Blue Copper Proteins as Honorary Cytochromes: The Structure and Evolution of Blue Copper Proteins

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Summary: The blue copper proteins are among the most beautiful macromolecules that we know, and the intensity of the colour of azurin per copper atom is eighty times as that of the cuprammonium ion. They occur in the periplasm of Gram positive bacteria and in the photosystems of blue green algae (cyanobacteria) and in algal and higher plant chloroplasts. The function of these proteins in electron transport is very similar to those of the soluble cytochromes c_6 ("algal cytochrome f") and c_6 ("*Pseudomonas* cytochrome c-551"), so much so that one thinks of them as 'honorary cytochromes'. Several different sequence classes have been recognized, including azurin, plastocyanin, amicyanin, pseudoazurin and rusticyanin. There is considerable three-dimensional similarity in the copper binding sites of the proteins, and have as ligands two histidine, one cysteine and one methionine side chains.

The proteins have been co-opted to function in a wide range of metabolisms, and azurin is often (but not always) associated with denitrification. The gene has been adapted to produce an outer membrane protein in gonococci, while azurin, amicyanin and pseudoazurin are involved in methylotrophy in different organisms -- though in other closely related bacteria, cytochromes *c*8 play equally versatile roles.

While the overall structures of each of the proteins are very similar wherever they are found, when duplicate genes occur in the same organism, the difference between the isoforms are large, even if no functional difference between the forms has been detected in vitro.

The time is ripe and the techniques now available for the function and relationships of these proteins to be elucidated genetically, which has not been possible before because of the multiplicity of alternative pathways in bacterial electron transport systems.

Introduction

In 1958 Crick [1] announced that a subject called 'Protein Taxonomy' had been born, and the next year Anfinsen [2] published his book "The Molecular Basis of Evolution". It had become possible to determine and compare the amino acid sequences of small proteins relatively easily, and several different systems began to be explored because of their evolutionary potential, such as insulin, haemoglobin, cytochrome c and the fibrinopeptides. The first volume of Margaret Dayhoff's *Atlas of Protein Sequence and Structure* [3] contained just 66 sequences of ten amino acids or longer. However, this was the start of a vast effort by laboratories all over the world, which accelerated with the development of DNA sequencing, and has already produced the complete genome sequences of several organisms.

The blue copper proteins azurin and plastocyanin were both discovered by the start of this phase (Table 1), and the first azurin sequence appeared in Volume 1 of "The Atlas". These and other blue copper proteins have proved very suitable probes to use to explore molecular evolution. Small blue copper proteins have been found across much of phylogenetic space -- in cyanobacteria, chloroplasts, higher plant cytoplasm, Gram negative bacteria, green nonsulphur photosynthetic bacteria, and archaebacteria -- and their similarities in structure are great (Fig. 1). Is this similarity due to their first having arisen in the remote common ancestor to all these organisms, or because the genes for their synthesis have spread laterally from a more recent source?

Many of these proteins are abundant and easy to prepare, and over the last forty years have been studied for a variety of purposes. They are beautiful proteins, with a pure azure colour about eighty times as intense as that given by the same amount of copper as cuprammonium ion. The nature of the copper binding site has fascinated two generations of

Table 1: History of Blue Copper Proteins
[for a Review, see 4]

AZURIN	
Denitrification [5]	Verhoeven & Takeda [5]
[RPA's 1st preparation: Oct58]	
Purification from Pseudomonas [6]	Horio [6]
Name; distribution	Sutherland & Wilkinson [7]
Amino acid sequence	Ambler & Brown [8,9]
Inter species sequence comparisons	Ambler [10,11]
First crystals	Strahs [12]
Immunological comparisons	Champion et al. [13]
3A X-ray crystal structure (an azurin-II)	Adman et al., [14]
1.8A X-ray crystal structure	Baker [15][16]
DNA sequence of gene	Arvidson et al. [17]
Site-directed mutagenesis	Karllson et al., [18]
PLASTOCYANIN	
Purification from Chlorella; - in higher plants	Katoh [19]
[RPA's 1st preparation (parsley); Dec60]	
	Kelly & Ambler [20]]
Amino acid sequence	Ramshaw et al. [21]
Higher plant phylogeny	Boulter [22]
Cyanobacterial protein homologous	Aitken [23]
Immunological comparisons	Wallace & Boulter [24]
2.7A X-ray crystal structure	Colman et al. [25]
1.6A structure refinement	Guss & Freeman [26]
DNA sequence of gene	Smeekens et al. [27]
Solution structure by NMR	Moore et al [28]
PSEUDOAZURIN	
Achromobacter protein	Iwasaki & Matsubara[29]
Partial sequence	Ambler [30]
[1976; RPA lost the culture]	
Two proteins in Ps.AM1	Tobari & Harada [31]
Ps AM1 protein sequences	Ambler & Tobari [32]
Crystal structure 2.9A	Petratos et al. [33]
[1995; A. cycloclastes sequence (Ambler & Suzuki)]	

spectroscopists, the X-ray crystallographers have worked out several three dimensional structures [14,25,34] genes have been isolated and characterized [16], and the perturbations caused by site-directed mutagenesis have been explored [18,35]. Nevertheless there is still a question mark over their purpose. In several cases the organism can produce a cytochrome c that can apparently perform the same function, and the blue copper proteins have some characteristics of an unnecessary luxury. It is important that the pheno-types of organisms in which the genes have been knocked out are determined, and so a real assessment made of why they have evolved.

An interest in these blue copper proteins has lasted throughout my scientific career. The work with bacterial electron transport proteins started because it was hoped to use them to prove one of the fundamental hypotheses of molecular biology, the colinearity of chromosome and the protein gene product. The power of bacterial genetics was already such that fine-structure mapping of mutations [36] was reaching the scale of amino acids in the coded polypeptide, even though the genetic code was not yet known. Sydney Brenner was searching for a protein that was genetically accessible but small enough to be a manageable amino acid sequencing project in those early post-insulin days. The first attempt was with *Salmonella* flagellin [37] but the protein proved to be too difficult, having a molecular weight more than double the 17kD expected from the flagellin from other bacteria. The next attempt used the cytochromes of denitrifying pseudomonads [38], but although the protein sequencing proved feasible [39,40], the system did not prove to be genetically accessible. Brenner then went on to try to use a system based on osmotic sensitive head mutants in bacteriophage (but no protein changes could be found), and the hypothesis was finally confirmed by Yanofsky *et al.,* [41] using the *E. coli* tryptophan synthetase system.

While purifying the cytochromes for the colinearity project in 1958, the blue protein that Verhoeven & Takeda [5] (Table 1) and Horio [6] had found in *Pseudomonas* made itself obvious, and was irresistible, so I started to explore its chemical properties. Meanwhile, Katoh [19] (Table 1) had discovered plastocyanin in algae and higher plants, and Robin Hill, in the next laboratory to me, seeing columns blue with the *Pseudomonas* protein, suggested we looked for plastocyanin in parsley (*Petroselinum sativum*). We made some, and its 1961 amino acid analysis on a very early automatic analyser, agrees well with that subsequently deduced from the sequence [42].

Crick [1] and Anfinsen [2] had suggested several evolutionary questions that might be answered by protein sequencing. Mitochondrial cytochrome c was the test system [47] that confirmed that a phylogeny derived from a single gene

H	C H M	residues	
46-MGHNW-	108-YMFFCTFPGHSAL-MKGTLTLK	128	Azurin
40-KGHNV-	74-YGFKCA-P-HYMMGMVALVVVG-	123	Pseusoazurin
47-LPHNV-	82-YDYICT-P-HPF--MKGKVVVE	99	Amicyanin
37-PPHNV-	80-YTFYCE-P-HRGAGMVGKITVAG	105	Plastocyanin
70-VQHNW-	133-YLYICTFPGHYLAGMKGTLTVTP	155	Auracyanin (1)
83-FGHSF-(?)	134-YYYVCQIPGHAATGMFGKIIVK	155	Rusticyanin (2)
44-RFHNV-	83-KYYICGVPKHCDLGQKVHINVTV	105	Stellacyanin (3)
84-GAHNV-	120-ALYVCT-P-HRAQGMYGAVIVE	139	Halocyanin (4)

(1) McManus et al.,(1992).
(2) Cox & Boxer (1978) and see Table 7.
(3) Bergman et al.,(1977).
(4) Mattar et al.,(1994).

Fig. 1: Copper Ligands of Blue Proteins

conformed to classical ideas from morphological and fossil evidence. But would a bacterial protein show similarity in sequence to proteins fulfilling the same function in more complex organisms? With simple morphology and a sparse and uninformative fossil record, there was (and is) very little information about the long-term history of bacteria. Despite the "chemical unity of life", there might not have been any genetic connection between eukaryotes and prokaryotes since the pre-cellular stage.

The first bacterial sequence determined was our *Pseudomonas* cytochrome c-551 [39,40]. There were sequence similarities to mitochondrial cytochrome c, but they were not great enough to show that the proteins were homologous rather than analogous. In the next few years several papers were published comparing the sequences, with some support for an independent origin for the bacterial protein, and other for divergence from an ancestor of the 'higher' sequence. It required tertiary structure determination [48,49] to provide convincing evidence for a common origin. Many other cytochrome c's have an α-band maximum at 551nm, so it is preferable to designate these proteins by their sequence class (Table 2). Proteins with similar sequences to *Pseudomonas* cytochrome c-551 have been found in a wide range of bacteria [50,51] and I think it best if all these are now called cytochromes c_8. Other prokaryotic cytochromes have since been characterized which are much closer in sequence to the mitochondrial ones [(52,53,54] though the search for the bacterial ancestor of the mitochondrion by looking at modern organisms is probably hopeless.

My subsequent research has continued to concentrate on bacterial evolution, and in particular the question of lateral gene transfer across species boundaries. When selection pressure is strong enough (e.g. for the acquisition of resistance to a new antibiotic), lateral transfer is very quick and effective. Our work with the sequences of beta-lactamases [summarised in 55] showed that proteins conferring penicillin resistance have spread right across the bacterial world, though they are only found in organisms that benefit by their possession. Similar effects occur with cytochromes c [50, 56] and blue copper proteins, but the selective advantage of gaining a new electron transport component are not as immediate as being able to survive a new antibiotic.

Evolution and function of bacterial electron transport proteins

It has proved much easier to isolate and purify the soluble proteins than it has been to determine their specific functions. In many organisms there are several different proteins that may be present in the periplasm under different growth conditions, and electrons seem to flow along the strands of a web rather than a single string. This accounts for the long lack of success at elucidating the mechanisms by genetics, as when a single component is knocked out

Table 2: SMALL BACTERIAL COPPER PROTEINS and SOME BACTERIAL CYTOCHROMES C COPPER PROTEINS

name	function	location	residues
Azurin	denitrification methylotrophy oxygen respn	periplasm/OM(1)	128
Plastocyanin	photsynthesis	cyanobacteria	105
Pseudoazurin	denitrification oxygen respn	periplasm	123
Amicyanin	methylotrophy	periplasm	99
Rusticyanin	sulphate respn	periplasm?	155
Auracyanin	photosynthesis	?	155
Halocyanin	oxygen respn	?	139
[Nitrite reductase]	denitrification	periplasm/OM(1)	340

CYTOCHROMES C

Cytochrome	function	residues	haems
c-2	photosynthesis oxygen respn	95-130	1
c-3	sulphate redn oxygen respn	85-115	4 (2)
c-4	oxygen respn	190	2 (3)
c-5	oxygen respn	115	1 (4)
c-6	photosynthesis	85-90	1
c-7	sulphur redn	68	3 (2)
c-8	denitrification oxygen respn photosynthesis	80-90	1
c-L(5)	methyltrophy	170	1 (4)
CCP(6)	oxygen respn	340	2
c'	oxygen respn photosynthesis	c.130	1 (7)
c#(8)	oxygen respn photosynthesis	c.130	1 (7)

(1) In *Neisseria gonorrhoeae* azurin [57] and nitrite reductase [58] occur attached to the outer membrane.
(2) Haems evenly distributed through sequence; proteins with eight and more haems are known
(3) Sequence is a 'covalent dimer'
(4) Haem attached near middle of sequence
(5) See Beardmore-Gray et al.[59]
(6) CCP, Cytochrome c peroxidase
(7) Haem attached near C-terminus
(8) c#, low spin, but sequence like c

its site can often be by-passed. This was shown well by Jenney & Daldal [62] and Rott *et al.*, [63]; when the primary cytochrome c_2 gene from either *Rhodobacter capsulata* or *Rhodobacter sphaeroides* was knocked out, alternative iso-cytochrome c_2 genes were expressed, which allowed photosynthetic electron transport to continue.

A long region of DNA adjoining the nitrite reductase in *Pseudomonas stutzeri* has been

Table 3: Distribution of respiratory pigments among bacteria

For cytochrome c abbreviations see Table 3.
Other pigments: az, azurin; psaz, pseudoazurin; amic, amicyanin;
Cnir, copper nitrite reductase; cd1, cytochrome cd1 nitrite reductase
For details of strain numbers see Ambler (1996)

	c-2	c-4	c-5	c-8	c-L	c'	c#	CCP	az	psaz	amic	CNir	cd1
Pseudomonas aeruginosa		+	+	+					+				+
fluorescens ABDE (1)		+	+	-					+			?+(2)	
fluorescens C		+	+	+					+			<?>	
denitrificans 9496		+	+	+					+			<?>	
stutzeri		+	+	+					-				+
Azotobacter vinelandii		+	+	+		?			-				
Alcaligines xylosoxidans						+			++			+	
faecalis S-6										+		+	
faecalis NCIB8156									+				?
denitrificans						+			+				
Achromobacter cycloclastes										+		+	
Paracoccus denitrificans	+					+		+		+	+		+
Methylobacterium extorquens AM1 (3)	+				+					+	+		
Methylomonas J							++						
Methylophilus methylotrophus				+	+				-				
Rhodobacter (spp.)	+			+		+						+	
Rhodocyclus (spp.)	+												
Rhodopseudomonas palustris	+								+			+	?
Chromatium vinosum	+	+	+										
Neisseria gonorrhoeae	+	+											

1) For biotypes A-G of *Ps. fluorescens* see Stanier et al. [60].
2) For CNir in *Ps. fluorescens* see Zumft et al., [61].
2) Formerly known as *Pseudomonas* AM1

sequenced [64], and shown to contain several c-type cytochromes (including cytochrome c8). Some of these are periplasmic while others are membrane bound, but the role of the individual components has not been resolved, and what are probably functionally equivalent though non-homologous proteins have been found in closely related organisms.

Several distinct classes of soluble cytochromes and blue copper proteins can be recognized in Gram negative bacteria (Table 2), but little sense can yet be made of their distribution (Table 3). Their presence does not correlate with particular types of electron transport metabolism: thus cytochrome c8 is involved with cytochrome cd_1 nitrite reduction in *Ps. stutzeri*, but also occurs in oxygen-coupled metabolism in the nitrogen fixing *Azotobacter vinelandii*, the methylotroph *Methylophilus methylotrophus*, and the phototroph *Chromatium vinosum*. Azurin occurs, possibly associated with cytochrome c8 in denitrifying *Pseudomonas aeruginosa* (though apparently not in *Ps. stutzeri*) and in several other denitrifiers (e.g. the copper nitrite reductase containing *Alcaligenes* strains), but also occur in some methylotrophs, such as *Methylomonas* J [65]. This organism produces two azurins of which one accepts electrons from the methylamine dehydrogenase. Pseudoazurin (Table 1) is associated with copper nitrite reductases in *Achromobacter cycloclastes* and *Alcaligenes faecalis* S-6 [33,66] but is also present together with amicyanin [32] in the methylotroph "Pseudomonas AM1" (now called *Methylobacterium extorquens*). In this organism amicyanin accepts electrons from the methylamine dehydrogenase. Azurin has been isolated from all the biotypes of *Pseudomonas fluorescens* and *Pseudomonas putida* [60] but cytochrome c8 has only been found in strains from biotype C [67] the only biotype which in our hands denitrifies vigorously.

So far, azurin has not been identified in any organism producing a cytochrome c_2, nor a pseudoazurin in any organism producing a cytochrome c8, but cytochromes of the c' sequence class are found together with each (Table 3). The soluble cytochrome contents of many interesting organisms are as yet unknown. These include most methylotrophs and the *Achromobacter/ Alcaligenes* group. A blue copper protein has been found in a strain of the purple *photosynthetic* bacterium *Rhodopseudomonas palustris* (T.E. Meyer, personal communication), but it is not yet know to what class it belongs.

In chloroplasts and cyanobacteria, plastocyanin acts as a soluble link between photosystems I and II. However, in some organisms a small soluble cytochrome (cytochrome c_6, formerly known as soluble cytochrome f) takes its place. Thus plastocyanin has not been found in *Euglena, Porphyra* or (among the cyanobacteria) *Spirulina,* although both proteins are produced by the eukaryotic *Scenedesmus* and *Chlamydomonas* [68] and the prokaryotic *Anabaena* and *Plectonema* [69]. It has been suggested that the alternative carriers provide a way an organism can manage under either copper or iron limitation [68]. I do not know of any searches for unexpressed genes for plastocyanin in *Euglena* or cytochrome c_6 in *Chlorella.*

Common features among small blue copper proteins

The photosystems of eukaryotic algae and cyanobacteria are similar. Comparison of the sequences of both the cytochromes c_6 [70] and plastocyanins [20, 71] between the groups showed them to be convincingly homologous (Figure 2), and provided evidence for a common origin of prokaryotes and eukaryotes.

The feature, which first marked this class of proteins, was their possession of intense absorption at around 600nm. While there was speculation (Guterman, 1963, personal communication) that the colour could be due to a free radical (as can be formed from 2,4,6-tri-*tert*-butyl phenol), cupric copper was the obvious candidate. Experiments with returning copper to apoazurin showed that the amount of colour correlated with the copper added, and metal analysis showed one copper atom for the predicted amount of protein [39,40]. Although azurin is a robust protein, there is some variation in its properties. The best value of the ratio of absorption at 625nm to that at 280nm for *Pseudomonas aeruginosa* azurin (which contains two residues of tyrosine and one of tryptophan) is 0.59, but pure preparations often have lower values. This variation may be due to the presence of some protein denatured in a way such that optimum copper binding cannot be achieved, such as the (reversible) oxidation of some methionine to methionine sulphoxide.

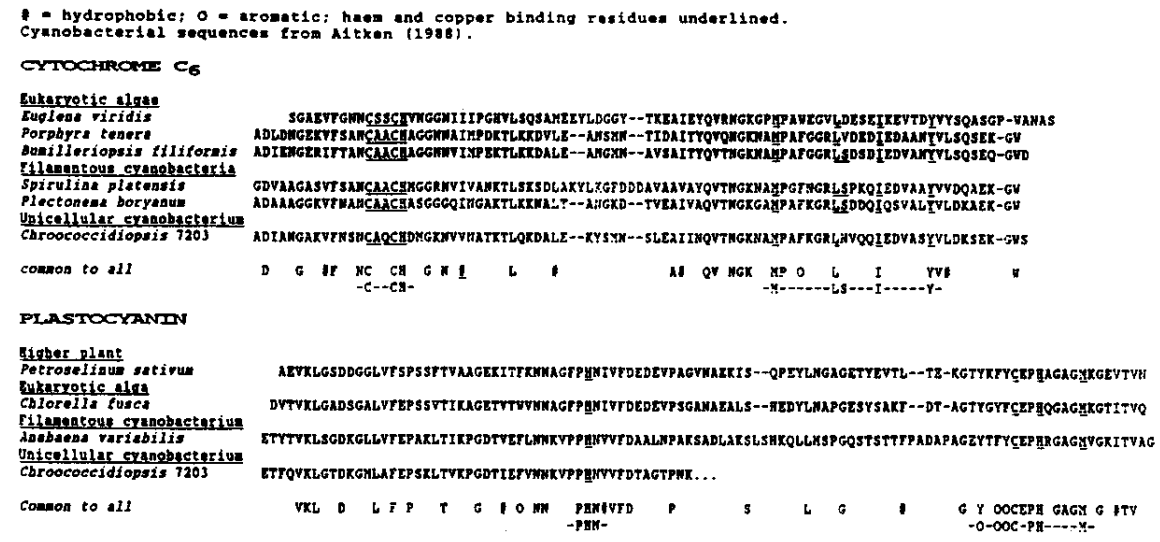

Fig. 2: Similar Sequences between Prokaryoyes and Eukaryotes

A plethora of spectroscopic techniques have been used to compare the epr, fluorescence, EXAFS and other properties of the blue copper proteins, though these studies have added little to our knowledge of their biological function.

Similarities between all the small blue copper proteins are obvious now that the primary and several tertiary structures are known. Katoh and Takamiya [72] suggested that thiol groups contributed to the copper binding site of plastocyanin, and sequence comparison [20] revealed similarities to a region that included the only free thiol group in azurins (Figure 1). With the first tertiary structures [14,25] the ligands could be seen to be a histidine residue near the N-terminus of the sequences, and a cluster of the cysteine, another histidine and a methionine near the C-terminus. This cluster also included several aromatic residues.

Interspecies sequence comparisons of azurins and plastocyanins

There are now about twenty complete azurin sequences known (Figure 3), as well as a comparable number of complete or partial plastocyanin sequences, at least three pseudo-azurins and amicyanins, and two rusticyanins. Within each type of protein there are very few internal insertions or deletions, and there is the expected conservation of the residues involved in copper binding. However, there is considerable interspecies variation in the sequences (Table 4), and it is noticeable that there is as much variation within the well-characterized genus *Pseudomonas* as there is between pseudomonads and *Bo. bronchiseptica*. The large differences between the isoazurins that have been found in the same organism are discussed below.

When Gotschlich and Sieff [57] found an azurin attached to the outer membrane of *Neisseria gonorrhoeae* I thought that this was a case of the co-option of a robust gene for an anti-immunological purpose by a pathogenic organism. However, now that a copper nitrite reductase has been found similarly located in the same organism [58], it is likely that they really are both involved in nitrite metabolism. Both proteins were only found because they contained an antigenic domain, and similar unsuspected membrane-bound copper proteins may be widely distributed. We know this to be the case for cytochromes c.

As well as the residues involved in copper binding, there are about thirty residues that are conserved in all the known azurins, or which are only changed in one or other of the sequences. The information about these conserved residues should be invaluable in the design of further site-directed mutagenesis experiments. The conserved sites are interspersed with highly variable sites (Figure 4), and designing an oligonucleotide that should locate an azurin of unknown sequence will be difficult.

The residues are numbered from the *Ps. seruginosa* sequence. Residues that form the copper ligands are marked ★. The evidence for residues shewn in lower case is weak. *Ps. seruginosa* NCPPB 1224 is probably identical to (1) except for I22V and I80V. *Ps. fluorescens* E-36 is very similar to (6), the only difference detected being E75P. In sequence (8) there may be a single residue insertion at about residue 95. *Alc. faecalis* NCTC 415 is the same as (15) except for V5I, S6T, V50I and I86L. *Alc faecalis* S-6 produces a pseudoazurin rather than an azurin (Hormel et al., 1986). Sequence (17) is preceded by a lipoprotein signal sequence and an alanine/proline-rich 39-residue domain.

(1) *Pseudomonas seruginosa* P6009
(2) *Pseudomonas fluorescens* A-126
(2a) *Pseudomonas fluorescens* A-192^{T}
(3) *Pseudomonas fluorescens* B-93
(4) *Pseudomonas fluorescens* B-400
(5) *Pseudomonas fluorescens* C-18
(6) *Pseudomonas fluorescens* D-35
(7) *Pseudomonas fluorescens* G-149
(8) *Pseudomonas putida* A-76
(8a) *Pseudomonas putida* (no strain number given)
(9) *Pseudomonas denitrificans* NCIB 9496
(10) *Bordetella bronchiseptica* NCTC 8344
(11) *Alcaligenes xylosoxidans* NCIB 11015 iso-1
(12) iso-2
(13) *Alcaligenes denitrificans* NCIB 8582
(14) *Alcaligenes faecalis* NCIB 8156$_{T}$
(15) *Methylomonas* sp. J NCIMB 13224 iso-1
(16) iso-2
(17) *Neisseria gonorrhoeae* H.3 outer membrane protein

References:
(1) Ambler & Brown,1967; (2,4,7) R.P.A. unpublished; (3,5,6,9,10,11,13,14) Ambler,1971;
(8) R.P.A. & L.McLellan unpublished; (15,16) Ambler & Tobari,1989; (17) Gotschlich & Seiff,1987;
(2a)Lee et al. (1996); (8a) Barber et al. (1993)

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Residue No.
          1         2         3         4         5         6         7         8         9         1         1         1
          0         0         0         0    ★    0         0         0         0         0         1         2
1         0                                                                                   0 ★       ★ 0★
(1)   AECSVDIQGNDQMQFNTNAITVDKSCKQFTVNLSHPGNLPKNVMGHNWVLSTAADMQGVVTDGMASGLDKDYLKPDDSRVIAHTKLIGSGEKDSVTFDVSKLKEGEQYMFFCTFPGHSALMKGTLTLK
(2)   AECKVTVDSTDQMSFNTKEITIDKSCKQFTVELTHSGNLPKNVMGHNWVLTTQADMQPVATDGMAAGIDKDYLKAGDERIIAHTKIIGAGEKDSVTFDVSKLKADEKYSFFCSFPGHISMMKGAVVLK
(2a)  AECKVTVDSTDQMSFNTKAIEIDKSCKTFTVELTHSGSLPKNVMGHNWVLSSAADMPGIASDGMAAGIDKNYLKEGDIRVIAHTKIIGAGEKDSVTFDVSKLAAGTDYAFFCSFPGHISMMKGTVTVK
(3)   AECKTTIDSTDQMSFNTKAIEIDKSCKTFTVELTHSGSLPKNVMGHNWLVISKQADMQPIATDGLSAGIDKNYLKEGDTRVIAHTKVIGAGEKDSLTIDVSKLNAAEKYGFFCSFPGHISMMKGTVTLK
(4)   AECKVTVDSTDQMSFNTKEITIDKSCKTFTVELTHSGSLPKNVMGHNWvvstdalmqpVATDGMAAGIDKNYLKEGDDRIIAHTKIIGAGEKDSVTFDVSKLAAGTDYAFFCSFPGHISMMKGTVVLK
(5)   AECKVTVDSTDQMSFDTKAIEIDKSCKTFTVDLKHSGNLPKNVMGHNWVLTTQADMQPVATDGMAAGIDKNYLKEGDTRIIAHTKIIGAGZTDSVTFDVSKLKADGKYMFFCSFPGHIAMMKGTVTLK
(6)   AECKVDVDSTDQMSFNTKEITIDKSCKTFTVNLTHSGSLPKNVMGHNWVLSKSADMAGIATDGMAAGIDKDYLKPGDSRVIAHTKIIGSGEKDSVTFDVSKLTAGESYEFFCSFPGHNSMMKGAVVLK
(7)   AECKVTVDSTDQMSFNTKAIEIDKSCKTFTVELTHSGNLPKNVMGHNWVLTSAANMQPVATDGMAAGIDKDYLKPGDDRIIAHTKIIGAGEKDSVTFDVSKLAAGTDYAFFCSFPGHISMMKGTVTVK
(8)   AECSVTVDSTDQMSFNTKEITVDKSCKEFTVKLTHSGSLPKNVMGHNLVISkTADMQGIATEGMSQGLDKDYIKADNAAIIAHTKMIGPAEKE?VKFDATKLEAGGDYSFFCTFPGHISMMKGKVVvk
(8a)  AECKVTVDSTDQMSFNTKDIAIDKSCKTFTVELTHSGSLPKNVMGHNLVISKEADMQPIATDGLSAGIDKQYLKDGDARVIAHTKVIGAGEKDSVTFDVSKLAAGEKYGFFCSFPGHISMMKGTVTLK
(9)   AECSVDIQGNDQMQFSTNAITVDKSCKTFTVNLSHPGSLPKNVMGHNWVLTTAADMQGVVTDGMAAGLDKNYVKDGDTRVIAHTKIIGSGEKDSVTFDVSKLKAGDAYAFFCSFPGHSAMMKGTLTLK
(10)  AECSVDIAGTDQMQFDKKAIEVSKSCKQFTVNLKHTGKLPRNVMGHNWVLTKTADMQAVEKDGIAAGLDNQYLKAGDTRVLAHTKVLGGGESDSVTFDVAKLAAGDDYTFFCSFPGHGALMKGTLKLVD
(11)  AECSVDIAGNDQMQFDKKEITVSKSCKQFTVNLKHPGKLAKNVMGHNWVLTKQADMQGAVNDGMAAGLDNNYVKKDDARVIAHTKVIGGGETDSVTFDVSKLAAGEDYAYFCSFPGHFALMKGVLKLVD
(12)  AQCEATVESNDAMQYNVKEIVVDKSCKQFTMHLKHVGKMAKVAMGHNLVLTKDADKQAVATDGMGAGLAQDYVKAGDTRVIAHTKVIGGGESDSVTFDVSKIAAGENYAYFCSFPGHWAMMKGTLKLGS
(13)  AQCEATIESNDAMQYNLKEMVVDKSCKQFTVHLKHVGKMAKavMGHNWVLTKEADKQGVATDGMNAGLAQDYVKAGDTRVIAHTKVIGGGESDSVTFDVSKLTPGEAYAYFCSFPGHWAMMKGTLKLSN
(14)   ACDITIEGNDSMQFNTKSIVVDKTCKEFTINLKHTGKLPKAAMGHNVVISKKSDESAVATDGMKAGLDNNYVKAGDERVIAHTDVVGGGESTSVTFDVSKLKEGEDYAFFCSFPGHWSIMTGZIKLGS
(15)  AGCSVDVEANDAMQYNTKNIDVEKSCKEFTVNLKHTGSLPKNVMGHNLVITKTADFKAVMNDGVAAGEAGNFVKAGDARVVAHTKLVGGGZKDSVKVDVSKLAAGEKYTFFCSFPGHATMMRGTVTVK
(16)  ASCETTVTSGDTMTYSTRSISVPASCAEFTVNFEHKGHMPKTGMGHNWVLAKSADVGDVAKEGAHAGADNNFVTPGDKRVIAFTPIIGGGZKTSVKFKVSALSKDEAYTYFCSYPGHFSMMRGTLKLZE
(17) -GNCAATVESNDNMQFNTKDIQVSKACKEFTITLKHTGTQPKASMGHNLVIAKAEDMDGVFKDGVGAA-DTDYVKPDDARVVAHTKLIGGGEESSLTLDPAKLADGD-YKFACTFPGHGALMNGKVTLVD
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Fig. 3: Azurin Sequences

Table 4: Percentage differences between azurins

When the sequences are aligned (Fig.1), no internal insertion/deletion events need to be postulated. The *Alc. xylosoxidans* and the *Bo. bronchiseptica* sequences are not separated from those from the pseudomonads. *Alc. denitrificans* is now commonly included with *Alc. xylosoxidans*. While the azurin sequences for *Ps. aeruginosa* and *Ps. denitrificans* 9496 are similar, the cytochrome c_8 sequences are quite distinct (Ambler, 1996). The results are given for *Alc. faecalis* NCIB 8156T; the protein from strain NCTC 415 differs in four positions (3%). *Ps., Pseudomonas; Alc., Alcaligenes; Bo., Bordetella.*

	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)
(A) *Ps. aeruginosa*	0	13	29	27	42	37	38	30	40	53
(B) *denitrificans* 9496	13	0	23	24	35	32	38	27	35	48
(C) *fluorescens* C	29	23	0	34	39	37	43	32	40	50
(D) *Alc. xylosoxidans* iso-1	27	24	34	0	33	31	36	21	37	50
(E) iso-2	42	35	39	33	0	11	33	36	38	47
(F) *Alc. denitrificans*	37	32	37	31	11	0	34	34	41	47
(G) *Alc. faecalis*	38	38	43	36	33	34	0	38	41	52
(H) *Bo. bronchiseptica*	30	27	32	21	42	34	38	0	36	53
(I) *Methylomonas* J iso-1	40	35	40	37	38	41	41	36	0	48
(J) iso-2	53	48	50	50	47	47	52	53	48	0

Boulter [21] tried to answer phylogenetic problems in flowering plants by comparing sequences of plastocyanins and cytochromes c, but found [75,76] that their rate of change was too slow, and the mathematical methods available for comparing sequences inadequate. Twenty years later, there is much more information available for other genes through DNA sequencing, but the relationship of the higher plant orders seems just as obscure

Duplicate genes for blue proteins

Two distinct but homologous azurins or plastocyanins are produced by some organisms.

In *Methylomonas* J [65] only one azurin is produced when the organism is growing on methanol, but a second one is also synthesized when growth is on methylamine. This azurin-II functions as the electron acceptor from methylamine dehydrogenase[31,77]. The proteins from *Methylomonas* J are among the most diverse azurins, and their difference (48%) between each other is more than that of the Azurin-I from the other known sequences (Table 4), so adaption to the new function as an acceptor from methanol dehydrogenase appears to have required considerable change.

Alcaligenes xylosoxidans NCIB [78] and *Alc. xylosoxidans* GIFU 1051 [79, S. Suzuki and R.P.Ambler, unpublished results] under some as get undefined growth conditions also produce two distinct azurins. A sample of azurin received from Dr H. Iwasaki in 1966 yielded peptides that can now be recognized as coming from both isoforms, but preparations at Edinburgh in 1969-71 [80] produced only the Azurin-I [81]. Much the closest sequence to the azurin-II from *Alcaligenes xylosoxidans* NCIB 11015 is the only azurin yet found in *Alc. denitrificans*, for which the three-dimensional structure is known [34]. The azurin-I is considerably closer in sequence to *Ps. aeruginosa* (the other known three-dimensional structure) so functional differences between azurins I and II may be recognisable. The nomenclature and taxonomy of *Alcaligenes* strains is not in a stable state at present [82,83] and the recommendation that *Alc. denitrificans* should be considered the same species as *Alc. xylosoxidans* has been retracted.

The plastocyanin used for the three dimensional structure determination [25] came from the Black Poplar (*Populus nigra* var.*Italica*). Freeman [84] has told the long saga that at last led to plastocyanin crystals worthy of an X-ray beam. Two different plastocyanins were isolated from the leaves of a stand of urban trees, and one protein crystallized. While the crystallographic analysis was in progress we sequenced the proteins (R.P. Ambler, unpublished results 1977-78), finding them to differ in ten positions. The sequence of the iso-2 plastocyanin has been completed and properly published by Dimitrov *et al.*, [85] There is as much difference between the iso forms (12%) as between plastocyanins from different orders of higher plants, so a functional difference seems likely.

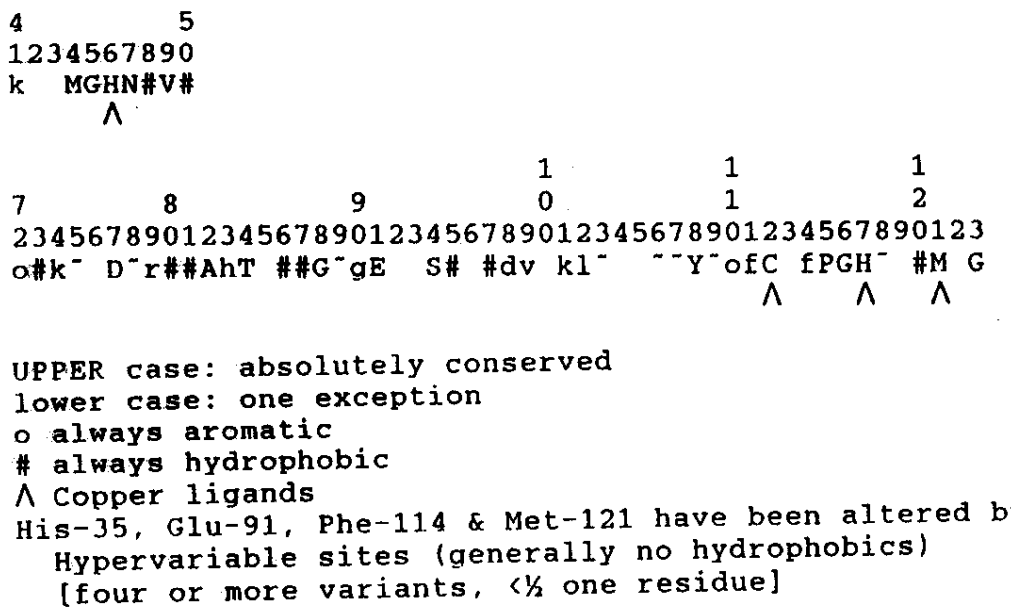

Fig. 4: Conserved Residues in Azurines

How perfect are electron transport proteins?

Dogma has it that natural selection can act upon changes in proteins or nucleic acids that have arisen through mutation, and if the change is favourable to the organism the change will tend to be perpetuated. The question then is "how small need a change be before it is favoured?". The approaches that have been made towards answering the question are:

(1) Kinetic measurements on natural proteins from different organisms

(2) Kinetic measurements on isolated mutant proteins

(3) Viability of organisms containing a mutated protein

(4) Estimation of polymorphism for a protein within a species.

Early kinetic studies with mitochondrial cytochromes c were unable to detect differences between proteins as diverse as those from yeast and mammals [86]. However, when the assay conditions were changed, kinetic differences were found between very similar cytochromes [87]. It has not been shown that these kinetic differences are of sufficient significance to enable natural selection to act upon them. These doubts have been strengthened by site-directed mutagenesis (SDM) studies with many proteins, where (in laboratory culture) the organism remains viable despite changes that have had a major kinetic effect on the functioning of the protein.

These results are relevant to our study of blue copper proteins because Wood [68] has suggested that cytochromes and blue copper proteins are functionally interchangeable in algal photosynthesis. Much work has also been done on the kinetics of electron exchange between azurin and cytochrome c8 [88,89,90]. If proteins are approaching perfection, the prediction would be that the blue copper protein and the cytochrome will have each been 'tuned' to the environment and their electron donors and acceptors in their own organism. Thus if the kinetic measurements have biological significance then a comparison between homologous and heterologous systems would show this. The structural changes in 'sequence space' [51] that have happened to the pair of carriers during the divergence of the line from other organisms should have kept their interaction optimal. For pseudomonads that express both azurin and cytochrome c8, there is around 30% difference in azurin sequence (Table 4) and nearly 50% in cytochrome c8, so ample scope for their surfaces to be 'out of tune' between species exists.

Protein polymorphism is rare in higher organism species, where sexual and population processes will regulate the fixing of neutral mutations. The situation for a clonally-propagated organism will be different. We have looked for intra-species differences in cytochrome c8 and azurin in pseudomonads, choosing cultures isolated from the wild in different ecological and geographical conditions [67]. *Pseudomonas aeruginosa* is a versatile and widely-distributed soil organism, able to be an opportunistic pathogen of animals, plants and fungi. However, the cytochromes c8 of nine out of ten strains examined have identical amino acid sequences, and no differences have been detected between their azurins. However, the tenth strain, isolated as a pathogen on the plant *Aglaonema commutatum* in Puerto Rico, has a single change in the cytochrome [A2D], and two changes in the azurin [I22V and I80V]. Are these changes functionally linked?

I interpret all these observations as meaning that natural selection in the real world -- not the microbial paradise of a laboratory culture medium -- polishes and maintains interacting proteins to a state of near perfection.

Aromatic amino acids in blue copper proteins

Pseudomonas aeruginosa azurin has an unusual feature in its ultraviolet absorption spectrum, a very sharp peak at 292nm, on the edge of the broad aromatic 280nm peak [39,91]. This peak remains if the copper is removed from the protein (e.g. by precipitating the protein with trichloroacetic acid, washing the precipitate, and redissolving to pH 7), but disappears with acid or urea denaturation. It also disappears if the apoprotein is treated with a protease -- the native protein is resistant to proteolysis. The peak has been explained as being due to the only tryptophan residue (W48) being sited in a hydrophobic environment in the interior of the molecule, with the ring parallel to the conserved phenylalanine residue F111, very close to the copper binding site.

Azurins are known with zero, one or two tryptophan residues, though these only occur at positions 48 and 118 (Table 5). The proteins with no tryptophan [e.g. those from *Pseudomonas fluorescens* strain B93, ATCC 17467, from *Ps. fluorescens* ATCC 13430 [92], and from some strains of *Pseudomonas putida*] lack the 292nm peak. This is also the case with the single tryptophan at W118 (those from *Alcaligenes faecalis* or the azurin II from *Alc. xylosoxidans*). However, the peak seems to be present in even the most sequence-divergent azurins which contain W48 & F111, including those from *Bordetella bronchiseptica*, *Alcaligenes* strain 11015 Azurin-I and *Pseudomonas fluorescens* A126, which spans 30% of sequence difference.

The only plastocyanins in which tryptophan occurs are in some from green algae, including *Chlorella, Scenedesmus* and *Enteromorpha*. The *Chlorella* [93] and *Enteromorpha* [94] proteins show notches on the '280nm' UV peaks, and the NMR structure [95] shows that in the *Scenedesmus* protein W29 and Y80 are close together. Residue 80 in the plastocyanin is two residues before the copper binding C82, and should be compared with F111 and C113 in azurin (Figure 1). If this structural feature is functionally important, it is surprising that it occurs in some plastocyanins and azurins, but not in all species of either protein.

Biological problems for protein structure/function studies

I would like to make a plea to biochemists and biophysicists to use biologically well defined material for their experiments. Inevitably, we collaborate with other specialists in the course of our work, but each laboratory has a responsibility to ensure that the protein under study comes from a defined and reproducible source. In my protein studies I have wasted a lot of time because of problems with the source of the material, and much published work is uninterpretable because of doubts about what organism the protein discussed actually came from. Even when using vertebrate material problems can arise, particularly if the biochemist does not meet the intact animal. For instance, there are worries from early sequencing days about two whale insulins having supposedly identical sequences but were immunologically different.

Problems are much more frequent with material from microorganisms, where a variety of difficulties can arise (Table 6), and several of our studies with blue copper proteins have been affected.

The strain used for our original azurin preparations was received in 1958 called

Table 5 Aromatic amino acids in azurins

Tryptophan residues are at position 48 and/or 118. Azurins having Trp-118 are marked *. The 6F 2Y 1W proteins listed below have the 292nm peak, but the *Alc. faecalis* azurins (6F 2& 1W*) do not.

6F 2Y 1W: *Ps. aeruginosa*
Ps. fluorescens (all except B-93)
Ps. denitrificans 9496
Bordetella bronchiseptica
6F 2Y 1W*: *Alc. faecalis* NCTC415 & 8156
6F 2Y 0W: *Ps. putida* A-76 & A-100
Methylomonas J iso-1
5F 2Y 0W: *Ps. fluorescens* B-93
6F 3Y 1W: *Alc.xylosoxidans* iso-1
4F 4Y 1W*: *Alc.xylosoxidans* iso-2
4F 4Y 2W*: *Alc.denitrificans*
7F 4Y 1W: *Methylomonas* J iso-2
5F 2Y 0W: *N.gonorrhoeae* OMP

Table 6: Problems with protein preparations

(1) Misidentification
(a) Animal or plant species
(b) Bacterial names change
(c) Wrong strain name
(d) Mislabelling

(2) Culture contamination
(a) Growth conditions allow contamination
(b) Stock culture contamination during maintenance
(c) Cross-contamination by related strains before or after growth
(d) Mislabelling

(3) Unrecognized impurity
(a) Duplicate genes in organism
(b) Impurity not detected by assay used

(4) Contamination with interfering substance
(a) Protease
(b) Salt or sugar

Pseudomonas fluorescens. Rhodes [96] examined more than 150 pseudomonad strains and concluded, with the help of an electronic computer, "*Ps. aeruginosa* may be regarded as a variety of *Ps. fluorescens*". It was not until a few years later that Jessen [97]) and Stanier *et al.*, [60] clearly separated the species, a differentiation confirmed by our sequence work with cytochrome c8 [11,98, 29% different] and azurin [67; 34% different). Stanier [cited in 98] confirmed that the Lenhoff & Kaplan [38] was a typical *Ps. aeruginosa*. Unfortunately Finazzi-Agro *et al.*, [91] did not give a strain number for the material used for their fluorometric studies of azurin, although they called it *Ps. fluorescens*. If a few milligrams of the azurin survived it would be easy to find out which it was. Another example of problems caused by bacterial names is the case of *Alcaligenes faecalis*. Azurin has been characterized from the type strain (NCIB 8156, = ATCC 8750) and from NCTC 415 (= ATCC 19108), but in another strain (S-6) called by the same name a pseudoazurin is found instead [66]. Unfortunately strain S-6 has not been included by the bacterial taxonomists in their studies of the *Alcaligenes/Bordetella* family [82,83], so we do not know if (by their criteria) it is an aberrant *Alc. faecalis* or a member of another group.

A recent example, with the well-known bacterium Paracoccus *denitrificans*, of how the literature and the field of electron transport proteins can be confused by inadequate care over strains and their names has been publicized by Goodhew *et al.*, [99].

In some cases organisms are grown under conditions when contamination with a similar organism is possible. Thus the algal preparations that Kelly [93] used for preparing plastocyanin were grown in a 40,000l outdoor culture unit. Large quantities of both *Chlorella* and *Scenedesmus* cells were produced, and plastocyanin isolated from the dried cells. However, two plastocyanins with different sequences were found in each of the initial batches of cells. A further batch grown after the heterogeneity problem had been recognized allowed the *Chlorella* plastocyanin sequence [100] to be determined. Subsequent work with *Scenedesmus* plastocyanin [prepared by Dr. R. Powles, Liverpool [quoted in 42] showed that the second "Chlorella" plastocyanin isolated and partially sequenced by Kelly [93] had come from *Scenedesmus*.

Rusticyanin [44,101] is a small blue copper protein produced by Thiobacillus *ferrooxidans*. The organism grows slowly, but requires such an acid medium (pH 2) that few laboratory contaminants would survive. We started to sequence the protein in

Table 7: Difference matrix for rusticyanins

	(a)	(b)	(c)	(d)
(a) Yano et al.(1991)	0	14	9	1
(b) Ronk et al.(1991)	14	0	10	12
(c) Scotland B	9	10	0	9
(d) Nunzi et al.(1993)	1	12	9	0

(Sequences (a) and (e) only differed at residue 5 (Ser/Gly)
Scotland A squence (see text) was identical to (b).
Thiobacillus ferrooxidans strains:
(a) isolated in Japan (no number given)
(b) ATCC 23270^T (NCIB 8455)
(c) Scottish strain was Porton Tf3 (Cox & Boxer, 1978)
(d) isolated in France (no number given)

1977, but got inconsistent results between batches, and it eventually became clear that we were trying to decode two different but very similar sequences. We finally produced a complete sequence of the most common form in 1989, but were too slow in preparing a manuscript, and three independent sequences have since been published [102,103,104]. Our main sequence was identical to that of Ronk *et al.*, [102]; but our second sequence was different to that of both Yano *et al.* [103], and Nunzi *et al.*,[104], which are nearly identical (Table 7). It seems likely that the cause of our trouble was contamination by another strain of *Th. ferrooxidans*.

Problems can arise when different batches of cells are stockpiled ready for a large scale preparation. I wasted much time when determining the sequence of a *Pseudomonas* amidase [105], because the protein being worked on turned out to be a mixture of amidases from *Ps. aeruginosa* and *Ps. putida*. Although the sequence difference is about 10%, the net charge of the protein from the two species is very nearly the same, and they were not separated under the standard purification conditions.

Thatcher [106] found that the yield and stability of *Drosophila* alcohol dehydrogenase depended on the order in which purification steps were carried out. This was caused by a protease present in the extract copurifying with the desired enzyme. If a step was used early on that separated the protease from its potential substrate, much better results were obtained. Comparable problems with a protease were found when preparing and sequencing the beta-lactamase II from *Bacillus cereus* strain 569 [107]. The sequences of peptides recovered from enzymic digests could not be reconciled with the specificities of the endopeptidases used until. It was then realized that the 'pure' beta-lactamase contained an aminopeptidase, whose activity had survived reduction with dithiothreitol in 8M urea and EDTA, and gel filtration at room temperature at pH 2.

Conclusions

The work on blue copper proteins discussed in this paper has largely used the experimental approach of amino acid sequence analysis. This approach has powers that are different to those of a gene cloning approach, which to a large extent can only find what is being looked for. While the small blue copper proteins from the widest range of sources have common structural features (Figure 1), these do not include sufficiently large, constant and continuous sequence motifs to be recoverable through DNA or immunological routes. This has meant, as Adman [108] has pointed out, that the blue copper proteins have mostly been discovered in the process of something else being looked for. The reasons for this is that the proteins are not obvious when in the reduced (Cu^+) form, and it is in this state that they are isolated from many cells. Progress would have been different if our copper proteins had a spectrum as intense as cytochromes, or if copper had a friendlier radioactive isotope, but ^{61}Cu, ^{64}Cu and ^{67}Cu all have half-lives of only a few hour.

There are still many interesting questions to be solved with and about these small blue copper proteins. Foremost are their functions, with the determination of phenotypes of strains from which genes have been knocked out being a way ahead. Good enzymic assays are necessary, and a comparison of the kinetics of electron exchange between azurin and cytochrome c_8 using proteins from different pseudomonad species should indicate whether this assay has any biological meaning. Measurement of the extent of silent ('third base') changes in the azurin and cytochrome c8 genes of *Ps. aeruginosa* from different strains will shed light on protein perfection. Searches with antisera or oligonucleotide probes may reveal the presence of azurins (or additional isoazurins) in organisms in which they have not been found. It will be interesting to see how many unsuspected copper proteins are present in bacteria when more complete genome sequences become available, as the motif for their copper binding site will be detectable in the sequences (Figure 1).

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