Volume 325, number 1,2, 39–48 FEBS 12533 June 1993

Engineering type 1 copper sites in proteins

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Received 15 April 1993

The use of site-directed mutagenesis methods has revolutionalized the study of the so-called type 1 and type 2 copper sites in proteins. In particular our understanding of the relation between the structure, and the mechanistic and spectroscopic features of these sites is benefitting from the application of these techniques. Recent progress in the field is reviewed with emphasis on the study of type 1 sites. Topics covered comprise the characteristics of the natural type 1 and type 2 sites, the genetics of blue copper proteins, the modification of Cu sites, the spectroscopy of natural and engineered type 1 and type 2 sites, the effect of mutations on midpoint potentials and the mechanism of electron transfer as carried out by the blue copper proteins.

Blue copper; Site-directed mutagenesis; Electron transfer; Redox potential; Type 2; Azurin

1. INTRODUCTION

Copper sites in proteins come in a great variety. Interestingly their natural diversity appears refractive to a classification based on the types of ligand, the metal coordination, the activity of the metal site and the spectroscopic features of the metal. In this way the mononuclear type 1 and type 2 sites have been distinguished and the binuclear type 3 site [1]. New Cu-site features have recently emerged with the description of the trinuclear Cu site in ascorbate oxidase [2], while also the Cu_A sites in cytochrome oxidase [3] and nitrous oxide reductase [4,5] appear to belong to a new class of copper sites.

For the study of the intrinsic features of a metal site the chemist or biochemist would like to be able to make small well-defined alterations in the molecular structure close to the metal and to observe the effect of the changes on the metal site properties. Until recently he had to rely on nature in the sense that he was restricted to the study of the variations in primary and tertiary structure of a particular protein as found in different organisms. Unfortunately the variations in most cases surpass the level of a single point mutation and often occur not at the position in the structure that one is interested in.

The bio-inorganic chemist on the other hand likes to approach the problem from a different angle by modelling a protein's metal centre through the synthesis of relatively simple model compounds. It is hoped that the models mimick the biological template sufficiently close in order that their study unveil the essential features of the biological original. Depending on one's outlook this approach has met with abundant or limited succes. Biological metal centres have provided inspiration for new coordination chemistry that is fascinating both from a synthetic as well as a mechanistic point of view. In a number of cases insights derived from the realm of coordination chemistry have provided invaluable insights in the structure and function of metal sites in proteins. On the other hand, with very rare exceptions [6] no model compounds have been synthesised as of yet that truly mimick the structural and enzymatic properties of a particular metallo-enzyme or -protein.

It has gradually dawned that the function of the protein matrix of a metalloprotein is at least two-fold. It is able to hold the metal ligands in a well defined, more or less rigid conformation [6a], and secondly, by burying the metal site somewhat within its core it protects the metal ligands (like cysteines) from unwanted side reactions. With some exaggeration it might be said that the model builder who finally would succeed in mimicking a biological metal centre, stands a fair chance of finding that he has synthesised not only the metal centre but the protein matrix as well.

The situation has radically changed with the advent of genetic engineering techniques, which allow the introduction of precise well-defined point mutations in the primary structure of a protein. This technique has been turned to use for the study of metal sites in proteins during the last five years with remarkable success. It appears that the protein forms an almost ideal matrix to modify and engineer the coordination sphere of a metal and to study the dependence of the mechanistic properties on the structural and chemical details of a metal site.

For copper proteins most of the engineering efforts

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until now have been restricted to those of the type 1 category. They will be briefly reviewed here. Attention will be given to the characteristics of type 1 copper proteins, the spectroscopy of type 1 sites, their genetics, the results of site-directed mutagenesis experiments, the engineering of midpoint potentials and the electron transfer paths inside the protein.

2. TYPE 1 COPPER SITES

Type 1 copper sites occur in so-called blue copper proteins or in blue copper protein-like domains of multi-domain enzymes. Blue copper proteins are relatively small (10–15 kDa) stable polypeptides built out of β-strands that are arranged in two β-sheets. Together they form a β-sandwich. The α-helical content is low and is restricted to a single α-helix or no α-helix at all and to turn-like structures in the loops connecting the β-strands. Blue copper proteins contain a single Cu atom that is located in an excentric position in the hydrophobic core between the β-sheets, at a distance of 2.5–7 Å from the surface (for a recent review see [7]). The spectroscopic features of type 1 Cu sites have intrigued coordination chemists for a long time as they could not be understood on the basis of the then current knowledge of the coordination chemistry of copper. The EPR spectrum has an axial appearance with $g_e < g_\perp < g$; it sometimes exhibits a small rhombic distortion [8]. The relative order of the g-values indicates that the unpaired electron in the Cu(II) ground state resides in the d_{x2-y2} orbital. One of the puzzling observations was the very small value of the Cu hyperfine splitting, $A_{||}$, in the $g_{||}$ region of the EPR spectrum, which amounted to $\leq 70 \times 10^{-4}$ cm^{-1} and for which there was no precedent [8]. The other striking feature was the strong blue colour of the proteins in the oxidized state which corresponds to a strong absorption around 600 nm with an extinction (3000–6000 $M^{-1} \cdot cm^{-1}$). This is about an order of magnitude (or more larger) than that of an ordinary Cu coordination compound in this spectral region [8].

After a number of crystal structures of blue copper proteins became available, it transpired that the anomalous spectroscopic properties of the type 1 site are related to its unusual geometry and coordination [7]. The Cu appears to reside almost in the plane of three strong equatorial ligands: two histidines both coordinating with their N^δ nitrogens, and a cysteine coordinating with its thiolate sulfur. A fourth axial ligand is provided by a methionine that weakly binds with its thioether sulfur to the Cu. The ligand configuration is best described as distorted tetrahedral or trigonal pyramidal (see Fig. 1).

The ligand configuration described above has been proven by X-ray investigations for plastocyanin, pseudoazurin and amicyanin [9–12]. For azurin the geometry is slightly different in that a second axial group provided by the backbone carbonyl oxygen of Gly^{45} interacts with the Cu [13–15]. The distance between the Cu and the carbonyl oxygen is too large for the interaction to have substantial covalent character, but the electrostatic interaction between the Cu and the carbonyl dipole is non-negligible. The coordination geometry in this case is best described as trigonal bipyramidal (see Fig. 1) and the position of the metal with respect to the N_2S-plane appears the result of a subtle balance between the two axial interactions as illustrated in Fig. 1. For instance, in the wild-type (wt) azurin the Cu is located at the Met^{121} side of the the N_2S plane, while replacement of the Cu by the 'harder' Zn results in the metal being pulled over to the other side by the carbonyl oxygen. On the other hand, when the Met^{121} is replaced by Gln the Cu is considerably displaced out of the N_2S plane and attracted by the O^ϵ of the Gln side chain (see Fig. 1). One might say that depending on the nature of the ligand at position 121 and the metal, the latter is pulled over either to the carbonyl-45 oxygen or to residue 121. This is in agreement with the results of extensive molecular dynamics simulations of azurin set up to design a proper force field for the copper [16–18]. It turned out that in order to obtain a stable Cu site the charge on the Cu had to be redistributed over the ligands to a considerable extent and the magnitude of the dipole moment on carbonyl-45 had to be reduced by 50% to prevent the Cu from being pulled over to the oxygen-45 by the strong Coulomb interactions between O45 and the Cu. In addition an induced dipole moment on the thioether of Met^{121} had to be taken into account and a weak binding force constant between the Met^{121} sulfur and the Cu had to be introduced. Recent Xα scattered wave calculations support this picture [19].

Variations on the type 1 Cu site structure as described above are found for stellacyanin and rusticyanin. Stellacyanin contains no methionines. Modelling studies, based in part on amino acid sequence comparisons [20], and site-directed mutagenesis experiments [21] have made it highly likely, that the position normally occupied by a methionine, in stellacyanin is taken up by a glutamine which coordinates with its side chain O^ϵ to the Cu (see Fig. 1). For rusticyanin one of the coordinating histidines could not be located in the primary sequence [22,23]. It has been suggested that its position might be taken up by an aspartate [23]. This suggestion is hard to reconcile with the high midpoint potential of rusticyanin (680 mV) unless the aspartate is protonated. In that case the Cu coordination may effectively reduce to 3-fold. Since a 3-fold coordination favours strongly the Cu(I) over the Cu(II) state, such a suggestion would be compatible with the high midpoint potential of rusticyanin.

Crystallographic studies have shown that the Cu(I) and Cu(II) site show minimal structural differences. This is a characteristic that favours rapid electron transfer since the reorganization energy that accompanies the transfer reaction will be low [23a]. In plastocyanin,

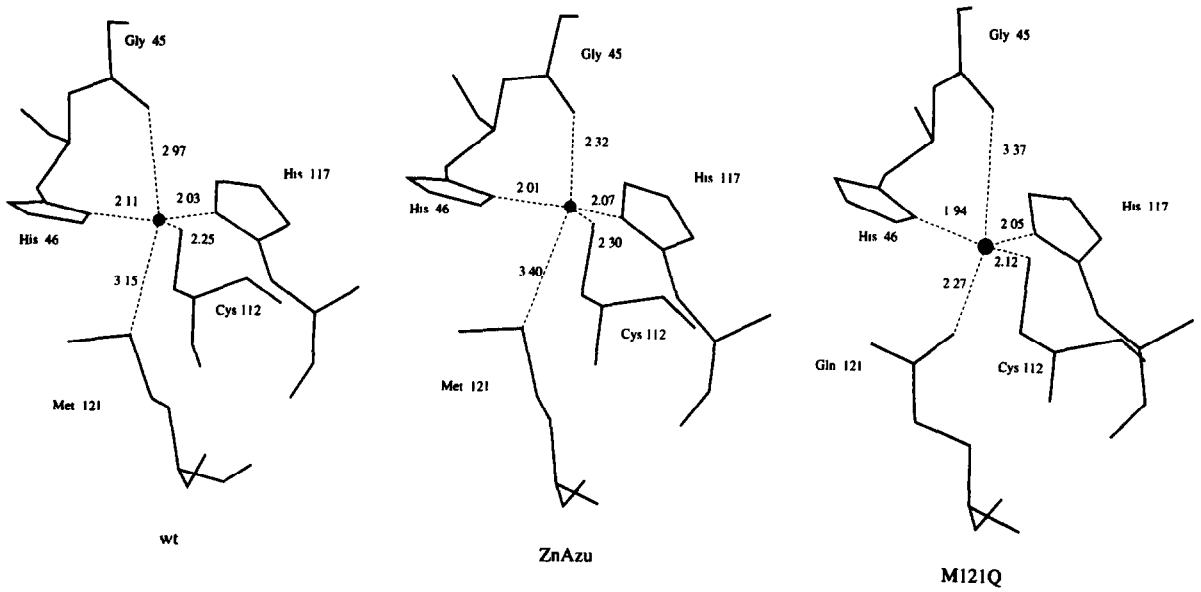

Fig. 1. Metal sites in azurin. (A) Cu site in wild type azurin from *P. aeruginosa* [15]. (B) Zn site in wt Zn-azurin from *P aeruginosa* [50]. (C) Cu site in M121Q mutant of azurin from *A. denitrificans* [21]. Metal ligand distances are indicated in Å.

amicyanin and pseudoazurin, however, this statement does not hold when the pH is lowered [10,11,24–26]. At low pH the histidine that protrudes through the protein surface becomes protonated at the N^{δ} position, as a result of which the Cu–histidine bond is broken and the coordination geometry of the Cu is reduced to three-fold. Subsequent to this the histidine adopts a new conformation by rotating about its C^{α}-C^{β}-C^{γ} bonds. This has been observed for the *reduced* but not for the *oxidized* protein. In the oxidized protein the histidine remains attached to the Cu and the coordination remains 4-fold. Since a 3-fold coordination favours the Cu(I) over the Cu(II) state, the midpoint potential increases as the pH is lowered. Moreover, the change in geometry considerably raises the activation barrier for electron transfer at low pH [26].

3. SPECTROSCOPY

Knowledge of the ligands and the coordination geometry of the Cu has formed the basis for a further understanding of the spectroscopic features of the type 1 site.

The small Cu hyperfine splitting in the $g_{||}$ region of the EPR spectrum was initially thought to be due to mixing of the $4p_z$ orbital into the $3d_{x2-y2}$ orbital (under effective D_{2d} symmetry this is group-theoretically allowed). The contributions from spin density in the $4p_z$ and $3d_{x2-y2}$ orbitals to the Cu hyperfine splitting are of opposite sign and cancel largely, which would explain the small $A_{||}$. Later it was found that the required amount of $4p_z$ mixing was too large to be realistic. It is now thought that the strongly covalent nature of the interaction of the Cu with its ligands and the concomitant dispersion of the electron spin over the ligands is responsible for the reduction of $A_{||}$ [8]. One of the attractive features of the earlier ligand field description of the type 1 site was that it provided a simple explanation for the experimentally observed linear correlation between $A_{||}$ and $g_{||}$ [27]. In the nowadays current numerical approaches this insight has been lost.

The trigonal pyramidal or bipyramidal configuration of the type 1 site fits in nicely with the axial character of the EPR spectrum. The usually small rhombicity must mean that the shape of the electronic distribution in the N_2S-plane is fairly insensitive to the detailed geometry and nature of the in-plane ligands. The effective symmetry is thus best approximated as C_{3v}. A sizeable rhombicity is observed for stellacyanin and some of the mutated type 1 Cu sites (vide infra). The current view is that in these cases the rhombicity is connected with a strong axial ligand field component [8,21,28–30]. The symmetry is effectively lowered from C_{3v} to C_{2v}, with the C_{2v} mirror planes running approximately through the two nitrogens and the Cu, and through the thiolate sulfur, the Cu and the axial ligand. Concominant with the rhombic distortion the Cu may be pulled out of the N_2S-plane in the direction of the axial ligand.

In the visible region of the spectrum type 1 sites show two bands, a strong one around 600 nm and a weaker one around 450 nm. It is well established now that the strong absorption around 600 nm, which is responsible for the strikingly deep blue color of the blue copper proteins, is mainly due to a sulfur to Cu ligand-to-metal charge transfer (LMCT) transition. As the cysteine thiolate sulfur has three lone pairs available, in principle three LMCT transitions may be expected in the UV-vis region of the optical spectrum. While the sp-hybrid ap-

pears too deeply buried to produce a transition in the visible region of the spectrum, the other two lone pairs do give rise to such transitions. According to recent theories the electron involved in the 600 nm transition originates from a $p\pi$-orbital on the sulfur. Strong overlap with this orbital causes the d_{x2-y2} orbital on the Cu to orient its lobes so that they are bisected by the Cu–S bond [8]. Apart from the main 600 nm band at least 5–7 weaker, partly overlapping bands have been identified. The more prominent of these at around 450 nm, has been assigned to a histidine to Cu LMCT transition. Recent resonance Raman studies on wt and mutant azurins and on superoxide dismutase show that more likely this band corresponds with the second sulfur to Cu LMCT transition [28–30]. The transition would involve the so-called pseudo-σ orbital on the sulfur [8].

It is not difficult to see that slight changes in the geometry of the Cu site may severely influence the relative intensities of the 450 nm and 600 nm bands. A rhombic distortion due to an enhanced axial interaction, for instance, may introduce d_{z2} character into the d_{x2-y2} state [8] and this may affect the overlap with the two sulfur orbitals alluded to above in a different manner. This may explain why increased rhombicity in the EPR spectrum often appears to be accompanied by an increase in intensity of the 450 nm over the 600 nm band. In the absorption spectrum of nitrate reductase from *A. cycloclastes* the 450 nm band is even stronger than the band at 600 nm [31]. The refinement of the type 1 Cu site in this protein is therefore eagerly awaited.

4. GENETICS

The first genes of bacterial blue copper proteins to be isolated were the azurin encoding gene of *P. aeruginosa* [32] and the gene coding for pseudoazurin from *A. faecalis* [33]. They appeared to code for a pre-protein in which the mature protein is preceded by a so-called signal peptide. This approximately 20 amino acids long peptide serves as a signal for the translocation of the protein over the cytoplasmic membrane into the periplasmic space. The signal peptide is cleaved off during or after translocation. In subsequent years the genes of azurin from *A. denitrifricans* and of various amicyanins have been cloned [34–38]. Also the genes of various plastocyanins have been isolated [39,40]. The signal peptides of the plant plastocyanins have a more complicated structure because plastocyanin is encoded in the nucleus of the cell and has to cross more than one membrane to reach its destination in the chloroplast. The information for crossing more than one membrane is hidden in the plastocyanin signal sequence. Apart from the cloning of naturally occurring genes there is also a report of a complete in vitro synthesis of the genes of an azurin and a plastocyanin [41].

In the production of a mutated protein three stages can be discerned: (a) construction of the mutated gene; (b) expression of the gene; (c) purification of the protein. For the first stage standard techniques ranging from casette mutagenesis to the PCR (polymerase chain reaction) and Kunkel's method, have been applied successfully [42–44]. Also expression of the protein in sufficient quantity usually is fairly straightforward unless the mutation results in a protein that is intrinsically unstable or does not fold properly. *E. coli* has been used as a host with general success for the heterologous expression of cloned 'blue copper' genes [45–47]. In the case of the plastocyanins the original signal sequence had to be replaced by the signal peptide of azurin to obtain reasonable expression. Also tobacco plants, after transgenic infection with *Agrobacterium tumefaciens*, have successfully been used for the heterologous expression of plastocyanin [48a]. Overall, *E. coli* appears to be the vehicle of choice for the expression of the blue copper protein encoding genes. The third stage, that of protein isolation, often represents a non-trivial effort. Properties that are of importance for the isolation procedure, such as electrophoretic characteristics, stability, sensitivity to ionic strength and pH, and pI point may have changed slightly but decisively by the mutation. As a result, a new mutant often requires adaptation of existing purification protocols or sometimes even the design of a completely new procedure. A case in point is the contamination that results from the partial incorporation of Zn instead of Cu into heterologously expressed blue copper proteins. This already occurs on a small scale (10%) in the natural situation when the wt protein is isolated from the parent organism [48b]. With heterologous expression the contamination may reach levels of 50% or more. The Zn protein is often difficult to remove and it requires special care to obtain preparations of Cu protein that are free from the contaminating Zn analog [49,50].

5. ENGINEERING COPPER SITES

The application of site-directed mutagenesis (sdm) techniques to the study of copper sites in proteins at the moment is considerably amplifying our understanding of the spectroscopic and mechanistic features of these sites in relation to their structure. Type 1 and type 2 Cu sites as they occur in nature belong to clearly distinct classes. The distinguishing features of the type 1 site have been dealt with above. Those of the type 2 site comprise [1,8] a square planar coordination by N or N/O donor ligands and an axial EPR spectrum ($2.0023 < g_{\perp} < g_{\parallel}$) with a Cu hyperfine splitting in the axial region of the spectrum ($A_{\parallel}$) of $\geq 130 \times 10^{-4}$ cm^{-1}; this is the regular value of $A_{\parallel}$ as observed for many coordination compounds of Cu. Lack of thiolate sulfur ligands is responsible for the lack of salient features in the optical spectra of type 2 sites. Type 1 sites are usually found in proteins that function as electron carriers in biological electron transfer chains. Type 2 sites occur

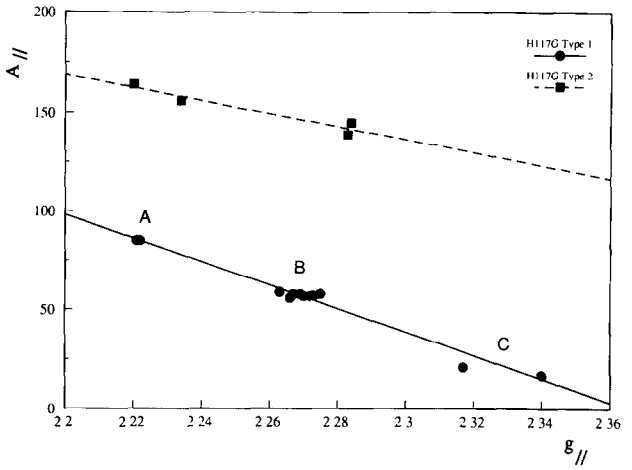

Fig. 2. Plot of $A_{\parallel}$ (in $cm^{-1} \times 10^4$) vs. $g_{\parallel}$ as observed for the $His^{117}Gly$ mutant of *P. aeruginosa* [29,30] with various external ligands. The points for the type 1 sites cluster in the regions A, B and C and correspond with the following external ligands (increasing $g_{\parallel}$): (A) imidazole, thiazole; (B) wild type, *N*-polyvinylimidazole, *N*-Me-imidazole, 2-Me-imidazole, 1-NH_2,[1-imidazole]butane, N^{α}-acetyl-histamine, *N*-butylimidazole, 1-Cl,[1-imidazole]ethane, Br^-, 4-methylimidazole; (C) N_3^-, SCN^-, Cl^-. For the type 2 sites these are: histamine, histidine H_2O and 4-imidazole-acetic acid (in order of increasing $g_{\parallel}$).

in enzymes at positions where they assist (often in conjunction with another redox co-factor) in catalysis of oxidations or oxygenations.

It has recently become clear that for engineerd Cu sites the natural borderline between type 1 and type 2 sites may became blurred. Type 1 sites may acquire some or most of the characteristics of a type 2 site and vice versa. Especially the presence of a cysteine ligand may make a type 2 site accessible for optical investigations and this can add a new dimension to the study of these sites. It also helps making resonance Raman effect (RRE) spectroscopy an important and useful tool in establishing the character of the optical transitions and the nature of the Cu coordination. A further classification of engineered Cu sites must be partly based on EPR characteristics. In general, for a particular type of site a roughly linear correlation is found between $A_{\parallel}$ and $g_{\parallel}$ [51,52]. Different types of site have been found to correspond to correlation lines in different regions of an $A_{\parallel}$ vs. $g_{\parallel}$ diagram. An example is provided by Fig. 2. The more extensive studies of engineered Cu sites have been confined so far to the azurins and superoxide dismutase (SOD) and they will be dealt with here in this order.

The four ligands of the type 1 site in the azurins (His^{46}, Cys^{112}, His^{117} and Met^{121}) have now all been mutated one by one. Of those only Cys^{112} appears necessary for maintaining the type 1 character of the site. When the latter residue is replaced the Cu binding is lost altogether ($Cys^{112}Ser$) or the site is converted into a type 2 site ($Cys^{112}Asp$) [53,54]. According to their EPR, UV-vis and RRE features the sites resulting from mutations in the other three ligands can be distinguished in three categories.

5.1. *(a) 'Blue type 1' sites*

The EPR, optical and RRE features of sites in this category very much resemble those of the wt. In the optical spectrum a strong band occurs around 590–680 nm and a weaker band or shoulder is seen around 450 nm. The EPR parameters fall close to the correlation line of the azurin type 1 sites (see Fig. 2) [29], while frequencies and intensities in the RRE spectrum (excitation in the 600 nm band) are like those in the RRE spectrum of the wt [30]. Presumably the mutation has not significantly changed the electronic properties of the site. Examples are provided by mutants in which Met^{121} has been replaced by a non-polar residue (Ala, Val, Ile, Leu) [55] and by the mutants $His^{117}Gly$ and $His^{46}Gly$, when the gap in the structure created by the mutation is filled by external ligands like substituted imidazoles or anions (see Fig. 2) [29,56,57].

5.2. *(b) 'Yellow type 2' sites*

Engineered sites in this category exhibit EPR features which resemble those of the natural type 2 sites ($2.0023 < g_{\perp} < g_{\parallel}$; $A_{\parallel} \geq 130 \times 10^{-4}$ cm^{-1}). However, the presence of a Cys-thiolate ligand produces optical features which are absent in the spectra of the natural type 2 sites. The 600 nm absorption of the original type 1 site has disappeared and it has been replaced by a strong absorption around 350–420 nm, which is responsible for the sometimes yellowish appearance of solutions of proteins in this category. The spectrum is reminiscent of that of square planar model compounds in which one of the Cu ligands is a thiolate or mercaptide sulfur [56,58]. The RRE spectrum clearly identifies the 350–400 nm band as deriving from a sulfur to copper LMCT transition, although a number of main bands in the RRE spectrum have dropped their frequency considerably (50–100 cm^{-1}) compared to the wt RRE frequencies [28–30]. The decrease in frequencies is diagnostic of a lengthening of the Cu–S bond, compatible with the presence of a fourth strong ligand.

The combined spectroscopic evidence points to a Cu site with a geometry that is distorted toward square planar and in which one of the ligands is a thiolate sulfur, the other three being provided by N/O donors. Possibly a fifth (axial) position is occupied by a strong field ligand like a water molecule or an OH^- group. Yellow type 2 sites have been observed in the case of the $His^{117}Gly$ and $His^{46}Gly$ mutants in which possibly one or two water molecules have entered the coordination sphere of the copper to compensate for the loss of the histidine. This type of site is also observed when the $His^{117}Gly$ protein is provided with externally added bidentate ligands like histamine or free histidine (see Fig. 2) [29,30].

5.3. *(c) 'Green type 1' sites*

The third category of mutated type 1 Cu sites has EPR features, that are intermediate between those of the first two categories. The $A_{\|}$, $g_{\|}$ values lie in a region of the $A_{\|}$ vs. $g_{\|}$ diagram that falls in between the correlation lines of the blue type 1 and the yellow type 2 sites. This category has therefore also loosely been referred to as 'type $1^1/_2$'. The EPR spectrum often exhibits a sizeable rhombicity. The optical spectrum is unusual in that it shows two intense bands, one around 580–600 nm and one around 430–460 nm. The two bands give solutions of these types of mutant a greenish colour. The RRE observed upon exitation in either absorption band classifies the bands as sulfur to Cu LMCT bands. The frequencies and intensities of the RRE peaks resemble those of the blue type 1 site [28–30]. Taken together, the spectroscopic features are compatible with a classical type 1 site that exhibits a severe rhombic (C_{2v}) distortion caused by a strong axial ligand. Just as in the wt case, the 600 and 450 nm absorption bands can tentatively be ascribed as corresponding to sulfur to Cu $p\pi \rightarrow d_{x2-y2}$ and pseudo- $\sigma \rightarrow d_{x2-y2}$ transitions, respectively. When comparing the spectra of a series of mutants, the sum of the intensities of the two bands appears roughly constant. This observation is compatible with the assig nments suggested here. A change in geometry of the Cu site will affect the overlap of both the $p\pi$ and the pseudo-σ orbital with the d_{x2-y2} orbital of the Cu. As the two sulfur orbitals are approximately orthogonal a decrease in overlap of either one of the orbitals with d_{x2-y2} will in general be accompanied by an increase in overlap of the other orbital with d_{x2-y2}. The total oscillator strength of the two bands which is roughly proportional to the sum of the squares of the two overlap integrals must therefore remain approximately unaffected by the change in geometry.

Examples of green type 1 sites are provided by mutants in which Met^{121} has been replaced by a ligand like His, Lys or Glu (pH 7) [55]. Also the $His^{117}Gly$ mutant at pH 6 belongs to this category. In this case the coordination position at the Cu that is left vacant by His^{117} is probably occupied by a water molecule [29,30].

A schematic and tentative view of the three types of site described above is given in Fig. 3. Two additional remarks apply. First, the limited experimental evidence available at the moment seems to indicate that the 'blue' and 'green' type 1 sites (categories (a) and (c)) represent the two extremes on a continuous scale connected with the strength of the axial ligand. Lacking a thorough quantum mechanical description of the sites, the ratio between the 600 and 450 nm absorbances may for the time being be adopted as a convenient ad hoc measure for the position on this scale [28]. An A_{600}/A_{450} ratio of 5–10 or more seems to correspond with the 'blue' end of this scale, a ratio of 1 or less with the other end. Secondly, sometimes for a particular protein a Cu site may occur in more than one form, depending, for instance, on pH. From the optical spectrum it is not always immediately clear whether one deals with a single type of site (for instance a green type 1 site) or a mixture of species (for instance, of a yellow type 2 and a blue type 1 site). EPR spectroscopy and RRE excitation profiles may help to distinguish between possible alternatives. As EPR and RRE experiments are often carried out at low temperatures, it should be kept in mind that the equilibrium between different species in some cases has been found to be affected drastically by the temperature.

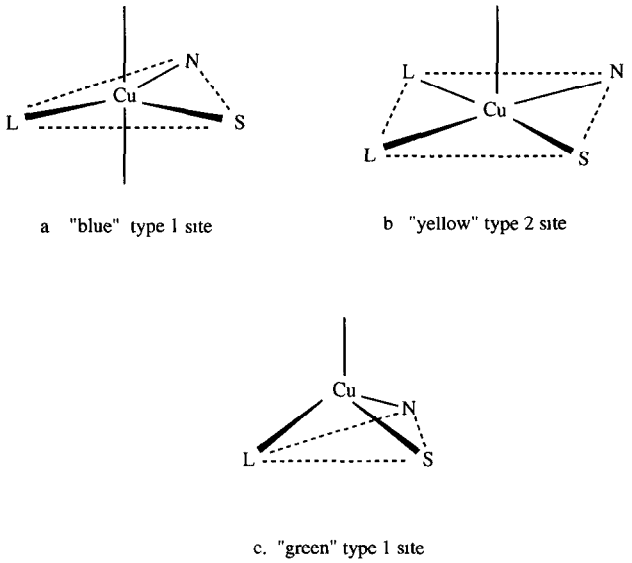

Fig. 3. Schematic representation of type 1 and type 2 Cu site geometries as discussed in the text.

The features observed for the mutated sites in superoxide dismutase (SOD) neatly fit within the classification scheme observed above. SOD has a type 2 Cu site with four histidine ligands one of which is bridging to the second site which is occupied by Zn. The zinc is coordinated in a tetrahedral fashion by three histidines and one aspartate [60]. Mutations have been made in the residues of both sites and their properties have been probed by filling either site with Cu. So far, efforts have concentrated on mutants in which a histidine was replaced by a cysteine, namely $His^{46}Cys$ (Cu site) and $His^{80}Cys$ (Zn site). The two sites, when occupied by Cu, exhibit the features of a yellow type 2 and a green type 1 site, respectively [61].

An interesting variation on the subject of constructing Cu sites is provided by insulin. This protein crystallizes as a hexamer. Two Zn ions are bound on opposite sides of the hexamer at positions that lie on the trigonal axis. Depending on the solvent conditions the residues lining the channels may adopt a tense (T) or relaxed (R) conformation. In the R-form each Zn is coordinated by three histidines from three different monomers and by an anion from the solvent. Brader et al. [62,63] have

succeeded in replacing the Zn by Cu ions in the R-form. It was found that various thiolates can enter the channel and bind to the copper, giving rise to various types of site, of which it is not directly clear how they would fit into the scheme presented above. (It should be kept in mind, however, that the scheme was developed for natural Cu sites that have been modified by sdm).

Special mention deserve the efforts of Hellinga et al. [64] who with the help of a specially written computer routine are able to design type 1 Cu sites in proteins. In their experimental work they have concentrated on thioredoxin from *E. coli*, an enzyme that has no natural transition metal binding sites. They first designed a number of possible Cu sites in thioredoxin, and then proceeded by constructing a number of the proposed structures by site-directed mutagenesis. Although some of the newly created sites accept metals, such as cobalt, in the manner of type 1 Cu sites, none of them have given rise, yet, to a truly type 1 Cu centre when copper is incorporated. Nevertheless, the efforts have highlighted the constraints on the 3D protein structure to which natural type 1 sites and their surroundings must conform.

6. MIDPOINT POTENTIALS

The question of which factors determine the midpoint potential (E_0) of the metal site in a redox protein has intrigued many researchers over the years. At least two factors hamper a direct application of coordination chemical concepts to the analysis of variations in midpoint potentials of metal centres in proteins. The low dielectric constant of a protein (often an effective value of 2–4 is applicable) makes the midpoint potential very sensitive to the charge distribution and the local polarisability inside the protein. Secondly, the protein matrix may confine type and geometry of the metal coordination in a manner not familiar from simple transition metal chemistry.

Initially attention focused on the coordination geometry of the metal, and the fairly straightforward concepts of ligand field theory were thought to provide adequate understanding of the naturally observed variation in midpoint potentials among redox proteins and enzymes. For instance, as it is known from the coordination chemistry of Cu that a tetrahedral geometry favours the Cu(I) over the Cu(II) state the distorted tetrahedral nature of the metal site in the blue copper proteins was thought to be responsible for the generally high midpoint potential of these proteins as compared to the aqueous Cu(I)/Cu(II) couple (150 mV). However, this does not account for the large variation (185 mV (for stellacyanin)–680 mV (for rusticyanin)) observed experimentally for the midpoint potentials of the type 1 sites. At one point the ligand at the axial position (usually a methionine) was thought to have a decisive influence on the tuning of E_0 [65]. Although modification of this residue certainly turned out to affect the redox characteristics of the Cu site [21,66], similar effects were observed when the coordinating histidines were replaced individually. In addition modification of residues outside the first coordination shell of the Cu sometimes had an effect of comparable size [67,68]. A revealing observation concerned the replacement of methionine-121 by a glutamine in azurin [21]. On good grounds it could be assumed that the Cu site in this mutant mimicks the Cu site in stellacyanin which has a very low E_0 of 185 mV. There was reason to expect that the E_0 of the mutant would drop significantly below the wt value, since the replacement of a methionine sulfur as a ligand for the oxygen of a glutamine side chain would certainly stabilize the Cu(II) state. Yet, the midpoint potential of the $Met^{121}Gln$ mutant (263 mV) appeared to have changed only by −23 mV with respect to the wt. The reason became apparent upon inspection of the structures of the oxidized and reduced site. In the oxidized form there is indeed a strong axial ligation of the Cu by the O of Gln^{121} stabilizing the Cu(II) state, but in the reduced form the coordination geometry changes and instead of four-fold the ligation is rather two-fold, which stabilizes the Cu(I) state. Compared to wt, therefore, both oxidation states are stabilized, leading to an only modest change in E_0.

In summary the following observations obtain. Replacement of Met^{121} in the azurins tunes the E_0 over a range of −105 mV to +138 mV with respect to the wt value at pH 7.0 [21,69]. Replacing His^{117} or His^{46} in the azurins by a glycine and supplying the Cu site with external ligands can increase the midpoint potential by about 100 mV [57]. Replacement of His46 by an Asp causes a drop of only 52 mV in E_0 (pH 5) [70]. Replacements in the second coordination sphere of the Cu can have fairly drastic affects on E_0, the causes of which are not clearly understood. For instance, the $Pro^{80}Ala$ mutation in pseudoazurin causes a change of + 139 mV of E_0 [68], while the $Asn^{47}Leu$ and $Asn^{47}Asp$ mutations in azurin cause changes by +110 and +23 mV, respectively [67,69]. Study of the temperature dependence of the $Asn^{47}Leu$ mutant vs. the wt, moreover has shown that the observed ΔE_0 between wt and mutant can strongly vary with temperature due to a sizeable entropy contribution to the difference in E_0 [67]. Finally, titrating groups can have a marked influence on E_0. That is because the titration changes the overall charge on the titrating residue and the electrostatic interaction of this charge with the Cu has a direct bearing on the E_0 of the metal. Especially when the titrating group is buried in the protein, the effect can be large since the effective dielectric constant of the protein is low, and the Coulomb interaction between the metal and the titrating group is not strongly attenuated in that case. For titrating surface residues the effect is much less pronounced, because the residue is partly surrounded by water which has a larger dielectric constant. Thus, titra-

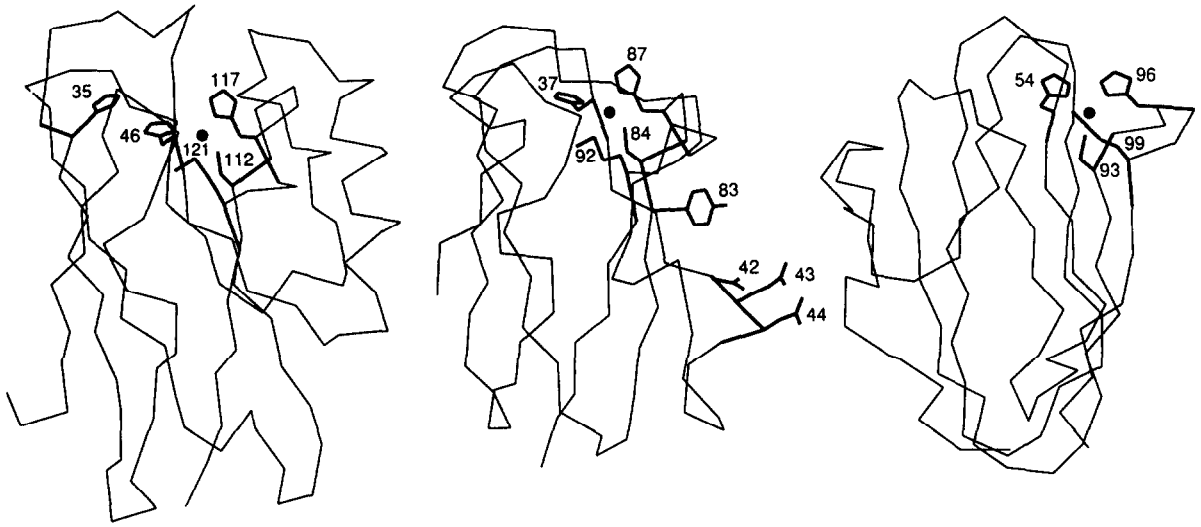

Fig. 4. C_α-backbones of azurin from *P. aeruginosa* [15], plastocyanin from *P. nigra* [9] and amicyanin from *T. versutus* [82]. The Cu ligands His^{46}, Cys^{112}, His^{117} and Met^{121} in azurin have been labeled together with residue His^{35}. In plastocyanin the Cu-ligands His^{37}, Cys^{84}, His^{87} and Met^{92} and the residues Asp^{42}, Glu^{43}, Asp^{44} and Tyr^{83} in the acidic patch have been marked. The preliminary amicyanin structure is based on NMR data and is the result of a distance geometry calculation with 642 distance constraints, 99 angle constraints and 16 stereo-specific assignments. Cu-ligands His^{54}, Cys^{93}, His^{96} and Met^{99} have been marked. The Cu atom in the structures is represented by a black circle.

tion of His^{35} in the azurins changes the midpoint potential of the Cu site by about 70 mV, while in the $Met^{44}Lys$ mutant the effect of the charged lysine on E_0 is reduced to 25 mV, the reduction being due to the location of Lys^{44} at the protein's surface [71].

Based on the foregoing considerations an adequate theory describing the effect of point mutations on the midpoint potential of metal centres should at least be able to account for: (a) the effect of mutations on the electronic wave function of the metal; (b) the effect that the residues in the first and second coordination shell of the metal have on E_0; (c) the effect of the mutation on the reduction entropy; and (d) the effect of titrating groups on E_0.

7. ELECTRON TRANSFER PATHS

Redox centres in proteins are usually buried some distance (5–10 Å) beneath the protein surface. From the point of view of the living cell this make sense as it allows the redox equivalents to be stored safely and prevents them from floating around at random, in which case the cell would loose control over its energy metabolism. A natural question is how electrons pass from the outside of the protein to the redox centre and vice versa. Two views are strongly debated in the current literature and a consensus appears not in sight, yet [72]. One view holds that the protein provides for special electron transfer pathways along particular residues and parts of the backbone, including covalent bonds, hydrogen bonds and through space jumps [73]. According to the second view the protein just acts as an organic matrix with a low dielectric constant which sustains through space jumps of electrons from one redox centre to another [74]. The experimental evidence, although in some cases abundantly available, is often ambiguous. What seems to be needed are experiments in which the effect of precisely engineered changes in purported pathways or in the protein as an organic medium, are established.

In the blue copper proteins the 'pathway' function of at least one of the copper ligands seems beyond doubt. Of the two histidines that coordinate to the Cu, the N-terminally located one is buried in the protein structure, but the C-terminally located one protrudes through the protein surface. In the azurins this residue is His^{117}, in plastocyanin it is His^{87} and in the amicyanins from *T. versutus*, *P. denitrificans* and *M. extorquens* AM1 it is His^{96}, His^{95}, and His^{89}, respectively. By studying the electron transfer reactions of site-directed mutants of azurin, it was convincingly established that His^{117} is the sole port of exit and entry of electrons to an from the Cu [46,75]. A second hypothetical pathway, involving His^{35} and His^{46} could be disproven. In plastocyanin His^{87} fulfills a similar role as H^{117} in azurin [76], although there is evidence for a second pathway involving the 'Eastern' (see Fig. 4) acidic patch. This patch functions as the docking site for cytochrome *f* and there are indications that electrons may reach the Cu via Tyr^{83} and Cys^{84}, the latter being a ligand of the Cu [77,78]. It has been estimated that this path is 10 times less efficient than the electron transfer path through His^{87} which is used by plastocyanin when it has to give off its electron to P_{700}^+, the oxidized reaction centre of photosystem I [79]. Finally, in amicyanin His^{96} appears a conduit for electrons in the reaction with methylamine dehydrogenase [12]. The existence of other possible pathways has not been looked into, yet,

in this case. It seems clear, therefore, that the histidine ligand of the Cu that protrudes through the protein surface has a crucial function for electron transport by the blue copper proteins. Interestingly, in cases where crystal structures have been solved at sufficient resolution one can see that close to this histidine a water molecule appears to be immobilized in the protein structure through various H-bonds, one of them connecting to the N^{ϵ} hydrogen of the histidine considered here [14,15]. NMR evidence shows that His $N^{\epsilon}H$ proton exhibits an abnormally slow exchange with the bulk water, indicating that also in solution a water molecule connects to this histidine [80]. Possibly this water molecule is part of the electron transfer pathway. Theoretical estimates show that in the absence of this water molecule electron transfer may be slowed down by as much as an order of magnitude [81].

8. CONCLUSION

Engineering of Cu sites in proteins in recent years has become a very active area of research. Insight into the structure and into the spectroscopic and mechanistic properties of these engineered sites is growing rapidly. Sites with new features not known to occur in natural copper proteins, have been created. The presence of a cysteine ligand appears responsible for a previously unwitnessed variety in the spectroscopic properties of those sites. At the moment three categories of engineered type 1 copper sites can be distinguished: (a) the blue type 1 site; (b) the yellow type 2 site; (c) the green type 1 site.

Acknowledgements: The authors gratefully acknowledge useful discussions with Profs. J. Sanders-Loehr, T. Loehr, B.G. Malmström and T. Vänngård, and with Dr. G. Karlsson. They thank Prof. E.I. Solomon and coworkers for sending them preprints of their work. They thank Dr. C.W.G. Hoitink for valuable comments, and Ms. G. van Pouderoyen and Ms. S.J. Kroes from the Metallo-Protein Group (Leiden University) for permission to use results from their work prior to publication.

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