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A study of the structure and redox properties of the copper site in azurins by means of EXAFS, NMR, redox titrations, potentiometry, equilibrium cyclic voltammetry and rapid scan voltammetry on protein films is reported. The results are discussed in light of existing theories on structure and function of type-1 copper sites. The exit and entry of electrons take place through the C-terminal histidine ligand of the copper. The hydrophobic patch through which this residue penetrates the protein surface plays an important role in partner docking (*cf.* The rim of the porphyrin ring sticking through the surface of the cytochromes-*c*). We find no experimental evidence for strain around the metal site. The active centre is able to maintain ET activity even in the presence of fairly gross disturbances of the site structure. The analysis of the thermodynamics of the redox reaction shows that the protein matrix and the solvent play an important role in 'tuning' the redox potential around a "design" value of around 300 mV at room temperature. The metal site appears "designed" to stabilise the Cu(II) instead of the Cu(I) form. The remarkable evolutionary success of the blue copper proteins is ascribed to the sturdy overall β-sandwich structure of the protein in combination with a metal site that is structurally adaptable because three of its four ligands are located on a loop. The electronic "gate" that occurs in the middle of a hydrophobic patch allows for fine tuning of the docking patch for recognition purposes.

I. Introduction

This contribution deals with small blue copper proteins, in particular the mechanistic, structural and spectroscopic aspects of their active centres, which consist of a so-called type-1 copper site (*vide infra*). Ever since the discovery of blue copper proteins in the late 1950's[1–3] coordination chemists have been intrigued by their unusual spectroscopic properties (strong optical absorption around 600 nm ($\varepsilon = 2000$–6000 M^{-1} cm^{1}); small $A_{\|}$ ($\sim$6.6 mT) in the $g_{\|}$-region of the EPR spectrum).[4,5] Their preoccupation with the subject has marked the research in this field and coined the language by which the properties of these sites are described and analysed. This includes the use of symmetry related concepts ("axial symmetry", "rhombic distortion", "Jahn–Teller deformation" [6,7] although in fact the protein surroundings of the Cu site do not display

symmetry, the concept of matrix-induced strain, the analysis of electron transfer (ET) processes in terms of speed and efficiency, and the focus on the ligand sphere of the metal in particular the "axial ligand" as the parameter that tunes the redox potential of the copper site. Biologists on the other hand might want to emphasise the influence of the protein matrix on the mechanistic properties of the active site, and to concentrate on physiologically relevant characteristics like metabolic rate and control.

Here we address the question of how the previously, partly intuitively formulated concepts relate to recent experimental evidence. In particular the following points are considered.

(i) There has been much speculation over the years about the mode of entry and exit of electrons on their way to and out of the copper site. Various patches have been identified on the surfaces of blue copper proteins that, supposedly, would be instrumental in mediating electron transfer. After a considerable amount of research there now appears to slowly emerge a consensus as to the mode of operation of blue copper proteins.

(ii) It has been argued that the configuration of the ligands in the active site of blue copper proteins is a compromise between the configurations "preferred" by Cu(I) and Cu(II). Although this leads to strain in the active site it also lowers the reorganisational energy connected with a change in valence state and, thus, the activation barrier for electron transfer (ET).[8,9] The delicate balance between two preferred conformations that evolved in the course of evolution, reflects, it has been said, the function of the blue copper proteins, which is the shuttling of electrons between different sites in an electron transfer chain.

(iii) The tetrahedral or distorted tetrahedral conformation of the ligands around the copper site in blue copper proteins stabilises, it has been argued, the Cu(I) state over the Cu(II) state compared to Cu in an aqueous environment.[9,10] This explains why the redox potential of blue copper proteins on average is higher than the redox potential of aqueous copper (150 mV *vs.* NHE at room temperature, pH 7[11]). The large variation in redox potentials observed for type-1 sites (190 mV for *Rhus Vernicifera* stellacyanin[12] to 780 mV for the type-1 site in laccase[13] has been ascribed largely to the 'tuning' effect of the ligand at the 'axial' position in the coordination sphere of the copper (normally a methionine, but a glutamine in the plantacyanins[14] and a leucine in the type-1 site of laccase[15,16]).

(iv) There appears to be a striking effect of pH on the structure of the reduced Cu site in some blue copper proteins. The C-terminal histidine ligand may become protonated at low pH and may dissociate from the copper. The differences that exist among blue copper proteins in this respect have been ascribed to differences in the length of the loop on which three of the four copper ligands are located. The precise mechanistic and physiological consequences of this phenomenon were not clearly perceived until recently.

II. Materials and methods

II.1 Protein chemistry and construction of protein variants

Proteins were heterologously expressed in *E. coli* and purified according to established procedures.[17–20] Protein variants were constructed at the genetic level by standard site-directed mutagenesis techniques.[17,20–28]

II.2 Redox chemistry

Redox potentials were determined either by titration with ferri/ferrocyanide,[29] by potentiometric titration or by cyclic voltammetry. For the potentiometric titrations an anaerobic cell was used that allowed the recording of the optical spectrum at equilibrated electrode potentials. The saturated Hg/Hg_2SO_4 reference electrode (Radiometer, Denmark) was calibrated with quinhydrone (Sigma).

For the fast-scan protein-film voltammetry an electrochemical cell as described before was used.[30,31] Analogue CV was recorded with an Autolab analyser (Ec-Chemie, Utecht) equipped with a PGSTAT 20 potentiostat and a fast analogue scan generator in combination with a fast AD converter (ADC750). Voltammograms were Fourier-filtered and background-subtracted[32] prior to analysis. To form a protein film a drop of buffered (20 mM MES, pH 6) protein solution was applied to a polished pyrolytic graphite electrode. Neomycin (2 mM) was added to the solution as a co-adsorbent to stabilise the protein film on the electrode.

II.3 NMR spectroscopy and determination of ESE rates

Samples for NMR measurements were prepared from aqueous protein solution by exchanging the water for D_2O and concentrating the solution at the same time by ultrafiltration (Amicon). Protein sample concentrations amounted to usually 1–2 mM and the solutions contained 20 mM phosphate buffer of the appropriate pH. The pH was adjusted by adding small aliquots of dilute NaOD or DCl. Protein was reduced by adding dithionite or ascorbate and washing away (Amicon) the excess reagent. To maintain reducing conditions solutions were kept under argon. Spectra were measured on 300 and 600 MHz Bruker NMR spectrometers at ambient temperature. Electron self exchange rates were determined from the broadening of the His46 C2H proton signal when a know (small) amount of oxidised protein was introduced in a solution of the reduced protein.[33]

II.4 EXAFS

Cu K-edge X-ray absorption spectra were measured at the D2 station of the EMBL Hamburg Outstation in the fluorescence detection mode. The storage ring operated at 4.5 GeV and 120–170 mA. Reference samples consisted of ZnS and Cu-phthalocyanin mixed with boron nitride. Samples were about 10 mM in protein concentration and buffered in 20 mM MES pH 6.8. Reduced samples were obtained by reducing oxidised protein solutions with a small excess of sodium dithionite. Reducing conditions during the EXAFS measurements were maintained by adding ascorbic acid up to 2 mM and flushing with argon. Measurements were performed at ambient temperature except for the case of oxidised His1117Gly azurin in the absence of external ligands. To push the equilibrium between the various species that co-exist in this case, to one side, T was lowered to about 20 K which drives the equilibrium to the type-2 form of the Cu-site. The data analysis was performed by the difference file technique according to standard procedures.[34–36] Protein raw data were Fourier transformed over a range from 3 to 11 $Å^{-1}$. Multiple shell fitting was performed in R-space over a range that covered between 1 and 2.2 Å of the FT spectrum.

III. Results and discussion

III.1 Pathway

The electronic link of the Cu site to the outside world that appears obvious from inspection of the available 3D-structures of blue copper proteins,[37,38] consists of the C-terminal histidine ligand. It protrudes through the protein surface, usually in an area with clear hydrophobic character (see Figs. 1 and 2). Its crucial role in mediating ET is corroborated by an extensive body of experimental data.[29,39–42] Apart from this link other electronic "pathway connections" have been envisaged. For azurin (Azu) a path containing His35 and Cu ligand His46 connecting to a surface area around Lys85 and Glu91 has been proposed as an alternative to the C-terminal His117 connection.[43,44] In plastocyanin a pathway linking Tyr83 on the protein surface to the Cu site has long been considered as providing a physiologically relevant link with the Cu centre.[45] Experimental evidence gathered over the past five years on surface modified blue copper proteins, among others, has demonstrated convincingly, however, that the hydrophobic patch around the C-terminal His is the critical area that mediates ET between the Cu site and all sorts of redox partners.[29,39,40,46] Strictly speaking this evidence does not prove the direct involvement of this His in ET, but circumstantial evidence[41,47–49] relating to mutants in which the C-terminal His was replaced by a glycine and to which an external ligand was added, point to the importance of the C-terminal His for ET activity. It is also this histidine that may become protonated at low pH in some blue copper proteins (*vide infra*).

III.2 Strain *vs.* ET rates

The ligand geometry around the copper in blue copper proteins deviates from the symmetrical configurations (octahedral or elongated octahedral for Cu(II) or tetrahedral for Cu(I)) that one encounters for copper coordination compounds that have four or six identical ligands. A deviation from such an idealised configuration can be achieved at the cost of a destabilisation of the electronic energy of the metal. Gray and Malmström have come up with a minimum estimate of 70 kJ mol^{-1} for this energetic effect in the case of a typical blue copper site.[9,50] More recently Pierloot

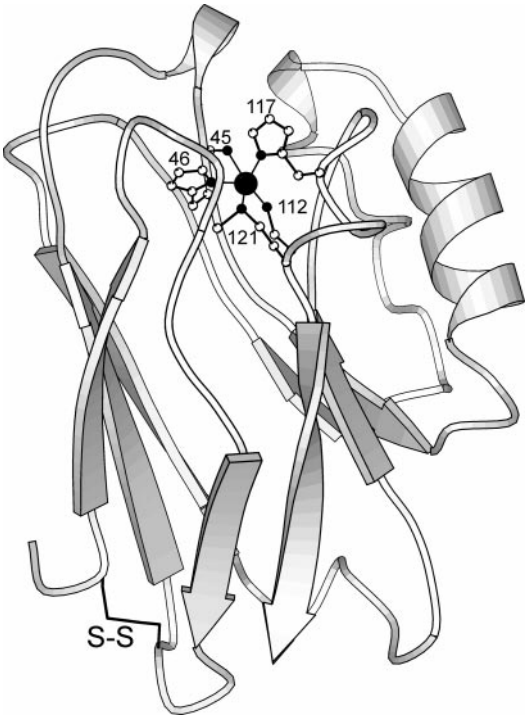


Fig. 1 Backbone representation of azurin from *Pseudomons aeruginosa*.[79] The copper is presented as a black sphere and the ligands are labelled according to their number in the amino acid sequence (H46, C112, H117, M121). The backbone carbonyl of Gly45, also indicated in the figure, has a mainly Coulombic interaction with the Cu. The Cys3–Cys26 disulfide bridge has been indicated in the lower left corner of the structure.

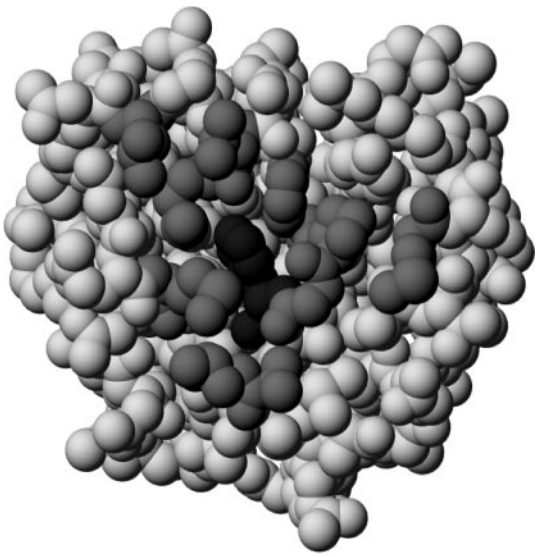


Fig. 2 Space filling representation of *P. aeruginosa*[79] as viewed from a 'Northern' direction. The shading is as follows: dark grey; Cu plus ligand His117 (side chain plus backbone); grey; hydrophobic residues (side chains plus backbone) in the 'hydrophobic patch'; light grey; rest of the protein.

and co-workers have argued on the basis of calculations on models of blue copper sites *in vacuo* that the electronic effect is small and that electronic energy is only little affected by the precise geometry of the ligands around the copper.[51]

Whatever the magnitude of the effect, if any, it is apparently compensated by the binding of the copper to the protein matrix. The metal is eagerly taken up by the apo-protein when the latter is incubated with Cu salts in solution.[52,53] Also the configuration of the ligands in the apo-protein is similar to their configuration in the holo-protein indicating that the protein matrix itself does not convert to a strained conformation upon binding of the metal (Fig. 3).[54,55] In fact, the only noticeable difference between holo- and apo-protein relates to the mobility of the side chains in the metal binding pocket, the mobility being significantly higher in the apo-form of the protein.[54,55]

Apart from the notion of matrix-induced strain, the idea has been advanced that the precisely tuned structure of the type-1 site, being as it is a compromise between the typical Cu(I) and Cu(II) conformations, lowers the barrier for ET.[8] The parameter which according to Sutin and Marcus[56] reflects the height of this barrier is the reorganisational energy, λ. To check the validity of this idea we have engineered a series of mutations in the active site of various blue copper proteins and tried to observe their effect on the ET activity. The problem with this approach is that the rate of ET with a redox partner not only depends on λ, but also on the formation constant of the (temporary) association complex, K_{ass}, the difference in redox potential between the redox partners, ΔE_0, and the electronic coupling between the active sites of electron donor (A) and acceptor (B), H_{AB}. These parameters may be affected by the mutation as well. By choosing the mutation such that the perturbation of the 'hydrophobic patch' and of the position of the C-terminal histidine ligand with respect to the protein framework are minimised, K_{ass} and H_{AB} will be affected minimally. By measuring, in addition, the electron self exchange (ESE) rate, possible changes in the redox potential become inconsequential.

In a first attempt we changed the methionine (Met121) ligand to the Cu into a glutamine (Q121) in Azu from *Alcaligenes denitrificans*.[17] The structures of the reduced and oxidised proteins have been determined.[57] Contrary to the wild type protein, there is a considerable difference in bond lengths between the oxidised and the reduced site (see Fig. 3), while the hydrophobic patch and the C-terminal histidine ligand, His117, are marginally affected. Yet, the change in ESE rate amounts to only two orders of magnitude, amounting to a change in λ of 400 mV.

In a second series of experiments the loops containing three of the four copper ligands were taken from a series of blue copper proteins and grafted onto the framework of the blue copper protein amicyanin (Ami) from which the native loop had been excised (see Fig. 4 and Table 1). The constructs were realised by standard mutagenesis techniques. Certainly one expects the ET properties to be seriously compromised when the structure of the ET site is seriously tampered with. However, the majority of the protein variants turned out to be quite competent in ET with ESE rates within two orders of magnitude from the wt value (see Table 2).[20,58]

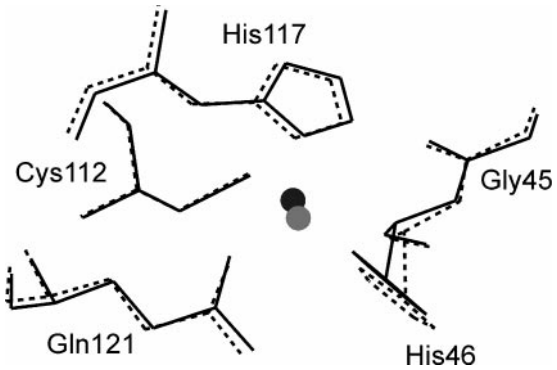


Fig. 3 Overlay of the structures of the Cu site in the reduced and oxidised M121Q variant of *A. denitrificans* azurin.[57] The solid black and grey spheres represent the copper in the oxidised and reduced forms, respectively. Solid and dashed lines refer to the structures of the oxidised and reduced protein.

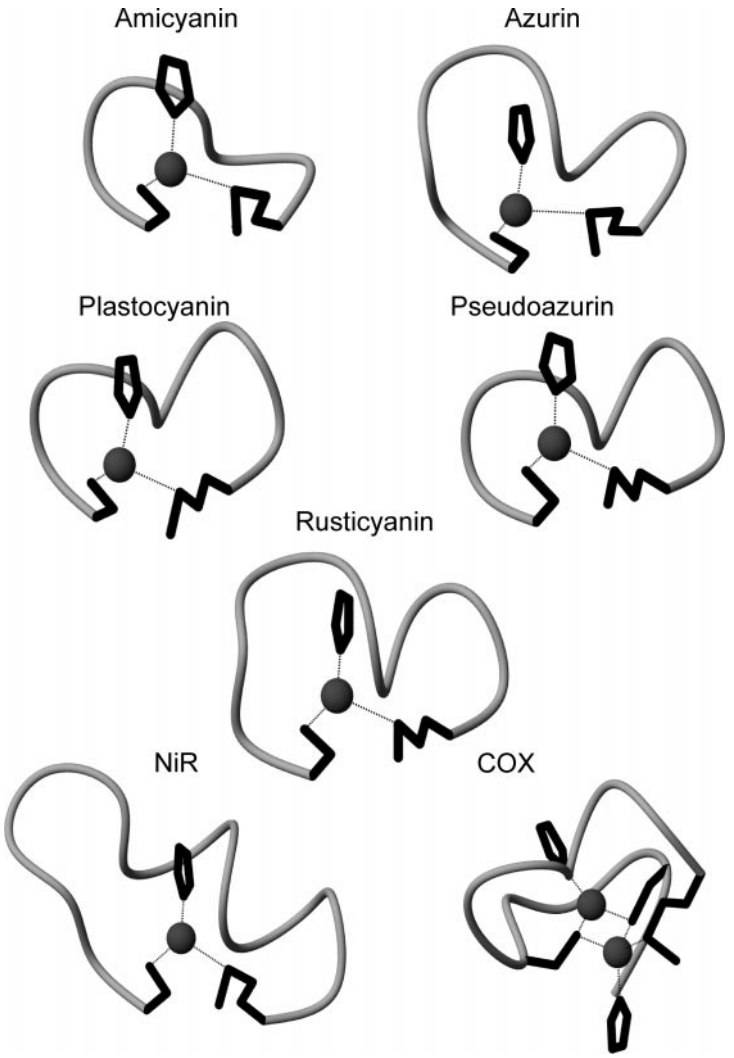


Fig. 4 Structures of the native amicyanin ligand loop and of the ligand loops[76–80] used for the loop-directed mutagenesis experiments. Although the loops of NiR[81] and COX[82] do not derive from small blue copper proteins they are included here for the sake of completeness. All loops were grafted successfully onto the β-sandwich structure of amicyanin to replace the native loop (shown top left).

The conclusion from the work reported in this section then must be that the active site in blue copper proteins is fairly robust when it comes to transferring electrons efficiently. It withstands rather course disturbances without loosing its capacity to function in ET.

III.3 Redox potential

The prediction of the redox potential of a redox protein by theoretical means is a challenge that has not been fully met yet. Many efforts have concentrated on finding proper ways to account for the Coulomb interaction between the redox centre and the charges within the protein matrix. The main complication thereby is how to properly account for the polarisabilities of bonds and atoms inside the protein. A parameter that is equally important is the entropy change that is connected with a change in redox state of the active site. That the entropy effect can be sizeable is illustrated by the example of the Azu mutant in which residue Asn47 is changed into a leucine. Asn47 in Azu occurs next to one of the histidine ligands of the copper (His46) and is one of the very few residues

Table 1 The partial amino acid sequence showing the C-terminal loops of five cupredoxins and two cupredoxin-like domains: amicyanin from *Paracoccus versutus*,[76] plastocyanin from *Populus nigra*,[77] pseudoazurin from *Alcaligenes faecalis* S-6,[78] azurin from *Pseudomonas aeruginosa*,[79] rusticyanin from *Thiobacillus ferrooxidans*[80] and the loops containing the blue copper centre in nitrite reductase (NiR) from *Alcaligenes xylosoxidans*[81] and the CuA centre in cytochrome *c* oxidase (COX) from *Paracoccus denitrificans*[82]

Amicyanin	**C**	T	—	—	—	—	—	P	—	**H**	P	—	—	F	**M**
Plastocyanin	**C**	S	—	—	—	—	—	P	—	**H**	Q	G	A	G	**M**
Pseudoazurin	**C**	T	—	—	—	—	—	P	—	**H**	Y	A	M	G	**M**
Azurin	**C**	T	F	—	—	—	—	P	G	**H**	S	—	A	L	**M**
Rusticyanin	**C**	Q	I	—	—	—	—	P	G	**H**	A	A	T	G	**M**
NiR	**C**	A	P	S	G	M	V	P	W	**H**	V	V	S	G	**M**
COX	**C**	S	—	E	L	**C**	G	I	N	**H**	A	—	—	Y	**M**

The copper ligands are shown in bold

that is strongly conserved in blue copper proteins.[59,60] To understand the reason for this, it is important to realise that in blue copper proteins the metal ligands are located on two loops. In Azu the N-terminal histidine occurs on a strand that connects β-strands 3 and 4, while the remaining three, C-terminal ligands are located on a second loop connecting β-strands 7 and 8. The conserved character of Asn47 derives from its crucial role in stabilising the two loops with respect to each other by means of hydrogen-bridges (see Fig. 5).[61] Surprisingly enough, the mutation does not affect the spectroscopic properties of the metal site (EPR, UV-Vis characteristics) nor the electron self exchange rate, but it does affect the E_0 which increases considerably with respect to wild type, *i.e.*, by 110 mV.[62] A study of the temperature dependence of the redox potential showed that this already sizeable effect is the sum of two even bigger, partly compensating changes in the enthalpy ($\delta\Delta H/F = 445$ mV) and entropy ($\delta(T\Delta S)/F = -335$ mV) of reduction.

This compensatory entropy/enthalpy behaviour was subsequently found to reflect a general trend among blue copper proteins, which was clearly borne out by a systematic and more elaborate study of the redox potentials of a number of blue copper protein variants.[12] There appears to be a linear correlation between ΔH° and ΔS°, the enthalpy and entropy of reduction (see Fig. 6). In the case of a perfect linear relationship between ΔH° and ΔS° there is a temperature, T_c, at which the free energy of reduction is a constant, independent of the individual protein. The least squares fit of the data presented in Fig. 6 corresponds with $T_c = 288 \pm 10$ K and a temperature independent contribution to the redox potential of 294 ± 10 mV. The deviation of the redox potential from this value at other temperatures is proportional to $(T - T_c)\Delta S^\circ$. Thus, although at room temperature the redox potential appears to be determined to a sizeable extent by ΔH°, the *variation* in E_0 is determined equally by the *variations* in entropy and enthalpy as is obvious from the lower part of Fig. 6 where the slope of the plot is close to 1.

Table 2 ESE rates of the loop mutants of amicyanin and those of corresponding native copper proteins

	$k_{ESE}/M^{-1}\ s^{-1}$ [a]	pK_a [b]	E°/mV[c]
Amicyanin (*Paracoccus versutus*)	1.3×10^5	6.80	248
Ami-Pcy	2.1×10^4	5.7	308
Plastocyanin (*Populus nigra*)	$\sim 10^3$ [d]	4.7	361
Ami-Azu	1.6×10^4	5.6	296, 298[e]
Azurin (*Pseudomonas aeruginosa*)	8.0×10^5	—	319[e]
Ami-Paz	1.6×10^4	5.4	282, 283[e]
Pseudoazurin (*Alcaligenes faecalis*)	2.8×10^3	4.8	270[e]
Ami-Rus	nd	nd	360
Rusticyanin (*Thiobacillus ferroxidans*)	nd	nd	680[f]

[a] Self exchange rate constants were determined at pH ~8.0, 298 K except azurin: pH 9.0, 309 K. [b] For the C-terminal histidine ligand.[20] [c] Determined at pH 7.0 by cyclic voltammetry. [d] Spinach plastocyanin. [e] Determined at pH 7.0 by redox titration. [f] Ref. 83.

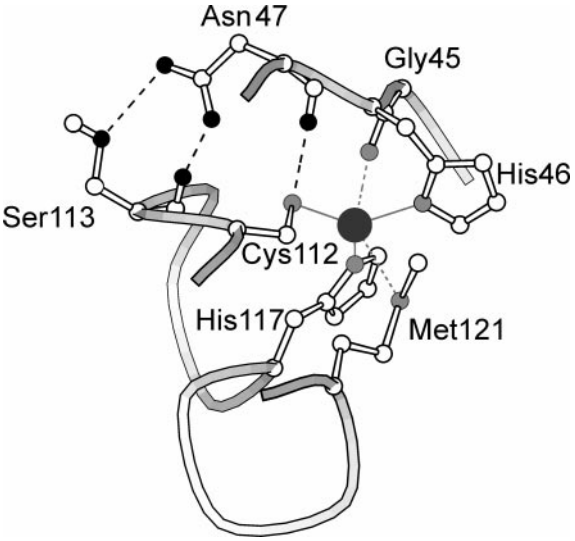


Fig. 5 Part of the structure of *A. denitrificans* azurin showing the participation of Asn47 in the hydrogen-bonding network connecting the two loops that contain the copper ligands.[61,79] Hydrogen bonds are indicated between Asn47 Oδ1 and backbone proton of Ser113, Asn47 Nδ2 and Ser 113 Oγ1 and between the backbone proton of Asn47 and Sδ of Cys112 (heavy dashed lines). The bonds between the copper and the equatorial ligands are inicated with the light solid lines, and the bonds between the copper and the axial ligands with light dashed lines.

The general mechanisms underlying possible compensation behaviour have been the source of extensive speculation.[63–67] Often enthalpy–entropy compensation is considered evidence that a single parameter has an overriding influence on the free energy driving a reaction or the free energy of activation determining its kinetics. It is difficult to imagine how the redox potential of the variety of proteins considered in Fig. 6 may be governed by a single parameter unless it relates to an overall property like solvation. Indeed, compensation phenomena in aqueous solution have

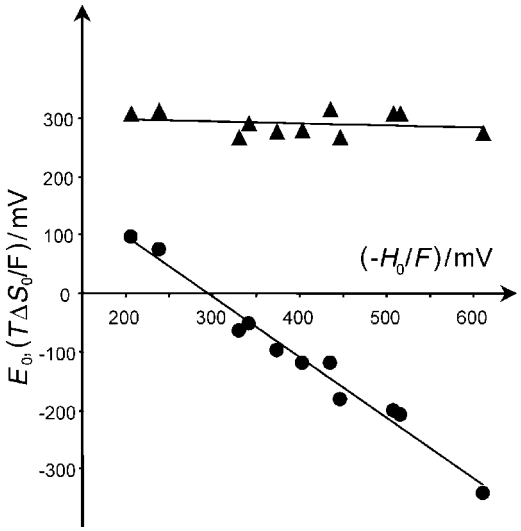


Fig. 6 Compensation plot for the thermodynamic data observed for a number of azurin variants (data from ref. 12 and this work). Plotted are the redox potential (triangles) and the reduction entropy (spheres) as a function of the negative reduction enthalpy. For the calculation of the points ΔS° was multiplied by $T = 298$ K.

been ascribed to the unusual solvating properties of water and often exhibit compensation temperatures in the range of 270–310 K. Thus there are at least three effects that have to be taken into account when analysing variations in redox potential. First there is the electronic effect that accompanies a change in redox state of the metal. This is confined to the metal and its ligands and ideally should be dealt with by quantum-chemical methods. Qualitatively, trends can be analysed by borrowing concepts from coordination chemistry of the transition metals ("hard and soft" ligands, electron donating power, *etc.*). A second contribution derives from the dielectric of the protein and a third contribution from solvent effects. It is a matter of debate whether the present data support the conclusion that within a particular class of proteins (*i.e.* the azurins) the solvation effect is damped out as a result of entropy–enthalpy compensation.

III.4 Active site conformation and pH

Freeman *et al.*, in a series of ground-breaking XRD structure determinations of plastocyanin (Pc) were the first to draw attention to the variations that may occur in the coordination sphere of the copper when the protein is reduced at low pH.[68] The C-terminal histidine (His 87 in the case of *Populus nigra* Pc) may become protonated at the Nδ position, as a result of which the His–Cu bond is broken. Moreover through a rotation mainly around the Cα–Cβ–Cγ bonds, the histidine moves away from the copper. When the copper is in the oxidised form, the normal 4-coordination of the metal is stable within the accessible pH-range. Subsequently this phenomenon has been found to be a general property of blue copper proteins, like the amicyanins[69,70] and the pseudoazurins,[71,72] the more notable exceptions being the azurins and the recently discovered Pc from the fern *Dryopteris crassirhizoma*. In an attempt to delineate the dynamics of the histidine dissociation and its effect on redox potential and ET reactivity, we have set out to study the histidine dissociation by means of spectroscopic techniques and rapid scan cyclic voltammetry on protein films. As a model system we chose amicyanin from *Paracoccus versutus*.

The signals of the C2- and C4-protons of the two histidine ligands of the Cu can be easily identified in the NMR-spectrum of the reduced amicyanin since there are only two histidines in the aa sequence. The signals of His96 titrate with pH as shown in Fig. 7, demonstrating that this histidine becomes protonated at low pH. The redox potential was determined by cyclic voltammetry at scan speeds where the system was in thermodynamic equilibrium. It varies strongly with pH, as shown in Fig. 7. The data were fit with the standard equation for the redox potential which is based on a pH-dependent equilibrium between two species with redox potentials E_l^o and E_h^o at low and high pH, respectively, and acid dissociation constants of pK_a^r and pK_a^o in the reduced and oxidised form, respectively.[73] The solid line in Fig. 7 represents the best fit with parameters presented in Table 3.

The dissociation and association kinetics of the C-terminal His ligand were investigated by means of rapid scan CV on films of the protein applied on glassy carbon electrodes. In the ideal case, at low scanning speed the reductive and oxidative wave of a cyclic voltammogram of a protein film have their peaks located at the position of the redox potential of the protein. However, when the speed by which the wave is traversed approaches the rate by which the protein exchanges electrons with the electrode, the peaks move apart.[30] This is illustrated for amicyanin by Fig. 8 (panels A and C). The peak separation is described by the Butler–Volmer equations[31] as

Table 3 Redox potentials connected with the protonation of His96 of *Paracoccus versutus* amicyanin as obtained by equilibrium potentiometry

	0–3 °C	22–25 °C
pK_a^{red}	6.4 ± 0.1	6.3 ± 0.1^a
pK_a^{ox}	$\leqslant 3.4 \pm 0.2$	$\leqslant 3.2 \pm 0.2$
E°_{alk}/mV	231 ± 3	221 ± 3
E°_{acid}/mV	$\geqslant 395 \pm 10$	$\geqslant 402 \pm 10$

[a] Value obtained by NMR in D_2O equals 6.8.

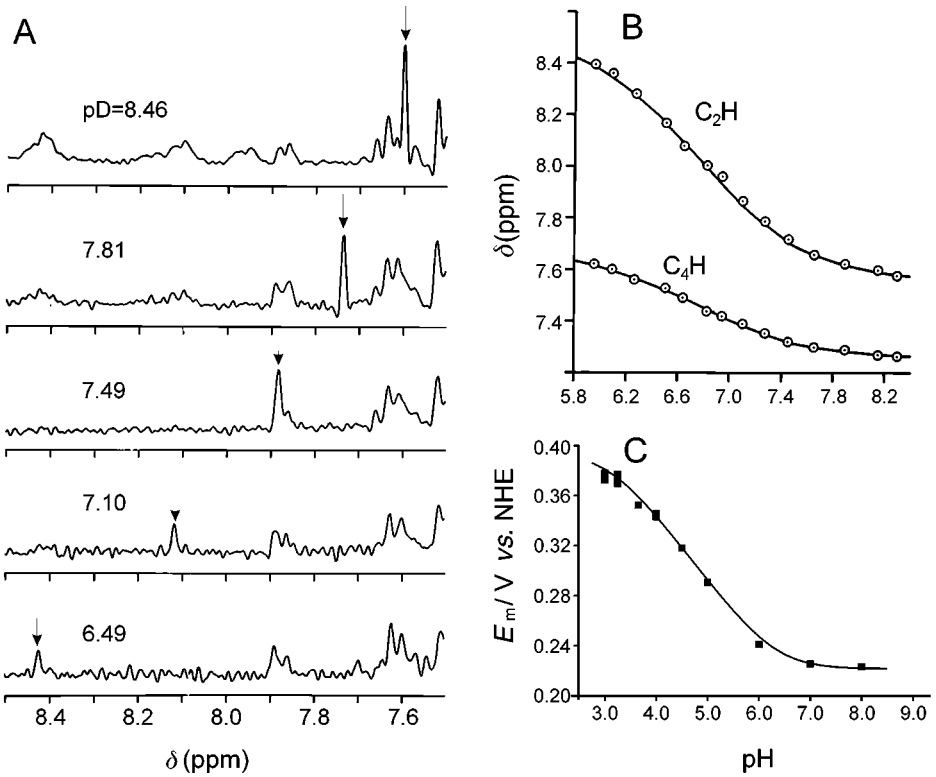


Fig. 7 Plot of the chemical shifts of the C2H and C4H proton signals of His96 (graphs A and B) and the redox potential (graph C) of Ami from *P. versutus* as a function of pD or pH, respectively. $T = 298$ K; NMR-spectra recorded at 300 MHz in D_2O.

a function of scan speed and the solid lines in Fig. 8 (panel C) are simulations on the basis of these equations. The situation becomes more complicated (see Fig. 8, panel B) when one of the species may occur in two forms, as is the case with reduced amicyanin, of which the redox centre may adopt different conformations depending on pH. The effect that the equilibrium between the two forms may have on the CV can be understood best by considering the oxidised Ami. Moving from an oxidative poise into the reducing direction, the Cu centre becomes reduced after which His96 dissociates. Moving back in the oxidative direction the centre will be oxidised again provided the scan speed is slow enough to maintain thermodynamic equilibrium during the scan. However, at sufficiently fast scan rates, the histidine has no time to dissociate and the protein is oxidised back before the histidine has had time to drop out of the first coordination sphere of the copper. At intermediate scan rates, a reductive wave is observed but the oxidative wave will be weak or absent. The data can be analysed again by the Butler–Volmer equations, now expanded with a term that accounts for the possible occurrence of two reduced conformations. From a fit of the data, redox potentials and the interconversion rate between the two species can be obtained (data not shown). The redox potentials appear in agreement with the data obtained from the redox titrations (see Table 3).

Contrary to the case of Ami, the Cu centre in Azu is stable at low pH in the reduced form. In an attempt to find the cause for this difference we removed the C-terminal ligand histidine (*i.e.*, a glycine was engineered at the position of His117) and replaced it with an externally added imidazole (Imz). When the Cu site is oxidised Imz spontaneously enters the gap created in the Cu coordination sphere by the mutation, but when the site is reduced the imidazole drops out. Similar to the case of Ami, the redox potential was determined, this time not as a function of pH but as a function of the Imz concentration. The results are summarised in Scheme 1. As in the case of Ami, the redox potential increases considerably in the absence of the C-terminal Imz ligand. Corre-

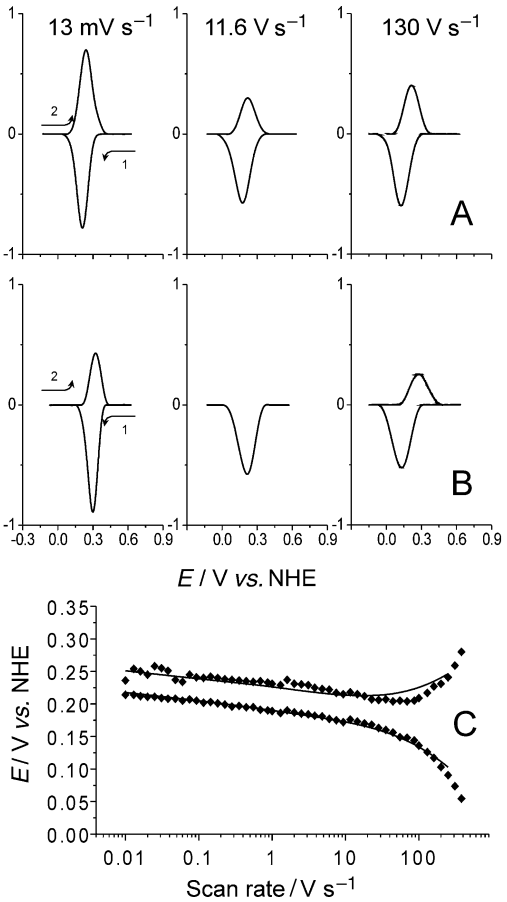
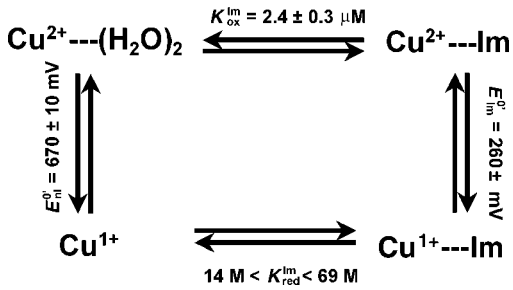


Fig. 8 Rapid scan cyclic voltammograms of protein films of Ami (*P. versutus*), $T = 298$ K, at pH 7.08 (panel A) and pH 5.04 (panel B). The peak positions of the reductive and oxidative waves have been plotted as a function of the scan rate in panel C. The solid lines are fits on the basis of the Butler–Volmer equations. A constant peak separation of 40 mV was included in the fit. The cause of the slope in the linear parts of the plots is unknown. This is at present the subject of further investigation.

sponding with this a very large increase in the Imz dissociation constant is observed when the Cu centre changes from the oxidised to the reduced form.

To investigate the nature of the Cu coordination in the His117Gly Azu variant, EXAFS experiments on reduced and oxidised His117Gly azurin in the absence and presence of a range of external ligands were performed. The EXAFS traces, which are reproduced in Fig. 9, were analysed by standard techniques; the results are summarised in Table 4. It is clear from these data



Scheme 1

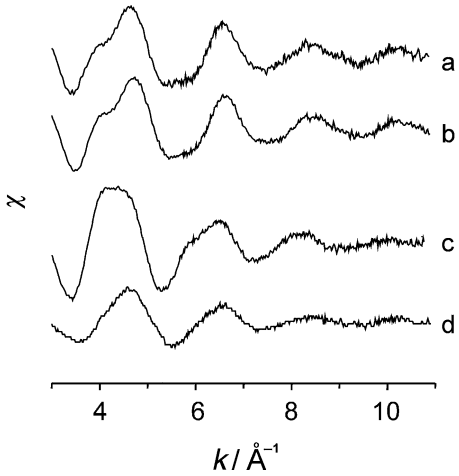


Fig. 9 Pre-edge modified, background subtracted and normalised raw EXAFS data obtained on variants of azurin from *P. aeruginosa*. (a) Oxidised wt Azu; (b) Oxidised H117G plus Imz; (c) Oxidised H117G in the absence of ligands; and (d) reduced H117G in the absence of ligands. Experimental temperatures: 20 K for trace (c), ambient for the other traces. The similarity between traces (a) and (b) demonstrates that in oxidised Azu the metal sites in wt and H117G + Imz are virtually identical. If the Imz is left out of the solution (trace (c)), the Imz is replaced by two water molecules or OH^- ions. When the protein is subsequently reduced the site reverts to a 3-coordination (see also Table 4). A more elaborate data analysis will be presented elsewhere.

that in the oxidised form the Cu centre is 4-coordinate with three ligands deriving from the protein (His46, Cys112 and Met121) and one from the solution, either in the form of a water molecule (in the absence of external ligands) or in the form of an Imz moiety. In the reduced form the Cu becomes 3-coordinate, with the Cu-S(Met121) bond considerably shortened.

IV. Conclusions

The results reported here can be summarised as follows.

The small blue copper proteins appear to operate by using a single port of entry and exit for electrons to get into and out of the active site. The C-terminal histidine, that ligates to the copper and protrudes through the hydrophobic patch on the protein surface, serves as such.

The idea that the ligand configuration around the copper in blue copper proteins carries conformational strain is interesting when viewed against the background of the coordination chemistry

Table 4 Structural data of the copper site in WT and the His117Gly variant of azurin from *P. aeruginosa* as derived from the EXAFS experiments reported in Fig. 9

	N^a	R/Å^b	Assignment
wt Azu(ox)	1 S	2.15	Cys 112
	2 N	1.90	His46, His117
H117G Azu(ox) + Imz	1 S	2.14	Cys 112
	2 N	1.89	His46, Imz
H117G Azu(ox)	1 S	2.26	Cys 112
	2 N/O	1.91	His46, H_2O or OH^-
	1 N/O	2.01	H_2O or OH^-
H117G Azu(red)	1 S	2.16	Cys 112
	1 N	1.86	His46
	1 S	2.29	Met 121

[a] N: coordination number after mean free path correction. [b] Distance, estimated error is ± 0.03 Å.

of copper. From the viewpoint of the protein there is no evidence pointing to strain in the structure. The peculiar ligand composition and conformation of the metal site may seem adapted to the function of the blue copper proteins, *i.e.* "fast" electron transfer. However, as we have seen above, the ligand sphere can be fairly thoroughly perturbed without the protein loosing its ability to transfer electrons at physiological rates. It may be interesting, therefore, to see if the data presented here allow one to expand this notion.

In light of the loop-directed mutagenesis experiments reported above, the first point to be noted, then, is that of the four ligands to the copper one is located on a loop inside the β-framework (the N-terminal His ligand). The three others are located on a second loop, two of which occur at the transition of the β-sandwich to the loop. Thus, three of the four ligands actually are associated with or anchored to the β-sandwich, *i.e.*, the N-terminal His, the Cys and the Met ligands. Only the fourth, C-terminal His ligand which provides for the electronic coupling with the Cu, is located in the middle of the C-terminal loop (see Fig. 10). As we have seen above, as long as the loop folds properly onto the β-sandwich, and the histidine binds to the Cu, the protein is able to function reasonably well as an electron carrier. This leaves room for the patch around the C-terminal His, to develop or maintain traits that are important for partner recognition. The remarkable evolutionary success of the blue copper proteins as electron carriers may therefore be related to the combination of a sturdy β-sheet framework onto which an active site is anchored that is malleable, yet is able to maintain its function under evolutionary pressure.

The redox potential depends on the electronics of the Cu site (metal plus ligands), the dielectric properties of the protein and the presence of the aqueous medium around the protein. The tuning of the redox potential appears to be provided in part by the ligand sphere of the metal (QM effects) and in part by the protein and the water environments. The latter contributions are marked by sizeable entropy and enthalpy contributions which to a large extent cancel. This, then,

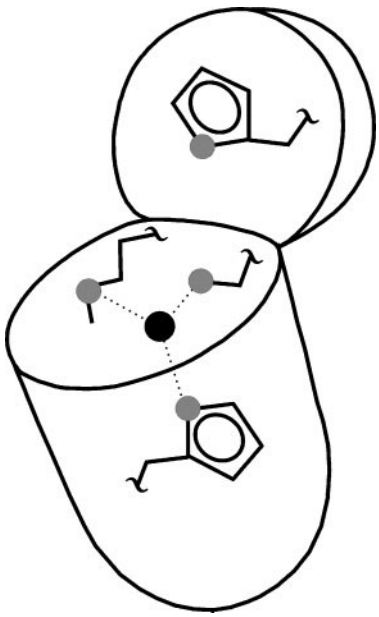


Fig. 10 Schematic drawing illustrating the structural properties of a blue copper site as discussed in the text. The protein is depicted as an elongated ellipsoid the main body of which consists of the β-sandwich housing the N-terminal histidine (His46 in the case of Azu). The top symbolises the hydrophobic patch containing the loop on which the C-terminal histidine ligand (His117 in the case of Azu) is located. The two sulfur containing ligands (Cys112 and Met121 in the case of Azu) are located at the transition from β-sandwich to loop. Changes in structure and composition of the 'lid' do not seriously compromise the redox activity of the protein as long as the lid is able to fold back onto the β-sandwich and the C-terminal histidine can be reached from the outside.

may provide for a second tuning mechanism. Variations in the protein interior and changes in the protein surface may affect mainly either ΔS°, ΔH° or both and, thus 'tune' E_0.

The idea that the redox potential of blue copper proteins is more positive than that of aqueous copper because the geometry of the Cu site favours the Cu(I) redox state, appears not to be supported by the experimental evidence reported here. In many blue copper proteins like the plastocyanins, the amicyanins and the pseudo-azurins, as well as in some mutants, the active site seems to be able to revert to a three-coordinated form when the metal is reduced. Further research is needed to establish whether this is an entropy- or enthalpy-driven process. Preliminary data indicate that the former may apply, stressing again the need to take the protein matrix into account when looking into the properties of the metal site.

The question why in some blue copper proteins the C-terminal histidine ligand does not dissociate from the metal upon reduction remains intriguing. The evidence available so far is compatible with the idea that steric hindrance is a major factor in this. The physiological relevance is likely to be related to the need to make the association with redox partners sensitive to the redox state of the protein. A case in point is amicyanin which in the oxidised form associates with its reduced partner methylaminedehydrogenase, but dissociates after electron transfer. The association constants differ markedly for the two redox states.[74,75]

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