 mL) of osmotic shock solution. These suspensions were shaken for 15 minutes and the cells were removed by centrifugation. The supernatant which should contain the azurin was decanted from the pellet.

On the second day frozen cells were removed from the -20°C freezer and were thawed at room temperature. They were resuspended in osmotic shock reagent, shaken for 15 minutes and cells were removed by centrifugation. The supernatant was decanted from the pellet. SDS-PAGE analysis was performed and the results are shown in Figure 3.18. The absorption values at 280 nm of the periplasmic fraction isolated from cells that were fresh and frozen are shown in Table 3.17.

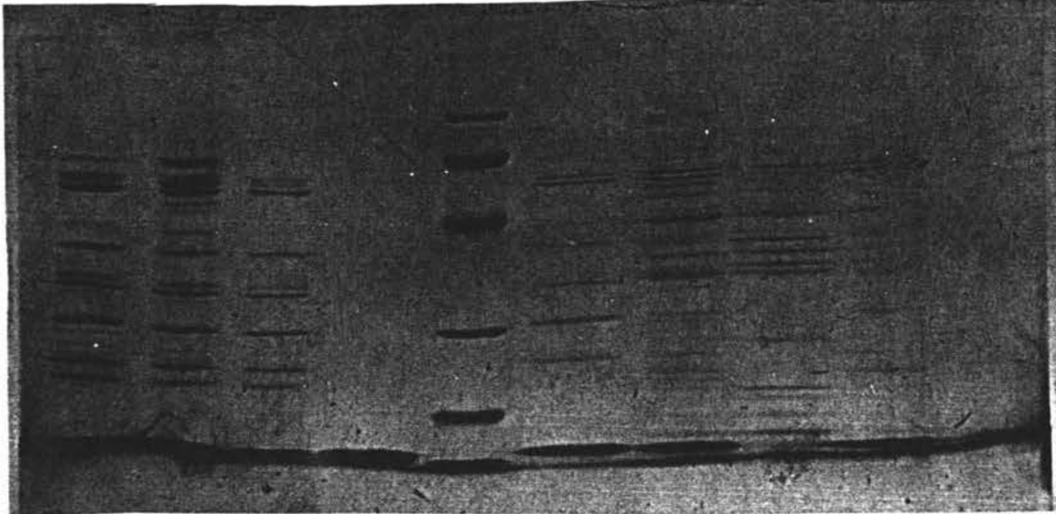
Table 3.17

Absorption Values at 280 nm of the Periplasmic Fraction when Azurin was Purified from Fresh and Frozen Cells		
	Fresh	Frozen
A	0.034	0.151
B	0.096	0.149
C	0.168	0.230
D	0.116	0.223

The 280 nm absorption values for the four fractions (A, B, C and D) were measured for azurin extracted from fresh and frozen cells. While there is considerable variance between the A^{280} values for the frozen sample is consistently higher than that of the fresh sample.

Figure 3.18

SDS-PAGE Analysis of the Periplasmic Fraction of Frozen and Fresh Azurin Containing Cells



SDS-PAGE analysis for the periplasmic fraction of *E coli* JM101 cells where azurin had been extracted from frozen and fresh cells. Lane 4 is a set of molecular weight markers, while lane 5 is a partially pure azurin sample which acts as a molecular weight marker. Lane 1 and 2 show the periplasmic fractions for fractions D, frozen and fresh respectively. Lane 3 and 6 show the periplasmic fractions for fractions C, frozen and fresh respectively. Lane 7 and 8 show the periplasmic fractions for fractions B, frozen and fresh respectively and lane 9 and 10 show the periplasmic fractions for fractions A, frozen and fresh respectively.

The results of the SDS-PAGE analysis shown in Figure 3.18 and of the absorption values at 280 nm for the periplasmic fractions shown in Table 3.17 suggest that more protein is present in the periplasmic fraction of the cells which were frozen. The results of the SDS-PAGE analysis appear to suggest that this is not due to the presence of more azurin but rather due to increased levels of impurities. The likeliest explanation for this is that freezing *E. coli* cells weakens the cell membrane and causes increased rupturing of the membrane releasing intracellular proteins into the periplasmic fraction.

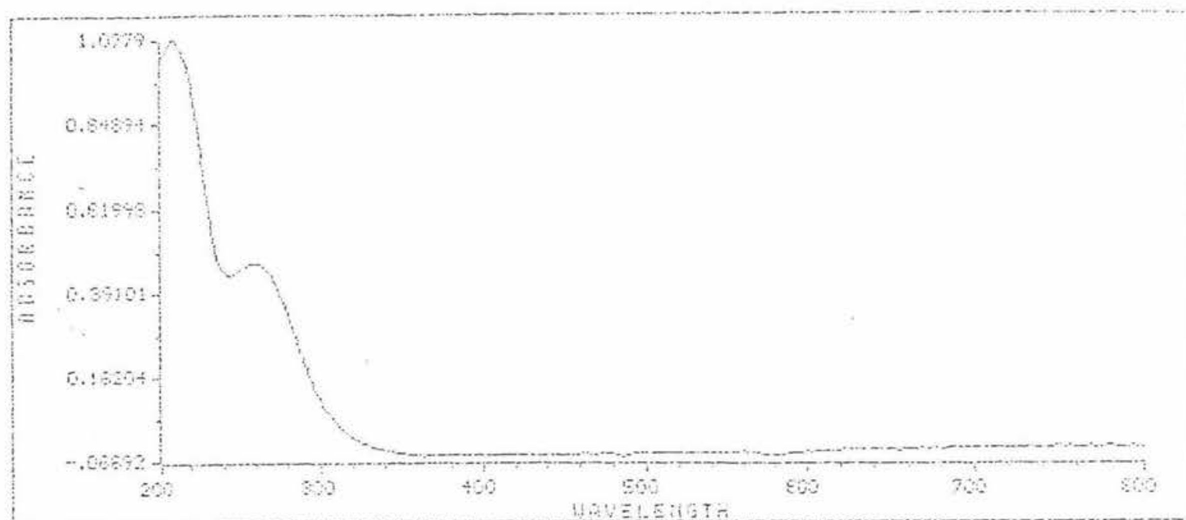
The low 280 nm absorption value and weak azurin band on the SDS-PAGE for fraction A suggests that this culture may not have been induced with IPTG and the only azurin expressed in this fraction was the result of a leaky promotor.

3.6.5 Absorption Spectrum of Azurin

Absorption spectrum of azurin preparations described to this stage in this thesis have possessed a maximum at 280 nm which is characteristic for most proteins. However no maximum was ever observed at 620 nm where a maximum is expected for blue copper binding proteins. This is demonstrated in Figure 19.

Figure 3.19

A Typical Absorption Spectrum for an Azurin Containing Sample Isolated from CM Sephadex Column



An absorption spectrum of an azurin containing solution eluted from a CM Sephadex column obtained from the azurin production described in Section 3.2.3.

SDS-PAGE analysis and silver staining of this gel indicate the presence of only one minor contaminant suggesting that this azurin sample is quite pure, as shown in Section 3.2.3. Protein sequencing of this sample agrees with the SDS-PAGE results regards the presence and purity of azurin as described in Section 3.2.4. The absorption maximum at 230 nm is believed to be due to cytochromes (Norris, G.E., pers. com).

The conflict between the absorption spectrum for azurin samples which suggest that copper binding azurin is not present and the SDS-PAGE and protein sequence results which indicate that azurin is present is best explained by supposing that while azurin is being produced, it is not binding oxidised copper. Other groups working with *P. aeruginosa* azurin expressed in *E. coli* have also reported difficulty in obtaining blue protein (Petrich *et al.*, 1987; Hutnik *et al.*, 1989 and Nar *et al.*, 1992b). In the standard

large scale azurin production a strong oxidising agent, $K_3Fe(CN)_6$, is added to the azurin containing solution simultaneously with copper following the osmotic shock step of purification. This would oxidise any copper bound to azurin as Cu(I) to Cu(II), deeming the possibility of azurin binding Cu(I) unlikely. A more likely explanation for the absence of the 620 nm maximum in azurin solutions produced from *E. coli* is that copper is failing to bind to azurin. Azurin has a very strong binding constant for both Cu(I) and Cu(II), so it is unlikely that the azurin would remain in the apo form. It is more likely then that some modification has occurred to the metal binding site which prevents the binding of copper. The explanations for the absence of a strong absorption maximum for azurin grown in *E. coli* hosts are discussed in detail in chapter four of this thesis.

3.7 INVESTIGATION INTO THE ZINC-BINDING AZURIN

In a recent publication Nar *et al.* (1992b) demonstrated a zinc binding form of azurin which was produced when azurin from *P. aeruginosa* was expressed in an *E. coli* host. This group were using a similar expression and purification system to that which has been used for this work. If the zinc binding form of azurin was being produced in this work this would explain the absence of the 620 nm absorption maximum, as the zinc binding species does not absorb at this wavelength. The possibility of the occurrence of the zinc binding form of azurin been produced in this work was investigated.

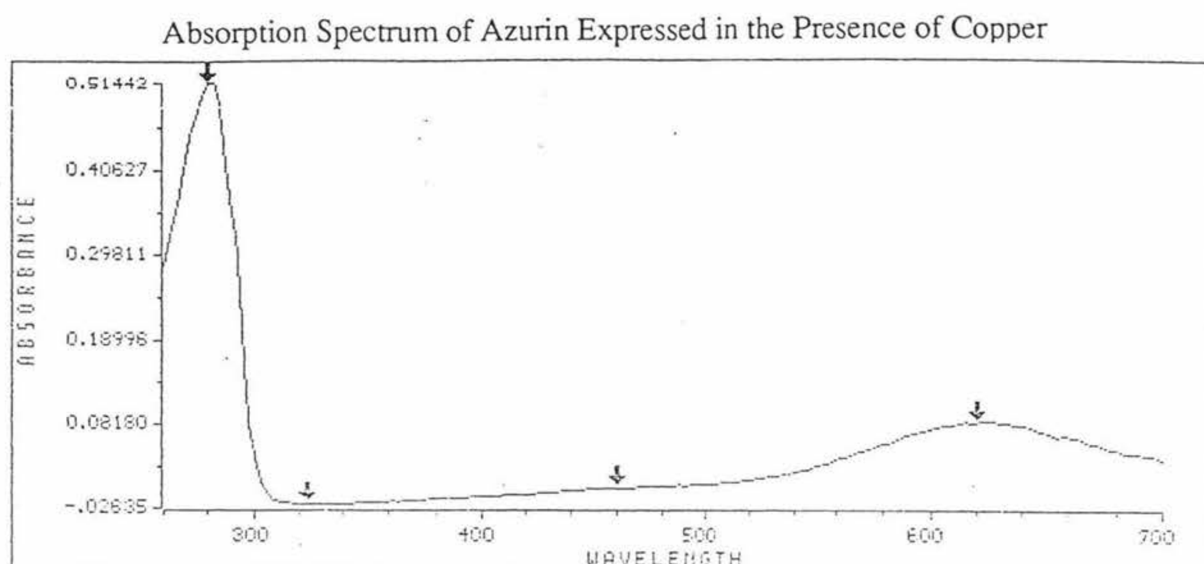
3.7.1 Azurin Production in High-Copper Media

An absorption spectrum for azurin expressed in the presence of high levels of copper is shown in Figure 3.20. A small maximum at 620 nm is observed. While this was encouraging, the maximum at 620 nm was quite small.

In an effort to optimise the conditions for the production of copper binding azurin the protein was expressed in various concentrations of copper. Four overnight cultures of JM101 cells containing pCH5 were grown with ampicillin selection and 1 mL of these cultures were inoculated into 8 x 500 mL of LB^{amp}. Copper sulphate was supplemented as shown in Table 3.18.

Figure 3.20

Absorption Spectrum of Azurin Expressed in the Presence of Copper



The pooled azurin containing fractions from azurin that was expressed in the presence of copper in the experiment described in section 3.6.3 reveals a small maximum at approximately 620 nm. This maximum, characteristic of copper binding proteins has not been observed for azurin fractions where the protein was expressed in the absence of supplemented copper.

Table 3.18

Amount of $CuSO_4$ Supplemented to 500 mL Cultures		
flask name	amount of 25 mM $CuSO_4$ added (mL)	concentration of copper in media
A	50	2.5 mM
B-D	5	0.25 mM
E-H	0	0.0 mM

The calculation of copper concentration does not consider the trace of copper that might normally be present in LB media. The only source of copper in the media is that which is present in the ingredients.

These flasks were incubated at 37°C with aeration until the cell density reached 1.0 at 600 nm. The cells grown in 2.5 mM $CuSO_4$ reached a cell density of 1.0 at approximately the same time as the other samples, indicating that cell growth had not been significantly inhibited by the addition of copper sulphate to this concentration.

These fractions were then treated as described in Sections 2.3.21 and 2.3.22. The resulting supernatant from flasks A-D were pooled, as were the supernatants from flasks

E-H. $CuSO_4$ and $K_3Fe(CN)_6$ were added to the later samples up to concentrations of 2.5 mM and 1 mM respectively. Neither $CuSO_4$ nor $K_3Fe(CN)_6$ were added to the pooled fraction of flasks A-D as the azurin present in this fraction has been produced in the presence of copper. If no blue colour was observed from this fraction then $K_3Fe(CN)_6$ could be added. If a blue colour appeared this would indicate where copper was binding in the oxidised or reduced state when azurin was grown in the presence of copper.

These two fractions were loaded onto two separate CM23 cellulose columns overnight. These columns were washed with acetate buffer at pH4.5 and then with acetate buffer pH4.8 to remove protein which comes off at a lower protein that azurin. Azurin was eluted with acetate buffer pH5.1. Eluate fractions were collected and examined by absorption spectra. Fractions that possessed a maximum at 280 nm in the region where azurin was expected to come off were pooled into two fractions one for azurin expressed in the presence of azurin and the other for azurin expressed in the absence of supplemented copper. These fractions were concentrated to approximately 1.5 mL as described in Section 2.3.31. SDS-PAGE was not performed at this stage but all fractions were kept until it was confirmed that these fraction did contain azurin.

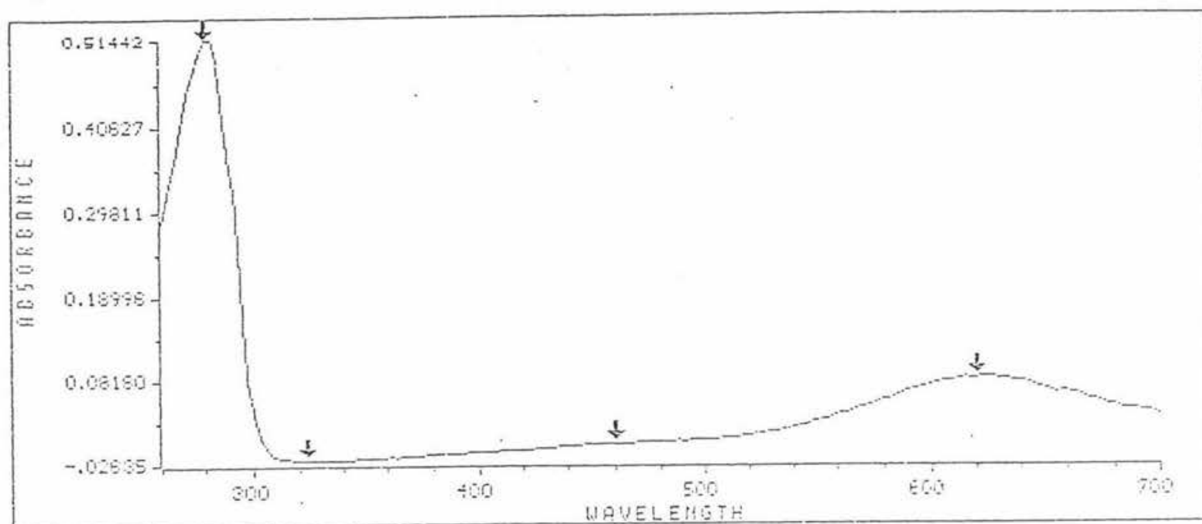
3.7.2 Absorption Spectrum of Azurin Produced in a High-Copper Media

An absorption spectrum for azurin that was expressed in the presence of copper is shown in Figure 3.21. Using calculation described in Section 4.1.3 the purity of azurin in sample was estimated to be 48%.

As a 620 nm absorption maximum had been observed when azurin was expressed in the presence of copper this calculation is considered more valid than earlier calculations where a maximum had not been observed. The yield however, which was estimated to be about 2.5 mg/ 2 litres was much lower than reported by Hoitink *et al.* (1990).

Figure 3.21

Absorption Spectrum for Azurin from *A. denitrificans* Expressed in a Copper Enriched Environment in an *E. coli* Host



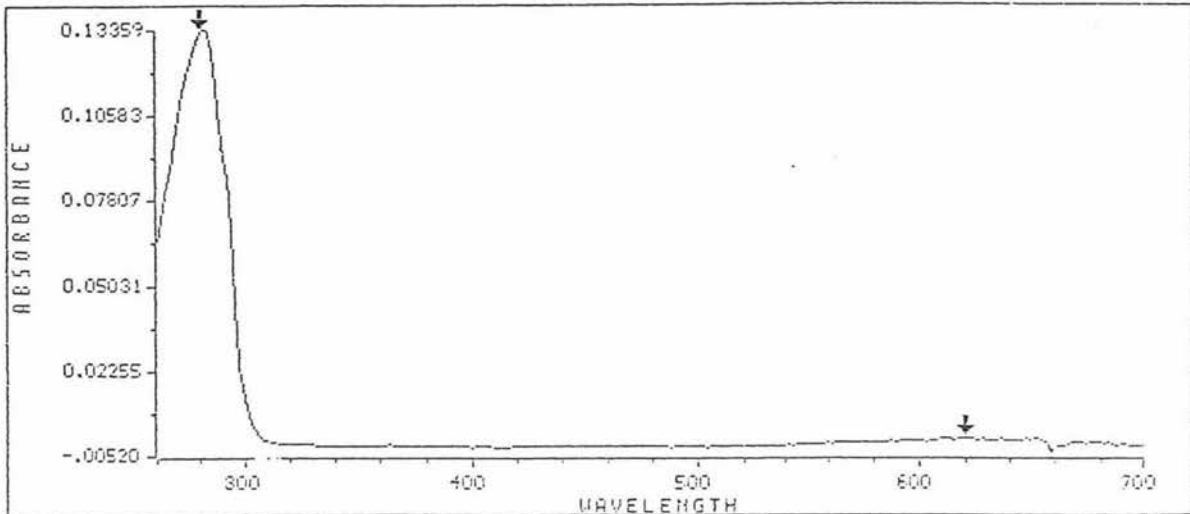
An absorption maximum was observed at 620 nm for azurin expressed in the presence of copper. This sample had been eluted from the CM cellulose column and calculations described in Section 4.3.1 suggest that the sample is 48% pure.

3.7.3 Absorption Spectra for Azurin Produced in Non-copper-enriched Media

An absorption spectrum for azurin that was expressed in the presence of copper is shown in Figure 3.22. The purity and yield can not be calculated accurately as there is no apparent maximum at 620 nm, which suggests that if azurin present is not present in the copper binding form.

Figure 3.22

Absorption Spectrum for Azurin from *A. denitrificans* Expressed in a non-Copper Enriched Environment in an *E. coli* Host



An absorption spectrum for an azurin solution was observed at 620 nm for azurin expressed in the absence of supplemented copper. This sample had been eluted from the CM cellulose column and concentrated to approximately 1.0 mL.

3.7.4 Preparation of Azurin by Gel Filtration

Protein was salted out of both azurin fractions which had been concentrated to approximately 1.5 mL. NaCl was added to 100% saturation and precipitates were observed to form for both samples. These precipitates should contain the majority of proteins in solution, including azurin.

These samples were transferred to microcentrifuge tubes and the precipitate was removed from solution by centrifugation at high speed for 10 minutes. The azurin grown in copper produced a rich blue pellet while the sample grown without copper produced a pale blue pellet. These pellets were resuspended in 200 µL of acetate buffer.

3.7.5 Gel Filtration Column

A gel filtration column was prepared as described in Section 2.3.30. The sample from azurin expressed in a copper enriched environment was loaded onto the column first and a blue band was observed on the gel filtration column. The column ran for eight hours and

0.5 mL fractions collected. Several fractions appeared to be a green/blue colour indicating the presence of azurin. These fractions that possessed an absorption maximum at 620 nm were pooled.

For the azurin expressed in the absence of supplemented copper no blue/green colour was observed on either the column or in the resulting fractions. Fractions which corresponded to the blue/green fractions of the previous column for the azurin supplemented with azurin were tested for the occurrence of an absorbance maximum at 280 nm. Those showing the presence of protein by this method were pooled.

SDS-PAGE analysis of these fractions revealed that azurin was present both cases. Azurin that was expressed in the presence of copper was concentrated to 2 mL while the azurin expressed in the absence of supplemented copper was concentrated to 1 mL using the methods described in Section 2.3.31. These volumes were chosen as the absorption maximum at 280 nm suggested that the sample with the azurin that had been expressed in the presence of copper contained approximately twice the amount of protein as the fraction of azurin expressed in the absence of copper.

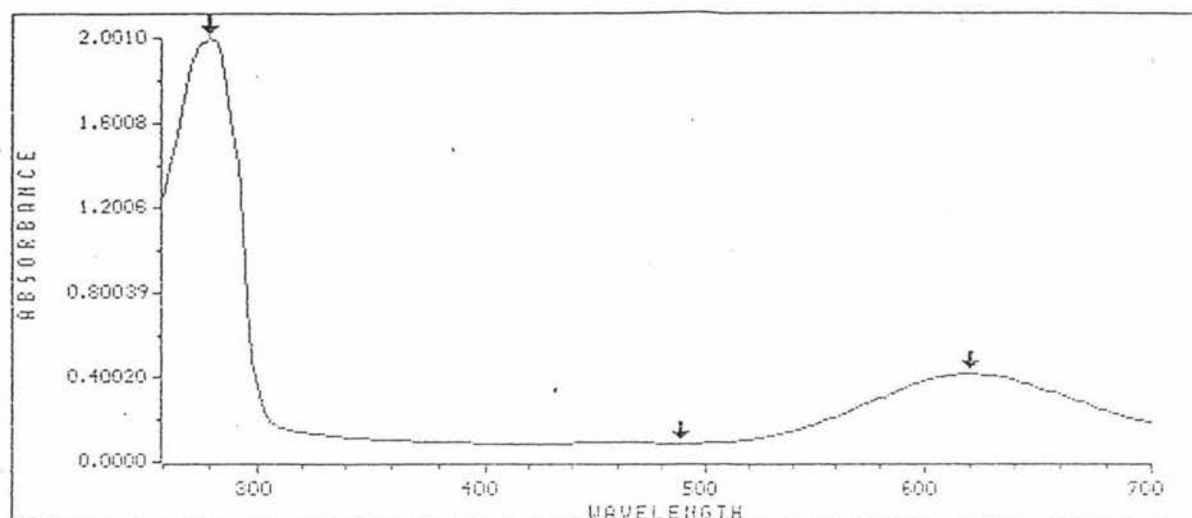
3.7.6 Absorption Spectrum of Azurin Expressed in the Presence and Absence of Copper, After Gel Filtration.

An absorption spectrum for azurin expressed in the presence and absence of copper after these samples were eluted from the gel filtration column are shown in Figures 3.23 and 3.24 respectively.

These two azurin samples were treated identically except that copper sulphate was supplemented to the growth medium of one, so that azurin folded into it tertiary structure in the presence of copper, while for the second copper was not added until after the azurin had been removed from the periplasmic space and the protein had already assumed its tertiary structure. The difference between the purity and yield of these two samples would appear to be directly correlated to the presence of copper available to azurin as it folds into its tertiary structure. The absorption values at 280 nm suggest that the difference in protein levels present in the two samples is approximately two fold while the absorption values at 620 nm suggest that the difference in the yield of copper binding azurin is approximately 29 fold.

Figure 3.23

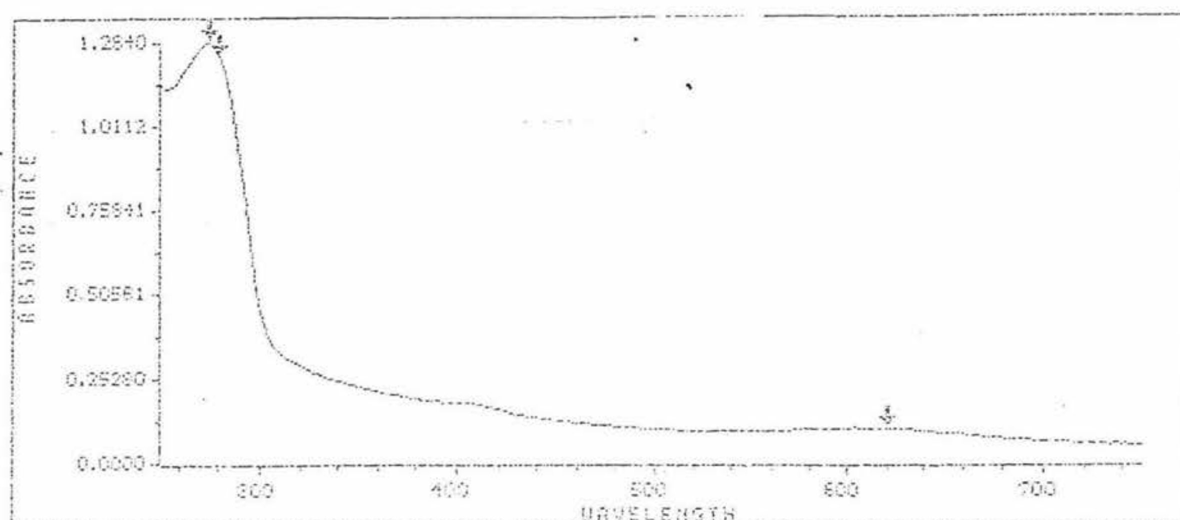
Absorption Spectrum for Azurin Expressed in a Copper-Enriched Environment after Elution from Gel Filtration Column



An absorption spectrum for azurin expressed in the presence of copper after this protein was eluted from the gel filtration column. The purity of this sample was be 69% using calculations described in Section 2.4.3. The quantity of azurin present in 2 mL of solution was 0.163 μmoles or 2.4 mg using the calculations described in Section 2.4.2.

Figure 3.24

Absorption Spectrum for Azurin Expressed in a None-Copper-Enriched Environment after Elution from Gel Filtration Column



As absorbance at 620 nm is dependant on the protein binding copper, it is obviously an unreliable indicator of both protein concentration and purity when copper is not bound to the protein.

3.7.7 Atomic Absorption

The difference in the apparent purity and yield for azurin which was expressed in the presence of copper compared to the azurin which was expressed in the absence of copper, suggests that either the amount of azurin produced increases when copper is supplemented to the *E. coli* host medium or that azurin does not bind copper when expressed in a none copper enriched environment. A combination of these two possibilities could also occur. These possibilities are discussed in detail in the chapter 4 of this thesis.

There are several possible explanations for the results if it is supposed that azurin expressed in the absence of copper did not contain this metal even when this is supplemented during the purification procedure. Firstly, the copper binding site of the protein may have been modified so that copper was unable to bind, resulting in apo-azurin. This is unlikely as the azurin appears to be processed correctly as shown by the protein sequence in Section 3.2.4, indicating that the 19 amino acid N-terminal leader sequence is cleaved correctly. If the proteins is processed correctly and can therefore fold into its correct tertiary structure then it will bind copper as it has a high binding affinity for copper. A second possibility is that copper is bound to the protein in the Cu(I) state. This however, is very unlikely as all purification procedures were carried out in the presence of oxygen (air) and an oxidising agent, $K_3Fe(CN)_6$, which was added to the azurin solutions during purification.

A third possibility is that the copper binding site is occupied by another metal. This hypothesis is consistent with the presence of a maximum at 280 nm and the absence of an absorption maximum at 620 nm. These three possibilities shall be considered further in chapter 4 of this thesis.

Nar *et al.*, (1992) reported the production of zinc binding azurin from *P. aeruginosa* when this protein was expressed in an *E. coli* host. To test the possibility of a zinc containing form of azurin in this work samples of azurin expressed in both the presence and absence of supplemented copper were analysed by atomic absorption and these results are summarised in Table 20.

Table 3.19

Levels of Zinc and Copper Present in Solutions of Azurin Expressed in *E. coli* in the Presence and in the Absence of Copper			
sample	amount of azurin	amount of copper	amount of zinc
azurin expressed with copper supplementation to culture	1.2 mg	5.5 μg	2.9 μg
azurin expressed without copper supplementation to culture	1.0 mg (approx)	0.28 μg	1.4 μg

Two azurin samples isolated from the gel filtration columns were analysed by atomic absorption, performed by Dr Roger Reeves of Massey University. Half of the sample (1 mL) prepared with copper present in the culture and all of the sample (1 mL) of azurin expressed without the presence of copper in the *E. coli* growth medium were tested for the presence of zinc by atomic absorption as described in Section 2.3.32.

The results of atomic absorption suggests that the azurin expressed in none-copper-enriched media is binding predominantly zinc with only low levels of copper binding while the azurin expressed with copper supplemented to the cultures is binding predominantly copper. These two elements have very similar molecular weights, therefore the quantity of each present in μg is an accurate reflection of how much is present in the azurin solution.

Percentage of copper binding for azurin expressed without copper supplementation
= 0.28/(0.28 + 1.4)
= 17%

When the azurin was grown in 0.25 mM $CuSO_4$ (with one quarter of the azurin grown in 2.5 mM $CuSO_4$) the binding of copper increases dramatically;

Percentage of copper binding for azurin expressed with copper supplementation
= 5.5/(5.5 + 2.9)
= 65%

These results suggest that even when azurin is expressed in the presence of 0.25 mM copper this protein still does not bind copper exclusively. It is likely that some azurin remains in the zinc form though it is possible that other metals may also be bound to the metal binding site preventing copper binding or that some azurin remains in the apo form. It appears that the azurin is still binding a significant amount of zinc though some of the

zinc could be present in the acetate buffer which the azurin sample was suspended in. Atomic absorption is a measure of how much copper and zinc are present in solution not how much of either metal is bound to azurin. However it is likely that acetate buffer would contain very little of either zinc or copper in the media.

3.8 SEQUENCING AND MUTAGENESIS OF AZURIN IN pT201

3.8.1 Oligonucleotides for Mutagenesis

Two oligonucleotides which would anneal in the azurin coding region but with slight changes in sequence were obtained from Massey University Separation Science Unit. These coded for the azurin mutants histidine 117 to glycine ($his_{117}gly$ or $H_{117}G$) and methionine 121 to glutamic acid ($met_{121}glu$ or $M_{121}E$). Both residues are found at the copper binding site where they are believed to act as copper ligands as discussed in Section 1.12.

The oligonucleotide for the histidine 117 to glycine is;

```
his 117       SER PHE PRO GLY HIS TRP GLY MET
              TCG TTC CCC GGC CAC TGG GCC ATG
                              * *
his117gly     TCG TTC CCC GGC GGC TGG GCC ATG
              SER PHE PRO GLY GLY TRP GLY MET
```

The oligonucleotide for the methionine 121 to glutamic acid is

```
met 121       HIS TRP GLY MET MET LYS GLY THR
              CAC TGG GCC ATG ATG AAG GGC ACG
                              * *
met121glu     CAC TGG GCC ATG CAG AAG GGC ACG
              HIS TRP GLY MET GLU LYS GLY THR
```

3.8.2 Yields of Mutagenic Oligonucleotides

The yield for the mutagenic oligonucleotide histidine 117 to glycine was;

stepwise yield 98.2%
amount 1.2 mg
amount 0.17 μmoles

The yield for the mutagenic oligonucleotide methionine 121 to glutamic acid was;

stepwise yield 97.4%
amount 1.21.mg
amount 0.17 μmoles

3.7.3 Amounts of Oligonucleotides Recovered after Isolation

Oligonucleotides were isolated by using Sep-Pak 18 as described in 2.3.17. Yields of recovery are indicated in Table 3.21.

Table 3.20

Results of Isolation of Mutant Oligonucleotides from Sep-Pak				
oligonucleotide	A_{260} 20 x dilution	A_{260} undiluted	μg of oligonucleotide per mL	percentage of oligonucleotide from that loaded
$his_{117}gly$	0.532	10.64	351	87
	0.082	1.64	54	13
	0.000	0.00	0	0
met 121	0.367	14.68	484	87
	0.109	2.18	72	13
	0.005	0.10	3.3	<1

Oligonucleotides were isolated by using Sep-Pak 18 which is described in Section 3.2.3. It was necessary to dilute these samples 20 fold (40 fold in the case of the first fraction of M 121) in order to measure the absorption at 260 nm. Theoretically about ninety percent of the oligonucleotide should be recovered in the first eluted fraction of a Sep-Pak, which was the case here. The concentration of oligonucleotide in μg/mL was calculated assuming that 1.0 O.D. at 280 nm corresponds to an concentration of 33 μg/mL.

The percentage total of oligonucleotide eluted from each fraction refers to the total amount eluted from the column, rather the amount applied to the column.

3.8.4 Calculations of the Amount Oligonucleotides Recovered

The yield and concentration of the mutagenic oligonucleotide histidine 117 to glycine was calculated to be 50 nmoles using the calculations described in Section 2.4.4. This was resuspended in 1 mL of water giving a concentration for the $H_{117}G$ oligonucleotide of 50 nmoles/mL, or 50 pmoles/μL.

The yield and concentration of the mutagenic oligonucleotide methionine 121 to glutamic acid was calculated to be 70 nmoles, using the calculation described in Section 2.4.4. This was resuspended in 1 mL of water. The concentration of this oligonucleotide was 70 nmoles/mL or 70 pmoles/μL.

3.8.5 Sequencing Primer

Before any sequencing could be done on any mutants the 0.2 kbp HincII/SmaI fragment had to be removed. This could be overcome by the acquisition of a oligonucleotide that annealed to the DNA internally to the azurin gene and only about 100-150 base pairs away from the mutant sites. This primer called azu 3 has the following sequence;

CGC GCA CAC CAA GGT CAT

This should allow any azurin mutants in this system to be sequenced easily in the future.

Chapter 4 : Discussion

4.1 Common Expression Techniques used for Azurin

Azurin is classified as a blue copper protein by virtue of its single Type I copper binding site. It has the typical intense blue colour of this class of protein which corresponds to a strong absorption maximum in the region of 600 nm. The only function so far attributed to these proteins is electron transfer and consequently these proteins have been termed cupredoxins, analogous to iron binding ferridoxins (Adman, 1985 & 1991). Azurins have been identified in several bacterial species (Ryden, 1988). The azurin used for the study in this thesis is based on that from *Alcaligenes denitrificans* .

In this work, the recombinant DNA was used to express the azurin of *A. denitrificans* in *E. coli* cells. Recombinant techniques allow greater amounts of protein to be expressed and purified, in shorter periods of time than would be obtained from the native host. Recombinant DNA is also easier to manipulate which is an important consideration as the original aim of this thesis was to produce mutants.

E. coli is a common host for expression of wild type heterologous proteins and mutant proteins derived from the wild type proteins. Azurins from a number of sources have been expressed in *E. coli* host-vector systems (van de Kamp *et al.*, 1990b; Pascher *et al.*, 1989; Nishiyama *et al.*, 1992; Nar *et al.*, 1992a; Karlsson *et al.*, 1989; Hoitink *et al.*, 1990; Chang *et al.*, 1991; den Blaauwen *et al.*, 1991; Arvidsson *et al.*, 1989). The expression system used in this thesis used *E. coli* and the expression plasmid pCH5, a pUC19 derivative. This was supplied by Hoitink and Canters, Leiden, The Netherlands, and is described in Section 3.1.1.

The original objectives of this thesis were to examine the nature and requirements of copper binding in azurin from *A. denitrificans*. To do this, mutants with modifications to several copper binding ligands were to be prepared and examined using crystallographic techniques.

4.2 Discrepancies in the Calculation of Protein Purity

Initial experiments in this study suggested that the pCH5-*E. coli* expression system produced only low yields of azurin. However, in these experiments quantitation of the azurin yield was based solely on the determination of the absorbance maximum at 620 nm. The low absorbance ratio at $A_{620:280}$ for the partially purified protein suggested

that the protein was very impure as seen in Sections 3.2.2 & 3.2.3. In fact in the early preparations there was no distinct A_{620} peak evident in the spectra.

This result was at variance with the results of polyacrylamide gel electrophoresis of the same protein sample which showed a Coomassie blue-staining band at the anticipated molecular weight as described in Section 3.2.3. The presence of only a single visible contaminant band suggested that the protein was quite pure by this criterion. The reason for the difference between these results was not pursued initially as the aim of the project was to produce mutants of azurin with altered copper binding properties. Consequently it was felt to be important to maximise yields of the blue copper-containing azurin.

At this stage an expression vector which had been reported to produce very high yields of recombinant proteins in *E. coli* became available to the laboratory. This expression vector was a pUC119 derivative and had been used for the large-scale production of T4 lysozyme (Poteete *et al.*, 1991). The DNA sequences coding for azurin were transferred from pCH5 to the new vector but this construct did not produce any azurin when expressed in *E. coli* as reported in Section 3.4.10. At this stage a series of experiments were performed to find the optimal expression and purification conditions for copper-containing azurin using the pCH5-*E. coli* expression system. The results obtained when *E. coli* cells which had expressed *A. denitrificans* azurin were frozen overnight following harvesting indicated that cells could not be frozen overnight without increasing the amount of contaminating protein released in the osmotic shock step of the purification. Otherwise these experiments confirmed that the reported (Hoitink *et al.*, 1991) expression and purification conditions were close to optimal with one exception as discussed in Section 4.3.

4.3 Addition of Copper to the Growth Medium

Pre-azurin has a 19 amino acid N-terminal presequence which ensures that the protein is transported into the periplasmic space of the host cell. Pre-azurin from *A. denitrificans* is processed correctly in an *E. coli* expression system as shown by the N-terminal sequence reported in Section 3.2.4. This mode of expression and translocation means that azurin will assume its tertiary structure in the periplasmic space. The copper concentration in the periplasmic space will reflect the concentration of Cu(II) in the medium in which *E. coli* is grown. Azurin which is expressed in *E. coli* is usually done so with no copper supplementation to the medium (van de Kamp *et al.*, 1990b; Nishiyama *et al.*, 1992; Nar *et al.*, 1992a; Karlsson *et al.*, 1989a; Hoitink *et al.*, 1990). However, a few groups have reported copper supplementation to the medium (Arvidsson *et al.*, 1989; Chang *et al.*,

1991). Apo-azurin has a very high affinity for copper and solutions of apo-azurin have been shown to extract copper from glass (Norris, G.E. pers. com). Because of this high affinity it had not been considered necessary to add copper to the growth medium; if it was added later it would easily bind to any apo-azurin.

Initial attempts to purify copper-binding azurin from *A. denitrificans* from an *E. coli* host produced low apparent yields of protein, with a low purity, as reported in Section 4.2. One of a series of experiments performed in an effort to increase the yield of blue copper protein obtained from the pCH5-*E. coli* expression system involved the addition of copper to the *E. coli* growth medium as described in Section 3.6.3. This copper was added in an effort to stabilise the protein by allowing azurin to bind copper as it assumed its tertiary structure. We anticipated that this would lead to an increase in the yield of blue copper containing azurin. Analysis on polyacrylamide gels suggested that the yield of the protein with a mobility corresponding to azurin was not increased significantly, so the protein did not appear to be more stable. However, an absorption maximum in the region of 620 nm which had been absent in the azurin produced in earlier experiments was apparent when the azurin was expressed in the presence of high copper levels in the *E. coli* growth medium as shown in Section 3.7.1.

This experiment revealed that *E. coli* JM101 cells would grow with no apparent inhibition of cell yield at a copper concentrations up to 0.25 mM. Two large-scale azurin productions were performed which differed at the point where copper was added to the azurin solutions as described in Section 3.6.6. To the first, $CuSO_4$ was supplemented to the standard *E. coli* growth medium to a concentration 0.25 mM, simultaneously with the addition of IPTG, which was added to induce azurin production. Copper was not added to the azurin sample following protein extraction from the periplasmic space of the host cells. To the second large-scale azurin preparation no copper was added to the standard *E. coli* growth medium but copper was supplemented to the protein solution following protein extraction from the periplasmic space, as in the original purification procedure described in 2.3.23. Subsequent purification identical for both preparations. Absorption spectra for the two samples of azurin demonstrated considerable differences as shown in 3.6.7 and 3.6.8. The azurin solutions obtained from the copper supplemented *E. coli* growth medium possessed a distinct peak in the region of 620 nm. An equivalent peak was not apparent for the azurin sample obtained from the non-copper supplemented *E. coli* growth medium. These two azurin samples were subjected to atomic absorption analysis as described in Section 3.6.12 and these results are discussed in Section 4.5.

4.4 Explanations for the Absence of the 620 nm Absorption Maximum

The absence of the blue colour and the corresponding 620 nm absorption maximum for azurin solutions from non-copper supplemented *E. coli* medium could be due to one or a combination of reasons. One possibility is that copper binds to azurin but does so in the reduced, Cu(I), state. Azurin containing Cu(I) does not absorb at 620 nm, so the an azurin solution with Cu(I) would not appear blue. As azurin is still produced it would be detectable on polyacrylamide gels. This corresponds with the results observed for azurin which is expressed without copper supplemented to the medium as described in Section 3.7.3. However, this explanation is unlikely as the strong oxidant $K_3Fe(CN)_6$ was added to the protein after its extraction from the periplasmic space of *E. coli* . This treatment has been shown to result in any Cu(I) bound to the protein being oxidised to the Cu(II) state and the blue colour and the corresponding 620 nm absorption maximum should become apparent.

A second possibility is that some modification had occurred at the copper binding site, which interferes with ability of azurin to bind copper. If azurin was unable to bind copper and therefore remained in the apo form, the 620 nm peak would not be observed. One explanation for the nature of this modification involves the 19 amino acid pre-sequence, which ensures that azurin is transported to the periplasmic space of the host cell. The azurin coding sequence used for this thesis was from the *A. denitrificans* but the azurin was expressed in *E. coli.* It is conceivable that the 19 amino acid pre-sequence was processed incorrectly in *E. coli* leading to the production of azurin which would have fewer or extra amino acid(s) at the N-terminus. This could conceivably interfere with the protein folding into its tertiary structure and may therefore deform the copper binding site. However, evidence clearly contradicts this possibility as several groups have reported evidence that the protein is found in the periplasmic fraction of the cells (Yamamoto *et al.*, 1987; van de Kamp *et al.*, 1990b; Nar *et al.*, 1992a; Chang *et al.*, 1991) and that the azurin pre-sequence is processed correctly in *E. coli* (Yamamoto *et al.*, 1987; van de Kamp *et al.*, 1990b; Nar *et al.*, 1992a). Correct processing was confirmed in this work using N-terminal sequencing as described in Section 3.2.4. The first eight amino acids of this protein were sequenced and these agreed with the first eight amino acids for correctly processed azurin from *A. denitrificans* (Hoitink *et al.*, 1990). While these results indicate that the 19 amino acid pre-sequence is processed correctly it does not rule out the possibility that copper is unable to bind to azurin due to some modification at the metal binding site.

A third possible explanation for the absence of 620 nm maximum when azurin is expressed in the absence of copper supplemented to the *E. coli* growth medium is that the copper binding site is occupied by another metal. If another metal was occupying the metal binding site, the 620 nm maximum would not be apparent while SDS-PAGE analysis would show that azurin was being produced. This is consistent with the results in this work. This possibility was initially thought to be unlikely as azurin has a very high affinity for copper.

The assumption that azurin selectively binds copper has been supported by evidence that copper binding is both thermodynamically and kinetically favoured over zinc (Tennent and McMillin, 1979; Engeseth and McMillin, 1986). However metal exchange experiments demonstrated that once zinc is bound to azurin an activation barrier is created which makes copper incorporation into zinc-azurin an extremely slow process, with a half-live of several days (Williams, 1982, 1983; Blaszak *et al.*, 1983; Engeseth & McMillin, 1986). These experiments indicated that zinc was the second most favoured element for azurin after copper. It has a similar atomic weight to copper and is situated next to copper on the periodic table.

4.5 Evidence for Zinc-binding Azurin

E. coli is a commonly used host for the expression of azurin and other blue copper proteins (Yamamoto *et al.*, 1987; van de Kamp *et al.*, 1990b; Nishiyama *et al.*, 1992; Nar *et al.*, 1992a; Karlsson *et al.*, 1989a; Hoitink *et al.*, 1990; Chang *et al.*, 1991). Several groups have reported a lower $A_{620:280}$ ratio when azurin was expressed in *E. coli* than when these proteins are produced in their native hosts (Petrich *et al.*, 1987; Hutnik & Szabo, 1989; van de Kamp *et al.*, 1990b). These findings are borne out in this work where a ratio $A_{620:280}$ of 0.3 (indicating 100% purity) was never attained. Using the original production and purification procedure no distinct 620 nm peak was obtained so calculations of quantity and purity based on A_{620} were impossible. Analysis of this material using 12% polyacrylamide gels indicated that the azurin had actually been purified considerably. A silver stained gel of purified azurin which had no apparent 620 nm absorption maximum showed only one minor contaminant as described in Section 3.2.2.

Chang *et al.* (1991) expressed the azurin from *P. aeruginosa* and another blue copper protein, plastocyanin from *Populus nigra* var. *italica*, in *E. coli* using modified XB media supplemented with 1.0 mM $CuSO_4$. The method of production and purification for azurin was similar to the procedures described in Sections 2.3.22 to 2.3.26. They

performed amino acid analysis, N-terminal sequencing, UV-visible spectroscopy and CD. The mass of the protein was determined by time-of-flight mass spectrometry. It was demonstrated using these criteria that the blue copper proteins produced in the native hosts were indistinguishable from those produced in the *E. coli* hosts. The authors did not report any difficulty in obtaining a blue protein. It is interesting to note that azurin produced in this experiment was expressed in the presence of copper supplemented to the *E. coli* growth medium.

Van de Kamp *et al.*, (1990b) reported the expression of the azurin from *P. aeruginosa* in *E. coli* JM101 and KMBL1164 strains. Azurin was produced and purified in a manner similar to that used in this thesis. The $A_{620/280}$ ratio for a sample of purified azurin was between 0.3 and 0.4, considerably lower than the ratio of 0.53 obtained when the protein was purified from *P. aeruginosa* (Karlsson *et al.*, 1989a). Van de Kamp *et al.* demonstrated the purity of azurin using polyacrylamide gels, which showed the presence of a single protein band. Isoelectric focusing however demonstrated the presence of two major bands, with pI's of 5.6 and 5.4 and a minor band with a pI of 4.6. The authors suggested that the latter band corresponded to apo-azurin. To separate the proteins with pI's of 5.6 and 5.4 anion exchange chromatography was implemented. The authors showed that copper-azurin has a pI of 5.6, while the colourless protein with a pI of 5.4 remained unidentified. Further investigation of this contaminating protein revealed that it was neither oxidised or reduced holo-azurin, apo-azurin or denatured azurin. It could not be reconstituted with cupric sulphate and it could not be oxidised using potassium ferricyanide. Its molecular weight and 1H-NMR characteristics were consistent with this protein having the same primary structure as holo-azurin. 1H-NMR spectra demonstrated that the protein had a structure similar to apo-azurin. The pK_a of His 35, the titratability of His 117, the fluorescence intensity and the absence of changes to the spectroscopic and electrophoretic properties of the protein when treated with potassium cyanide supported the idea that this protein was azurin with no metal ion at its active site. The authors tentatively concluded on this evidence that this protein was unable to bind copper due to a modification in the metal binding site.

Recently Nar *et al.*, (1992b) reported the crystallisation and X-ray structure solution of the colourless protein, previously reported by van de Kamp et al., (1990b). The structure showed that the metal binding site was occupied by a single zinc atom. Zinc and copper, which are situated next to each other on the periodic table of elements, possess similar atomic weights. However the presence of zinc in the azurin binding site is surprising as the binding of copper to azurin had been considered a highly selective process as shown by Nar *et al.* (1992b).

Yamamoto *et al.*, (1987) reported the cloning of the pseudoazurin from *Alcaligenes faecalis* and the expression of this protein in *E. coli.* Pseudoazurin was expressed in *E. coli* grown in LB media which was apparently not supplemented with copper. A blue protein was observed and this was purified. This protein gave a spectral ratio $A_{593:277}$ of 0.493 which is almost identical to the value of 0.509 obtained when this pseudoazurin was produced in its native host. These results indicate that this pseudoazurin bound copper when it was expressed in an *E. coli* host when copper is not supplemented to the *E. coli* growth medium. This result suggests that the zinc binding phenomenon observed to occur with azurin may not be relevant to other blue copper proteins. This is discussed further in Section 4.8.

The large-scale azurin preparations reported in Section 3.7 involved two azurin preparations which differed only the concentration of copper in the host cell growth medium. The first preparation had copper supplemented to the media in the form of $CuSO_4$ to a concentration of 0.25 mM while the second preparation had no copper supplementation. Copper was supplemented to the protein solution following the extraction from the periplasmic space of the host. Protein was extracted and purified and these protein samples were subjected to atomic absorption analysis as described in Section 3.7.7. The results of this analysis are summarised in Table 3.19 and suggest that when azurin is expressed in standard *E. coli* medium which is not supplemented with copper the zinc form of azurin is the predominant species. In contrast, when azurin is expressed in a standard *E. coli* medium which is supplemented with copper, the copper form of the protein is the predominant species. Copper and zinc have similar atomic weights and therefore the actual weight of each present in the azurin solutions should accurately reflect the proportion of each binding to azurin. Calculations described in Section 3.6.12 suggest that when azurin is expressed in standard *E.coli* medium which has not been supplemented with copper, only 17% of the azurin is present in the copper binding form, while the remaining 83% is present in the zinc form. When azurin was expressed in the presence of 0.25 mM copper sulphate the percentage of copper-azurin increased to 65%, while only 35% of the azurin molecules bound zinc. Atomic absorption results do not indicate whether the zinc and copper detected is bound to the protein or if it is present in solution. It is unlikely that a significant amount of either of these elements would be present in the acetate buffer which the azurin was suspended in. No buffer sample was examined by atomic absorption.

While these results appear to indicate that the level of zinc-azurin can be dramatically decreased by adding copper to the *E. coli* growth medium, they also appear to indicate

that a significant proportion of the azurin continues to bind zinc even under these conditions.

4.6 The Significance of the Rack Induced Model

As discussed in Section 1.8, Gray & Malmström (1983) introduced the concept of rack induced bonding in blue copper proteins. The authors proposed that the conformational energy of the peptide backbone structure forces the Cu(II) ion to adopt the special structure of a blue copper site. The authors also suggested that differences in the reduction potential of various blue proteins can be ascribed to differences in back-bonding of a blue copper site induced by the protein structure. The structural causes of the variation in back-bonding that have been considered include changes in strength of the Cu-S(Cys) and Cu-S(Met) interaction (Blair *et al.*, 1985; Ainscough *et al.*, 1987) and NH-S hydrogen bonding (Gray & Malmström, 1983). Karlsson *et al.* (1989), investigated the rack induced model by preparing the Met_{121}Leu mutant of azurin from *P. aeruginosa.* and expressing this mutant in an *E. coli* host. This mutant demonstrated that a small change in the primary structure of the protein can cause a large change in reduction potential while having only a small effect on spectroscopic properties of the oxidised copper site. These findings support the rack induced model. The structure of the apo-azurin from *A. denitrificans* (Shepard *et al.*, 1993) showed only minimal changes in the protein structure of the binding site when compared to the structure of the copper-containing proteins. This result provides further support for the rack induced model.

The rack induced model suggests that the structure of the copper binding protein is determined by the tertiary structure of the protein rather than by the metal ion. While this site appears to adopt a conformation which is specific for Cu(I) and Cu(II) there must be enough flexibility to allow some distortion of the polypeptide backbone and side chain atoms to accommodate the zinc atom. This is borne out by some of the parameters of the structure of the zinc-containing azurin shown in Table 4.1 which compares some parameters obtained for the tertiary structure of zinc-azurin from *P. aeruginosa* reported by Nar *et al.*,(1992b) with parameters obtained for copper form of azurin from *P. aeruginosa* reported by Nar *et al.* (1991b).

Table 4.1

Comparison of Ligand of Metal Bond Lengths for Copper-Azurin and Zinc-Azurin from *P. aeruginosa*		
Ligands	Copper	Zinc
His_{45}	2.11 Å	2.01 Å
His_{117}	2.03 Å	2.07 Å
Cys_{112}	2.25 Å	2.30 Å
Met_{121}	3.15 Å	3.40 Å
Gly $(O)_{45}$	2.97 Å	2.32 Å

A comparison of the metal binding site of azurin from *P. aeruginosa* which binds copper with that which binds zinc reveals that only minor changes occur in the bond lengths of His_{46} and Cys_{112} to metal, while the His_{117} to metal bond is almost identical in the copper and zinc form of azurin. However the Met_{121} residue which, at a distance of 3.15 Å is close enough to act as a weak ligand in the copper form of the protein moves 0.25 Å further away from the metal in the zinc form of azurin. At this distance it is probable that Met_{121} is not a significant ligand in the zinc form of the protein.

In contrast to the Met_{121} residue, the carbonyl group of Gly_{45} appears to move closer to the metal ion in the zinc form of the protein. The carbonyl group of Gly_{45} is believed to act as a weak ligand in copper-azurin while the shorter distance in the zinc-azurin suggests a considerably stronger interaction. This change in interaction causes structural causes adjustments of the polypeptide atoms in the immediate surroundings of the Gly_{45} residue with residues 44 and 45 moving significantly towards the zinc ion.

The movement of the Met_{121} ligand away from the metal and movement of the carbonyl group of Gly_{45} towards the metal, in the zinc form of the azurin combine to cause a change to the geometry of the binding site. The copper binding form of the protein forms a distorted trigonal bipyramidal conformation which is favoured by copper, while the zinc form of the protein forms a square planar geometry. This geometry is favoured by zinc and is a common conformation in zinc binding sites as described in Section 1.10. As discussed in Section 1.8, the rack induced model for metal binding conformation suggests that the polypeptide backbone of the protein has a major effect on the conformation of the metal binding site. These results would suggest that in some situations the metal can change that conformation of binding site as evidenced by the tertiary structure of zinc-azurin (Nar *et al.*, 1992b).

Shepard *et al.* (1993) reported that when the copper was absent from the binding site of azurin from *A. denitrificans* the copper binding ligands moved closer together. The radius of the cavity between the strong-binding ligands, His_{46}, His_{117} and Cys_{112} shrunk from 1.21 Å in reduced azurin and 1.24 Å in oxidised azurin to 1.16 Å in apoazurin. It appeared that one of the copper-binding loops was more flexible in the oxidised and apo forms of the protein but it was concluded that the copper site found in holo-azurin is a stable structure which is defined by the constraints of the polypeptide structure even in the absence of a bound metal, which supports the rack-induced model.

4.7 Properties of Zinc Binding Proteins

It has been demonstrated using the zinc binding enzymes carboxypeptidase and carbonic anhydrase, that removal of the zinc atom from the metal binding site allows other divalent metals, including copper, to bind to the site. Of all the divalent metals substituted for zinc in these proteins copper was found to make the structure the most chemically stable (Vallee & Galdes, 1984).

As discussed in Section 1.10 only four amino acids have been found to act as ligands for zinc. These are the sulphur group of cysteine, the nitrogen group of histidine and the hydroxyl from the carboxylate side chains of glutamate and aspartate. Water has been found to act as a ligand in some zinc-binding proteins. Methionine has not been found to act as a ligand in any zinc proteins reported to date (Struhl, 1987). The absence of methionine as a ligand is significant when considering the zinc form of azurin where Met_{121}, which acts a ligand in the copper binding form of the protein, is not close enough to act as a ligand to the zinc. While the Met_{121} interaction is decreased, the interaction between the carbonyl group of Gly_{45} and the metal is increased in the zinc-binding form of the protein. The interaction of the carbonyl group associated with Gly_{45} and the zinc metal may be analogous to the interaction of zinc with carboxylate side chains of glutamate and aspartate which are common in the binding sites of zinc-binding proteins.

Blue copper proteins which include azurin all possess two histidines, one cysteine and one methionine residue as ligands (the only exception is stellocyanin which does not have a methionine residue). Azurin and possibly stellocyanin are the only blue copper proteins that possess a carbonyl group associated with a glycine residue as an additional ligand (Ainscough *et al.*, 1987; Adman, 1991).

It is interesting to hypothesise that zinc binding sites require a tetrahedral binding site as the distorted tetrahedral geometry found in the copper form of azurin is distorted to a tetrahedral geometry in the zinc-binding form. The tetrahedral form is found in many zinc-binding proteins while the distorted tetrahedral form has not been found in any zinc-binding sites to date as discussed in Section 1.10. Furthermore it could be speculated that zinc can not utilise methionine as a ligand as no known zinc-binding protein possess this residue as a ligand. These observations are consistent with the observation that when azurin is expressed in an *E. coli* host, is grown in medium which is not supplemented with copper, the zinc-binding form of the protein appears to be produced as the predominant species. When other blue copper proteins are expressed in a similar expression system blue copper binding protein appears to be the dominant species (Yamamoto *et al.*, 1987). In azurin, the methionine ligand interacts only weakly with the copper, while in other commonly studied blue copper proteins (eg psuedoazurin and plastocyanin) the methionine-copper interaction appears to be much stronger (Adman, 1991).

4.8 Prominence of Zinc-Binding Azurin

E. coli has been used as an expression host for azurin for several years. Recently has it been found that zinc-azurin is produced as a significant contaminant when azurin is expressed and purified from *E. coli* hosts. As has been noted in this thesis and elsewhere (van de Kamp *et al.*, 1990a) this contaminant is difficult to identify because it possesses the same molecular weight and sequence as copper-azurin. Other groups that expressed azurin in an *E. coli* expression system noted difficulty in purifying azurin, failing to get the same $A_{620/280}$ ratio as was achievable when azurin was purified from its native host. This is discussed in Section 4.5. While these groups did not report the existence of zinc containing azurin some of their results may be best interpreted by the presence of zinc containing azurin.

Chang *et al.* (1991), stated the yield of azurin from *P. aeruginosa* and plastocyanin from *Populus nigra* var. *italica* increased when 1.0 mM $CuSO_4$ was supplemented to the standard *E. coli* growth medium. The authors suggested that this was due to the greater resistance to proteolysis of the holo-proteins when the site was occupied with a copper atom. The presence of azurin was detected by Western blots but they did not specify how they quantified the azurin yield. If their quantification method was dependant on the 620 nm maximum then the authors observation of increased yields of azurin when the blue copper protein were expressed in a copper rich environment may in fact be erroneous. The apparent increase in yield could in fact be due to an increase the

proportion of copper-azurin. This explanation of the results obtained by Chang *et al.*, (1991) would be in agreement with the findings of this thesis. If however, the authors are correct and the addition of copper to the growth medium did stabilise the copper binding proteins, their findings would contradict those reported in Section 3.6.3, where A_{280} absorption and polyacrylamide gel analysis of azurin expressed in the presence and absence of additional copper indicated that there was no significant difference in the yield of azurin. While these two methods would not allow accurate quantitation of the amount of azurin produced they would be expected to show any significant differences between two samples. Section 3.7 describes the expression of azurin in the presence and absence of copper supplemented to the media An apparent two fold increase in the yield of azurin was noted when the protein was expressed in the presence of copper. This could be due to the loss of the colourless zinc form of azurin which can only be detected using SDS-PAGE analysis. However the apparent two fold increase in azurin production could be real and this result may support the finding of Chang *et al.* (1991).

Nishiyama *et al.* (1992) reported the production of pseudoazurin from *A. faecalis* S-6 in *E. coli.* They quantified the amount of azurin produced using polyacrylamide gels which measures the amount of azurin produced independent of the metal occupying the metal binding site. The authors noted that the yield increased when the protein was expressed in the presence of 0.3 mM of $CuSO_4$ which was added to the host *E. coli* growth medium. These finding contradict the findings for azurin from *A. denitrificans* reported in 3.7, where the addition of copper to the *E. coli* medium, made no apparent difference to the yield of azurin expressed.

The presence of zinc binding forms of what are considered copper binding proteins has not as yet been reported in proteins isolated from their native hosts. However observations reported by Nar *et al.*, (1992) suggests that some zinc binding may occur in the native hosts. The authors reported the expression of the azurin from *P. aeruginosa* in *E. coli* and the separation of the copper-binding form of azurin from the zinc-binding form. A the bond lengths of the zinc-binding ligands to the metal atom were calculated and are shown in Table 4.2.

The $A_{628/280}$ for this purified copper containing azurin was 0.58, while the highest $A_{628/280}$ ratio for azurin purified from the native hosts was 0.53 (Hutnik & Szabo, 1989; Karlsson *et al.*, 1989). This result suggests three possibilities. Firstly it could indicate that the previously calculated spectral ratio for azurin purified from native host was performed on slightly impure azurin. Secondly some of the azurin present in the pure azurin sample from the native hosts could have been present in the apo form. A third

possibility is that the reduced spectral ration of azurin extracted from the native hosts could be due to the presence of a slight contamination of zinc-azurin.

Table 4.2

Summary of Bond Lengths of the Metal Binding Ligands in Azurin from *A. denitrificans* and *P. aeruginosa*			
Copper of Ligand Bond	Bond Length in *A denitrificans* at pH 5.0	Bond Length in *P. aeruginosa* at pH 5.5	Ligand Lengths for Zinc Azurin from *P. aeruginosa*
S(Cys_{112})-Cu	2.14 Å	2.25 Å	2.30 Å
N(His_{46})-Cu	2.06 Å	2.11 Å	2.01 Å
N(His_{117})-Cu	1.96 Å	2.03 Å	2.07 Å
S(Met_{121})-Cu	3.11 Å	3.15 Å	3.40 Å
O(Gly_{45})-Cu	3.13 Å	2.97 Å	2.32 Å

A comparison of the parameters of the binding sites of azurin from *P. aeruginosa* and *A. denitrificans* as shows that these two proteins are very similar. However they do have several significant differences which may be important when considering the occurrence of zinc form of azurin. One of these differences is the proximity of the carbonyl group associated with Gly_{45} to the copper atom in these two proteins. In *P. aeruginosa* the carbonly group is 0.16 Å closer to the metal than in *A.denitrificans*. Conversely the Met_{121} residue appears to slightly further away from the metal in *A. denitrificans*. The difficulties encountered in this thesis in obtaining suitable quantities of the copper form of azurin from *A. denitrificans* compared to the published results using azurin from *P. aeruginosa* would suggest that azurin from *A. denitrificans* binds zinc more favourably than does the azurin from *P. aeruginosa*. This is not supported by the position of the carbonyl group of Gly_{45} which is closer to the metal site in *P. aeruginosa*. This residue acts as a weak ligand for copper-azurin and a strong ligand for zinc-azurin. Its closer proximity to the metal in *P. aeruginosa* would suggest that this azurin may be more favourable for zinc binding if this residue is indeed significant in zinc-azurin. The backbone structure of azurin from *A. denitrificans* may favour zinc binding more that the backbone structure of azurin from *P. aeruginosa*.

Results obtained by den Blaauwen cited in Nar *et al.* (1992b) suggest that up to three times more zinc azurin may be produced than copper azurin for azurin from *P. aeruginosa*, while results from Hoitink and Canters also cited in Nar *et al.*, (1992) suggest that the ratio of copper azurin to zinc azurin depends on the time of bacterial growth following the induction of azurin expression. This suggests that the zinc is incorporated while the apo-protein resides in the periplasmic space, which would be

consistent with the observations made in this work that azurin expressed in a copper supplemented environment resulted in higher levels of copper-azurin than when azurin was expressed in standard *E. coli.* medium.

4.9 Copper Versus Zinc Binding

The results in Sections 3..6.7, 3.6.8 and 3.6.12 for azurin from *A. denitrificans* being expressed in *E. coli* suggests that zinc and copper may compete for the metal binding site of this protein. When copper is not supplemented to the medium it would appear that the zinc-azurin is more abundant, while an increase in copper concentration in the host growth medium leads to an increase in the proportion of copper-azurin produced.

There are several reasons why azurin may bind more zinc when produced in *E. coli* than when it is synthesised in its native species. One possibility is that azurin forms a different tertiary structures when in the different bacterial hosts. The tertiary structure in *E. coli* may lead to a change in the conformation of the copper binding site such that the geometry changes to being a tetrahedral shape, which favours zinc binding, rather than trigonal bipyramidal which is found in copper-azurin. This suggestion would be consistent with the rack induced hypothesis discussed in Section 4.6, which suggests that the tertiary structure of the protein determines the geometry of the copper binding site. However this explanation seems unlikely as results in this thesis suggest that when azurin is expressed in the presence of high levels of copper protein, the proteins binds copper and must therefore fold correctly.

A second possibility is that an ion exchange system operates in the *Pseudomonas* and *Alcaligenes* species which differs from that present in *E. coli..* A mechanism for regulation of copper versus zinc uptake by metal homeostasis has been proposed (Williams, 1983, 1985). This mechanism suggests that more Cu(II) than Zn(II) may be present in the periplasmic space of *P. aeruginosa* and *A. denitrificans.* This higher concentration of copper would promote copper incorporation into azurin. This possibility is suggested by Nar *et al.*, (1992b) and is supported by the findings in this work where azurin from *A. denitrificans* which was expressed in an *E. coli* host in a copper rich environment, where 65% of the azurin molecules appeared to bind copper compared to 17% of the azurin molecule binding copper when this protein was expressed in a standard *E. coli* medium with no copper added, as was discussed in Section 4.5. The existence of such a regulatory system would explain the observed results, as comparatively higher zinc levels present in the periplasmic space of *E. coli* would allow this atom to complete more successfully for the copper binding site. Nar *et al.* (1992b) suggests that over

expression of foreign protein-coding genes in *E. coli* might disturb metal homeostasis. Even if *E. coli* did have the same homeostasis control as exists in *P. aeruginosa* and *A. denitrificans*, it may unable to control the unusually large yields of metal binding proteins which are released into the periplasmic space.

Zumft *et al.* (1990) has reported a copper transfer protein in *Pseudomonas stutzeri*. Proteins were found in this organism which mediated the transfer of copper. If similar proteins were to exist in *P. aeruginosa* and *A. denitrificans* then a high level of copper may be present in the periplasmic space allowing azurin to bind a higher percentage of copper. The absence of these proteins in *E coli* would allow zinc to compete with copper for the metal binding site.

4.10 Summary

The results described in this thesis allow the conclusion that when azurin from *A. denitrificans* is expressed in an *E. coli* host using standard media with no copper added, then the predominant form of azurin produced is the zinc form of the protein. As mutants are going to made of this protein, conditions where the protein was binding copper only were needed. It appears that a high proportion of blue protein can be produced with the addition of copper to the medium, as reported for pseudoazurin by Nishiyama *et al.*, (1992) and for azurin from *P. aeruginosa* by Chang *et al.* (1991). The ideal conditions for this are still to be calculated but results from this thesis would suggest that copper concentrations in the vicinity 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 $CuSO_4$ which may lead to the production a much higher ratio of the copper-azurin compared to the zinc-azurin.

References

Adman, E.T. and Jensen, L.H. (1981) *Isr. J. Chem* **21** 8-12

Adman, E.T. (1985) In *"Topics in Molecular and Structural Biology, Metalloproteins"* (P.Harrison, ed.) Vol I 1-42 McMillan LTD. New York.

Adman, E.T. (1991) *Adv. Protein Chem* **222**, 145-197

Adman, E.T., Stenkamp, R.E, Sieker, L.C. and Jensen, L.H. (1978) *J. Mol. Biol.* **123**, 35-47

Adman, E.T., Turley, S., Bramson, R., Petratos, K., Banner, D., Tsernoglou, D., Beppu, T. and Watanabe,H. (1989) *J. Bio. Chem.* **264**, 87-99

Ainscough, E.W., Bingham, A.G., Brodie, A.M., Ellis, W.R., Gray, H.B., Loehr, T.M., Plowman, J.E., Norris, G.E. and Baker, E.N. (1987) *Biochemistry* **26**, 71-82

Ambler, R.P. (1971) in *Recent Developments in the Chemical Study of Protein Structures* (Previero,A., Pechere, J.-F. and Collette-Previero, M.-A., Eds) 289-305, Inserm, Paris

Ambler, R.P. and Tobari, J. (1989) *Biochemistry. J* **261**, 495-499

Arvidsson, R.H., Nordling, M. and Lundberg, L.G. (1989) *E. J. B.* **179**, 195-200

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Smith,J.A., Seidman,J.G. and Struhl,K. (1989) eds. *"Current Protocols in Molecular Biology "* vol I Greene Publishing Associates & Wiley Intersciences N.Y.

Baker, E.N. (1988), *J. Mol. Biol* **203**, 1071-1095

Blair, D.F., Campbell, G.W., Schoonover, J.R., Chan, S.I., Gray, H.B., Malmström, B.G., Pecht, I., Swanson, B.I., Woodruff, W.H., Cho, W.K., English, A.M., Fry, H.A., Lum, V. and Norton, K.A. (1985),. *J. Am. Chem. Soc.* **107**, 5755-5766

Blaszak, J.A., McMillin, D.R., Thornton, A.T. and Tennent, D.L. (1983) *J. Biol. Chem.* **258**, 9886-9892

Boas, F.J.F. (1984) in *"Copper Proteins and Copper Enzymes"* (Lontie,R., ed) vol I pp 5-62, C.R.C Press, Boca Raton, Florida

Branden, C. (1979), in *"Metalloproteins"* pp 220-245 (Ed; Ulrich Weser)

Canters, G.W., Lommen, A., van de Kamp, M., and Hoitink, C.W.G. In, *"Contribution to the Symposium on Biochemical and Biophysical Approaches to the Study of Copper Proteins"*. (1989)

Coleman, P.M., Freeman, H.C., Guss, J.M., Murata, M., Norris, V.A., Ramshaw, J.A.M. and Venkatappa, M.P. (1978) *Nature* **272**, 319-324

Cotton, F.A. and Wilkinson. G. (1988), *Advanced Inorganic Chemistry*, fifth ed. 1335-1376

Chang, T.K., Iverson, S.A., Rodrigues, C.G., Kiser, C.N., Lew, A.Y.C., Germanas, J.P. and Richards, J.H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1325-1329

den Blaauwen, T., van de Kamp, M. and Canters, G.W. (1991) *J. Am. Chem. Soc.* **113**, 5050-5052

Dower, W.J., Miller, J.E. and Ragsdale, C.W. (1988) *Nucleic Acids Res*, **16**, 6127-6145

Engeseth, H.R. and McMillin, D.R. (1986) *Biochemistry* **25**, 2448-23455

Ettinger, M.J. (1984) in *"Copper Proteins and Copper Enzymes"* (Lontie,R., ed) vol III pp 175-229 C.R.C Press, Boca Raton, Florida

Farver, O. and Pecht, I. (1981) *Isr. J. Biochem.* **21**, 13-17

Fee, J.A. (1975) *Structure and Bonding* **23**, 1-60

Fielden, E.M. and Rotilio, G. (1985) in *"Copper Proteins and Copper Enzymes"* Lontie ed (Vol II) 27-82 C.R.C Press, Boca Raton, Florida

Fields, B.A., Guss, J.M. and Freeman, H.C. (1991) *J. Mol. Biol.* **22**, 1053-1065

Gray, H.B. and Malmström, B.G. (1983) *Comments Inorg.Chem.* **2**, 203-209

Guss, J.M. and Freeman, H.C. (1983) *J. Mol. Biol.* **169**, 521-563

Guss, J.M., Merritt, E.A., Phizackerley, R.P., Hedman, B., Murata, M., Hodgson, K.O. and Freeman, H.C. (1988) *Science* **241**, 806-811

Haylock, S.J., Buckley, P.D. and Blackwell, L.F. (1983) *J. Inorg. Biochem.* **19**, 105-117

Hill, H.A.O. and Smith, B.E. (1979) *J. Inorg. Biochem.* **11**, 79-93

Hoitink, C.W.G., Woudt, L.P., Turenhout, J.C.M., van de Kamp, M. and Canters, G.W. (1990)*Gene* **90**, 15-20

Hutnik, C.M. and Szabo, A.G. (1989) *Biochemistry* **28** 3923-3934

Kagi, J.H.R., Kojima, Y., Berger, C., Kissling, M.M., Lerch, K.and Vasok, M, (1979) in *"Metalloproteins"* pp194-206 (Ed;Ulrich Weser)

Karlsson, B.G., Pascher, T., Nordling, M., Arvidsson, R.H.G. and Lundberg, L.G. (1989a) *FEBS Letters* **246**, 211-217

Karlsson, B.G., Aasa, R., Malmström, B.G. and Lundberg, L.G. (1989b) *FEBS Letters* **253**, 99-102

Lappin, A.G. *Metal Ions in Biological Systems.* 13 (1981) **23**, 15-71

Lever, A.B.P. (1984) in *"Inorganic Electronic Spectroscopy"* 203-208, Elsevier, New York.Proc.Natl.Acad.Sci.USA. **71**, 4760-4762

McManus, J.D., Brune, D.C., Han, J., Sanders-Loehr, J., Meyer, T.E., Cusanovich, M.A., Tollin, G. and Blankenship, R.E. (1992) *J. Bio. Chem.* **267**, 6531-6540

McMillin, D.B., Rosenburg, R.C. and Gray, H.B. (1974) *Proc. Natl. Acad. Sci. USA.* **71**, 4760-4762

McMillin, D.R. (1985), *Jour. of Chem. Edu.* **62**, 997-1001

Messerschmidt, A., Rossi, A., Ladenstein, R., Huber, R., Bolognesi, M., Gatti, G., Morchesini, A., Petruzzelli, R.and Finazzi-Agro, A. (1989) *J. Mol. Biol.* **206**, 513-529

Muchmore, D.C., McIntosh, L.P., Russell, C.B., Anderson, D.E. and Dahlquist, F.W. (1989).*Methods Enzymol* **177**, 44-73

Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., and Canters, G.W., (1991a) *J. Mol. Biol.* **218**, 427-447

Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., and Canters, G.W., (1991b) *J. Mol. Biol.* **221**, 765-772

Nar, H., Messerschmidt, A., Huber, R, van de Kamp, M. and Canters, G.W. (1992a) *FEBS Letters* **306**, 119-124

Nar, H., Huber, R., Messerschmidt, A., Filippou, A.C., Barth, M., Jaquinod, M., van de Kamp, M. and Canters, G.W. (1992b) *Eur. J. Biochem.* **205**, 1123-1129

Nishiyama, M., Suzuki, J., Ohnuki, T., Chang, H.C., Horinouchi, S., Turley, S., Adman, E.T. and Beppu, T. (1992) *Protein Engineering* **5**, 177-184

Norris, G.E. (1983) The Three Demensional Structure of Azurin; A Blue Copper Protein, At 3Å Resolution (Thesis), 1982.

Norris, G.E., Anderson, B.F. and Baker, E.N. (1986).*J. Amer. Chem. Soc* **108**, 2784-2785

Otsuka, S. (1988), *"Metalloproteins: Chemical Properties and Biological Effects"* pp1-14 (Ed;S.Otsuka, T.Tamanaka)

Ouzounis, C.and Sanders, C. (1991) *FEBS Letters* **279**, 73-78

Pascher, T., Bergström, J., Vänngård, T. and Lundberg, L.G. (1989) *FEBS Letters*. **258** 266-268

Petratos, K., Banner, D.W., Beppo, T., Wilson, K.S. and Tsernboglou, D. (1987) *FEBS Letters* **218**, 209-214

Petrich, J.W., Longworth, J.W. and Fleming, G.R. (1987) *Biochemistry* **26**, 2711-2722

Poteete, A.R., Dao-Pin, S., Nicholson, H. and Matthews, B.W. (1991) *Biochemistry* **30,** 1425-1432

Rees, D.C., Lewis, M ., Honzatko, R.B., Lipscomb, W.N. and Hardman, K.D. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3408-3412

Rydén, L., (1988) In *"Oxidases and Related Redox Systems* (T.E.King, H.S.Mason and M.Morrison, ed) 349-366 Alan.R.Liss, Inc, New York

Sambrook, J., Fritsch, E.F. and Maniatis, T. , *Molecular Cloning: A Laboratory Manual*

Shepard, W.E.B., Kingston, R.L., Anderson, B.F. and Baker, E.N. (1993), *Acta Cryst.* D**49**, 331-343

Shepherd, P.R., Elwood, C., Buckley, P.D. and Blackwell, L.F. (1992) *Biolog.Trace Elem. Research* **32**, 109-113

Solomon, E.I., Hare, J.W. and Gray, H.B.(1976), *Proc. Natl. Acad. Sci. USA.* **73**, 1389-1393

Solomon, E.I.,Rawlings, J, McMillin, D.R., Stephens, P.J. and Gray, H.B (1980a) *J. Am. Chem. Soc.* **98**, 8046-8048

Solomon, E.I., Hare, J.W., Dooley, D.M., Dawson, J.H., Stephens, P.J. and Gray, H.B. (1980b) *J. Am. Chem. Soc.* **102**, 168-178

Struhl, K. (1987) *TIBS* **14**, 137-140

Sutherland, I.W. and Wilkinson, J.F. (1963) *J. Gen. Microbiol* **30**, 105-112

Sykes, A.G. (1991). in *"Structure and Bonding"* **75**; Long-Range Electron Transfer in Biology. (Ed; Clarke.M.J., Goodenough.J., C.K.Jorgensen.C.K., Neilands.J.B., Reinen.D., Weiss.R.).

Tennent, D.L. and McMillin, D.R., (1979) *J. Am. Chem. Soc.* **101**, 2307-2311

Thompson, J.S., Marks, T.J. and Ibers, J.A., (1977), *Proc. Natl. Acad. Sci.USA.* **74**, 3114-3118

Tsuru, D. (1988), in *"Metalloproteins: Chemical Properties and Biological Effects"* (Ed;Otsuka.S., Tamanaka.T.) pp 343-355

Ugurbil, K. and Bersohn, R. (1977), *Biochemistry*, **16**, 3016-3023

Ugurbil, K., Norton, R.S., Allerhand, A. and Bersohn, R. (1977) *Biochemistry*, **16**, 3016-3023

van de Kamp, M., Floris, R., Hali, F.C. and Canters, G.W. (1990a) *J. Am. Chem. Soc.* **112**, 907-908.

van de Kamp, M., Hali, F.C., Rosato, N., Agro, A.F., and Canters, G.W. (1990b) *Biochemica et Biophysica Acta.* **1019**, 283-292

Vallee, B.L.and Galdes, A. (1984) *Adv. Enzy.* **56**, 283-430

Volbeda, A. and Hol, W.G.J. (1989) *J. Mol. Biol.* **206**, 531-546

Williams, R.J.P. (1982) *Pure Appl. Chem.* **54**, 1889-1904

Williams, R.J.P. (1983) *Pure Appl. Chem.* **55**, 35-46

Williams, R.J.P. (1985) *Eur. J. Biochem.* **150**, 231-248

Yamamoto, K., Uozumi, T. and Beppu, T. F (1987) *Journal of Bacterilogy* **169** 5648-5652

Yamanaka, T. (1988), in *"Metalloproteins: Chemical Properties and Biological Effects"* (Ed; Otsuka.S., Tamanaka.T.) pp 382-385

Yanisch-Perron, C. Vieira, J. and Messing, J. (1985) *Gene* **33**, 103-119

Zumft, W.G., Viebrock-Sambale, A.and Braun, C. (1990) *Eur.J. Biochem.* **192**, 591-599