

# Effects of protein folding on metalloprotein redox-active sites: electron-transfer properties of blue and purple copper proteins

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## Abstract

The tuning of the electron-transfer properties of the metal sites in blue and purple copper proteins by the protein fold is reviewed, with azurin and the *Thermus* Cu<sub>A</sub> domain as examples. These proteins have unique electronic and EPR spectra, which are ascribed to

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highly electron delocalized metal sites. The folding free energies of these proteins are higher in the oxidized compared to the reduced states, and as a consequence the reduction potentials of the unfolded proteins are higher than those of the native forms. The high potentials of the unfolded proteins are ascribed to maintenance of the core ligand structure of the metal site. In proteins with the folded forms having a higher potential than the unfolded forms, the potential is increased by hydrophobic encapsulation. In proteins with a lower potential of the folded forms, the high potential is down-tuned by different strengths of axial ligation, controlled by the protein fold. © 1999 Elsevier Science S.A. All rights reserved.

**Keywords:** Electron transfer; Azurin; Copper A; Protein folding; Reduction potentials

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## 1. Introduction

The folding of a protein into its conformation of minimum free energy modulates the reactivity and other properties of functional groups, such as polar amino-acid side chains or prosthetic metal ions. Means for this modulation are, for example, control of the polarity of the medium surrounding the reactive group and access to the solvent or external ligands, like substrates. The fold may also create local free-energy maxima by introducing strain at specific points in the structure, and this idea is part of ‘the rack’ [1,2] and the entatic state [3] hypotheses. The rack and entatic state descriptions have also been further developed specifically for redox-active metalloproteins [4–8].

The redox proteins in the electron-transfer chains of respiration and photosynthesis, the two main processes in the energy metabolism of living organisms, must span a range of reduction potentials from  $-0.45$  V (ferredoxin) to about  $+0.8$  V ( $\text{O}_2/\text{H}_2\text{O}$  at pH 7). This is in part accomplished by a variation in the redox-active prosthetic groups, which are flavins and iron–sulfur proteins in the low-potential parts of the chains but cytochromes and blue or purple copper proteins in the high-potential regions. The potential span can, however, be large even for a given type of redox-active group. For example, blue copper proteins can have potentials from about  $0.2$  V (stellacyanin) to about  $0.8$  V (fungal laccase) [9].

Here we will discuss how the protein fold controls the electron-transfer properties of blue and purple copper proteins, with azurin and  $\text{Cu}_\text{A}$  as examples. Azurin functions as an electron-transfer mediator in denitrifying bacteria, whereas  $\text{Cu}_\text{A}$  mediates electron transfer from cytochrome *c* to the proton-pumping machinery of cytochrome oxidase, the terminal enzyme of respiration. The electron-transfer properties of these proteins include, in addition to reduction potentials, facile electron-transfer pathways between electron donor and acceptor as well as a decrease in the electron-transfer reorganization energy by the protein rack minimizing structural changes on reduction of the metal sites. It is concluded that the protein fold leads to a hydrophobic encapsulation of the redox site and then tunes the site properties further by a strict control of the ligand interactions.

## 2. Spectroscopic properties and electronic and geometric structures

### 2.1. Blue copper proteins

The metal sites in blue copper proteins display unique spectral properties in their oxidized state compared to normal cupric complexes, as illustrated in Fig. 1. Their strong blue colors (600-nm band with extinction coefficients around 3500–5000  $\text{M}^{-1} \text{cm}^{-1}$ ) was noticed already in 1939 by Keilin and Mann in their work on laccase [10]. In 1960 it was discovered [11] that laccase and ceruloplasmin have unusual EPR spectra also, with very low  $g$  values and the parallel hyperfine ( $A_{\parallel}$ ) splitting reduced by more than a factor 2 compared with normal copper complexes, including nonblue copper proteins (Fig. 1). Since the two blue oxidases studied contain EPR-undetectable copper as well [12], it had been suggested [13] that this is in the Cu(I) state and responsible for the strong color, which would then not originate from the same copper ion as gives rise to the unique EPR signal. This possibility was, however, eliminated in 1963, when it was shown [14] that the blue bacterial protein, azurin, which has a single blue copper ion, gives an EPR signal with a narrow hyperfine splitting.

The unique spectral properties of blue copper proteins are, of course, a result of an unusual ligand coordination in the blue sites, as illustrated by the structure of the *Pseudomonas aeruginosa* azurin site in Fig. 2. There are three strong ligands in a trigonal plane, a thiolate S from Cys112 and two imidazole N from His46 and His112, as well as two weaker axial interactions, with a thioether S from Met121 and a carbonyl O from Gly45, resulting in a trigonal bipyramid structure. This structure is unusual in many ways. Thiol compounds usually do not form stable complexes with Cu(II), which instead becomes reduced to Cu(I), as, in fact, occurs with unfolded azurin [15]. Thus, the protein must impart some steric restraints, preventing such a redox reaction in the folded protein. In general, Cu(II) prefers a

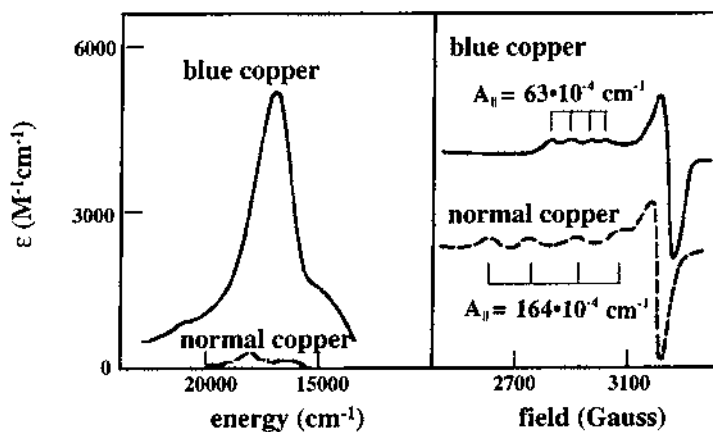


Fig. 1. Electronic and EPR spectra of blue copper proteins compared to normal Cu(II) complexes.

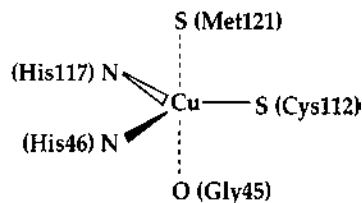


Fig. 2. The structure of the metal site in azurin.

square planar or tetragonal coordination, so it had been suggested [4,6] that the protein fold imposes strain (rack) at the metal site, leading to the unusual Cu(II) geometry.

The rack concept appears to be supported by crystal structure investigations, showing that there is very little structural change on reduction of plastocyanin [16] and azurin [17]. In addition, these findings have functional importance, since the lack of significant structural change on reduction means that the reorganization energy associated with electron transfer is minimal. Recently it has, however, been questioned that this is a result of the protein structure imposing the reduced geometry on the oxidized site, as suggested in the original rack description [4,5]. Electronic structure calculations [18–20] indicate that there is little or no strain imposed on the trigonal Cu(II)N<sub>2</sub>S unit in blue copper sites. Instead, it was proposed [18] that the protein fold imposes a long methionine S–Cu bond on the reduced site that leads to a short thiolate S–Cu bond, which results in a high reduction potential as well as in a minimal structural change on reduction.

The structure of the oxidized blue site can explain the unique spectral features. The strong electronic transition near 600 nm has long been recognized to be a S → Cu<sup>2+</sup> ligand-to-metal charge transfer (LMCT) band [21]. The thiolate S–Cu bond is highly covalent with only 42%  $d_{x^2-y^2}$  character [18]. This covalency reduces  $A_{\parallel}$  by delocalizing the electron spin off the metal center and reducing its interaction with the nuclear spin of the copper. The electron delocalization also results in the low  $g$  values characteristic of blue copper.

## 2.2. Purple copper proteins

The purple Cu<sub>A</sub> center found in cytochrome oxidase has EPR characteristics even more unusual than those in blue copper sites, since the relaxation time is so short that the EPR signal disappears at temperatures above 150 K, and the  $A_{\parallel}$  value is so small that the hyperfine lines cannot be resolved in X-band EPR spectra [22]. The interpretation of the spectral properties of Cu<sub>A</sub> centers has been greatly facilitated by the expression of small, soluble proteins containing such sites [23–25]. Multifrequency EPR spectra [26,27] of such soluble Cu<sub>A</sub> domains have demonstrated that the Cu<sub>A</sub> sites are dinuclear, mixed-valence [Cu<sup>1.5+</sup> ... Cu<sup>1.5+</sup>] centers, and this is also evidenced by paramagnetic NMR studies [28]. The dinuclear nature of the sites has been confirmed by X-ray structures of two oxidases [29,30] as well as of an engineered Cu<sub>A</sub> domain [31].

The structure of the *Paracoccus* Cu<sub>A</sub> site is shown in Fig. 3. The two Cu ions are bridged by two thiolate S from Cys216 and Cys220. Both are coordinated by one histidine, His121 and His224, respectively. They complete their coordination with one weak bond each, to the thioether S from Met227 and a carbonyl O from Glu218. Thus, the Cu<sub>A</sub> site can be regarded as a dimer of two blue sites in which one histidine on each Cu has been replaced by the second cysteine. EXAFS measurements [32] have demonstrated that the bond lengths changes on reduction (from the 1 + 2 + to the 1 + 1 + valence state) are extremely small, so that the reorganization energy associated with this electron-transfer step is minimized.

The electronic spectrum of the Cu<sub>A</sub> site in the whole oxidase is masked by the strong heme absorption bands, except for a band near 800 nm [33]. With the soluble domains it has, however, been demonstrated that there are two absorption bands of nearly equal intensity at 480 and 530 nm as well as two weaker bands at 360 and 790 nm. The electronic structure of the Cu<sub>A</sub> center has been derived from the spectroscopic properties [27,34–36]. The highest molecular orbital (HOMO) is a singly occupied antibonding orbital, in which each Cu ion is  $\pi^*$ -bonded to one S atom and  $\sigma^*$ -bonded to the other. There is direct Cu–Cu bonding [32], and an  $\sigma$ – $\sigma^*$  transition gives rise to the near-IR band [35]. The electronic structure calculations [36] have also revealed that on reduction the site would undergo significant distortion, which is prevented only by the site structure being maintained by the protein fold (or rack). In fact, according to these calculations, also blue copper sites would tend to lose a ligand and rearrange on reduction were it not for the rigidity of the protein fold [36].

The dinuclear nature of the Cu<sub>A</sub> center is important for its function, which is to transfer electrons from cytochrome *c* to cytochrome *a* in cytochrome oxidase. Because the protein subunit containing the Cu<sub>A</sub> site is anchored to the membrane, it cannot rotate. Thus, electrons must enter and leave the site by different routes. The structure of the Cu<sub>A</sub> site (Fig. 3) allows the electron from cytochrome *c* to be transferred His181 at the northern end of the molecule, close to the cytochrome *c* binding site. The Cu<sub>A</sub> site is completely delocalized [27,28,34], so the electron density is equally large on His224, which is hydrogen bonded to an inter-helix loop of the protein subunit binding cytochrome *a*. In this way an electron transfer path of 18 effective bonds with a tunneling length of 24 Å from Cu<sub>A</sub> to cytochrome *a* is provided [37,38]. For electron transfer to occur at the experimentally observed rate

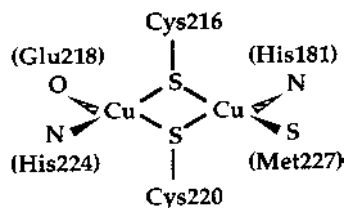


Fig. 3. The structure of the dinuclear metal site in the Cu<sub>A</sub> domain (*Paracoccus* numbering of the amino-acid residues).

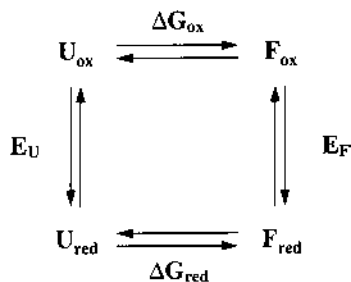


Fig. 4. Thermodynamic cycle for unfolding oxidized and reduced forms of a metalloprotein. F and U represent the folded and unfolded protein, respectively, the subscripts designating the redox states.  $\Delta G$  is the folding free energy and  $E$  is the reduction potential.

( $2 \times 10^4 \text{ s}^{-1}$ ) [39], the total reorganization energy of the site must be extremely small (0.3 eV). A reduction of the inner reorganization energy is, in fact, another consequence of the dinuclear nature of the  $\text{Cu}_A$  site [40].

### 3. Folding free energies

In redox-active metalloproteins, one of the electron-transfer properties tuned by the protein fold is the reduction potential of the redox site. If this is different for the folded and unfolded protein, the folding free energies for the oxidized and reduced proteins will differ by a corresponding amount, as can be seen from the reaction cycle in Fig. 4. This has, for example, been demonstrated with three heme proteins, cytochrome *c* [41], cytochrome  $b_{562}$  [42] and myoglobin [43]. In cytochrome *c* the heme reduction potential drops by about 0.35 V in going from the native to the unfolded state, and the folding free energy is correspondingly larger by about 34 kJ mol<sup>-1</sup> for the reduced compared to the oxidized protein [41]. Here we will discuss the corresponding situations for a blue (*Pseudomonas aeruginosa* azurin) and a purple (the soluble  $\text{Cu}_A$  domain from *Thermus thermophilus*) copper protein.

#### 3.1. Azurin

##### 3.1.1. Oxidized and reduced native azurin

The unfolding of oxidized and reduced azurin induced by guanidine hydrochloride (GuHCl) has been followed by CD in the far-UV region [15], which monitors the loss of secondary structure. The measurements were complicated by the thiol group of the ligand Cys112 slowly reducing the  $\text{Cu}^{2+}$  ion in the unfolded protein, as has already been mentioned (Section 2.1). Since this redox process does not take place in native, oxidized azurin, this is in itself evidence for the influence of the protein fold on the site properties. In addition, it is easily corrected for [15], so that the CD changes due to the unfolding reaction can be estimated. The unfolding of the reduced protein is completely reversible, and so are also the initial changes due

to unfolding with the oxidized protein. Thus, the equilibrium between the folded and unfolded protein can be regarded as a reversible two-state process.

The degree of unfolding of oxidized and reduced azurin as a function of the concentration of GuHCl is shown in Fig. 5a, which also gives the corresponding free-energy curves calculated for a two-state equilibrium (Fig. 5b). The fact that the free energy curves are linear functions of the GuHCl concentration supports the assumption of a two-state process. It also allows an estimation of the folding free

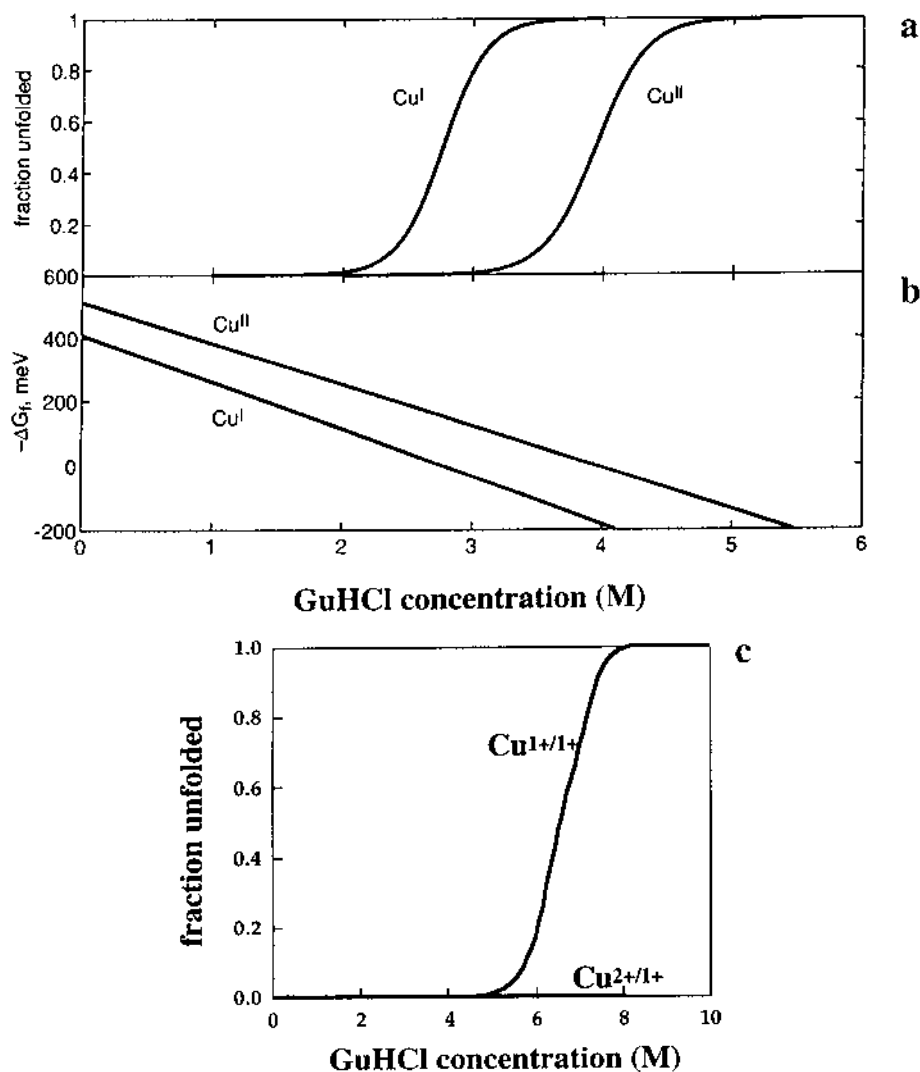


Fig. 5. Curves for GuHCl-induced unfolding of azurin and the  $\text{Cu}_A$  domain: (a) unfolding curves for oxidized and reduced azurin; (b) the corresponding free-energy plots; (c) unfolding curves for the  $\text{Cu}_A$  domain.

energies in H<sub>2</sub>O by extrapolation of the lines to zero concentration of GuHCl. This yields a folding free energy of  $-52.2 \text{ kJ mol}^{-1}$  for oxidized azurin and  $-40.0 \text{ kJ mol}^{-1}$  for the reduced protein. Thus, unlike the heme proteins mentioned earlier, oxidized azurin is more stable than the reduced form. As a consequence (cf. Fig. 4), the reduction potential of unfolded azurin should be 0.13 V higher than that of the native protein, and this has been confirmed by electrochemical measurements [44].

### 3.1.2. Apo- and zinc azurin

The paramagnetic nature of oxidized azurin makes it difficult to follow the unfolding by techniques other than CD, such as fluorescence and NMR, but these methods have been applied in investigations of the unfolding of zinc-substituted azurin [45]. It was then found that a fluorescence quenching occurred at lower GuHCl concentrations than the loss of the CD signal (i.e. loss of secondary structure). In addition, the unfolding monitored by NMR displayed two transitions in the signals from amino-acid residues close to the Trp residue mainly responsible for the fluorescence. The first transition occurs at the same GuHCl concentration as where the fluorescence quenching is detected (2.3 M). The second NMR transition, on the other hand, takes place in the same range of GuHCl concentration as the CD changes and thus reflects the main unfolding process. These data indicate that a folding intermediate involving small, local structural changes around the Trp residues is formed prior to complete unfolding. An intermediate state with native azurin has also been observed by electrochemical measurements at low GuHCl concentrations, where the secondary structure is still intact [46], as will be discussed later (Section 4). The free-energy plot constructed from the unfolding curve for zinc azurin (followed by CD) yields a folding free energy of  $-44.3 \text{ kJ mol}^{-1}$ , i.e. about  $8 \text{ kJ mol}^{-1}$  smaller than the folding free energy for native azurin [45].

The equilibrium unfolding of apoazurin displays two transitions, when monitored by CD [45]. This has been interpreted as being due to two folding domains. The one occurring at low GuHCl concentration (1.66 M) has been suggested to include the metal-binding site, because it is eliminated on the addition of zinc. This interpretation is also supported by the transition becoming irreversible at long times due to formation of dimers involving disulfide bonds; the transition is reversible in the presence of a reducing agent (dithiothreitol). The second CD transition, observed with apoazurin, represents loss of the remaining secondary structure, since it occurs at approximately the same GuHCl concentration as the unfolding of zinc azurin (3.09 and 2.75 M, respectively). Thus, the zinc ion stabilizes the fold of the protein, so that the partial unfolding seen with apoazurin is eliminated in zinc azurin, and the overall loss of secondary structure becomes a two-state process.

### 3.1.3. The effect of metal ions on the folding free energy

The folding free energy of native azurin ( $-52.2 \text{ kJ mol}^{-1}$ ) is unusually large for a protein of this size, since this energy generally falls in the range  $-20$  to  $-40 \text{ kJ mol}^{-1}$  for small proteins [47]. Even if this can in part be ascribed to the presence of a disulfide bond, it can undoubtedly also be attributed to the affinity of the folded protein for a metal ion ( $\text{Cu}^{2+}$ ,  $\text{Cu}^{+}$  or  $\text{Zn}^{2+}$ ). In accordance with this, the

metal binding domain in apoazurin is less stable than any of the metalloazurins [45]. The difference in folding free energy between oxidized and reduced azurin ( $12.2 \text{ kJ mol}^{-1}$ ) suggests that the affinity of the metal-binding site for  $\text{Cu}^{2+}$  is 138 times larger than that for  $\text{Cu}^+$ , provided that the energy difference entirely reflects the relative affinity for the two valence states. The affinity for  $\text{Cu}^{2+}$  can then also be estimated to be 24-fold larger compared with  $\text{Zn}^{2+}$  (folding free-energy difference of  $7.9 \text{ kJ mol}^{-1}$ ). This is in accord with the electronic structure calculations [36], suggesting that the structure of the metal-binding site is optimal for  $\text{Cu}^{2+}$  and not for  $\text{Cu}^+$ .

It has been shown that the metal remains bound to unfolded azurin [15,45]. Thus, there must be some residual structure maintained in the unfolded state, which has also been demonstrated by EXAFS measurements (unpublished results). These show that the Cu(I) ion in unfolded, reduced azurin has a trigonal coordination with one S and two N(O) ligands. The residual structure may be an important factor facilitating the folding process. Any such structure can have a kinetic effect by reducing the size of the conformational search for the state of minimum free energy. It also contributes thermodynamically by eliminating some of the entropy decrease associated with folding of the peptide chain. In addition, if the folded form has a higher affinity for the metal than the unfolded protein, a considerable contribution to the folding free energy results from forming the native metal site.

### 3.2. $\text{Cu}_A$ domain

In a study of a  $\text{Cu}_A$  domain, unfolding of the protein by GuHCl was monitored by CD as well as by electronic absorption (with the oxidized protein) and by fluorescence (with the reduced protein) [48]. Also, with this copper protein, the unfolding equilibrium can be regarded as a two-state process, as evidenced by the linear free-energy curves and the fact that the same unfolding curves were obtained with the different methods used. The *Thermus*  $\text{Cu}_A$  domain is so stable that its oxidized form cannot be unfolded at room temperature, whereas the reduced form unfolds around 6 M GuHCl, as shown in Fig. 5c. The folding free energy for the reduced protein is  $-65 \text{ kJ mol}^{-1}$ , which is higher than that for both oxidized and reduced azurin. As the free energy for the oxidized  $\text{Cu}_A$  protein is even larger, the unfolded  $\text{Cu}_A$  domain must, like azurin, have a reduction potential higher than that of the native protein. Electrochemically this was determined to be 0.45 V compared with 0.24 V for the folded protein [44]. The folding free energy for the oxidized form can then be calculated to be  $-85 \text{ kJ mol}^{-1}$  with the aid of the reaction cycle in Fig. 4.

The difference in folding free energy between the oxidized and reduced  $\text{Cu}_A$  domain is  $20 \text{ kJ mol}^{-1}$ , which is almost twice that for azurin ( $12 \text{ kJ mol}^{-1}$ ). This cannot only be related to the high absolute stability of the *Thermus* protein but must be a result of the unique structure of this copper site (see Section 2.2 and Fig. 3). The axially ligated  $\text{Cu}_2\text{S}_2$  diamond structure of this site is indeed expected to be less stable in the reduced compared to the oxidized form according to electronic structure calculations [36]. Thus, it is the native fold (or protein rack) that keeps the

Table 1

Reduction potentials (V) of the folded, intermediate and unfolded states of the Cu<sub>A</sub> domain and azurin

| Protein         | Folded | Intermediate | Unfolded |
|-----------------|--------|--------------|----------|
| Cu <sub>A</sub> | 0.24   | 0.41         | 0.45     |
| Azurin          | 0.32   | 0.42         | 0.46     |

site intact in both oxidation states, which accounts for the fact that there are minimal changes in bond lengths on reduction [32].

The high reduction potential of the unfolded Cu<sub>A</sub> domain must mean that also in this case the metal ions are still attached to the protein. Thus, there must again be some residual structure in the unfolded state, which facilitates the folding process.

#### 4. High-potential intermediate states

Electrochemical measurements on azurin and a Cu<sub>A</sub> domain have demonstrated that an intermediate state, with a different reduction potential compared with the native proteins, is formed at low denaturant concentrations, at which the proteins are still blue or purple [46], and all of the native secondary and tertiary structure is intact. These intermediate states have reduction potentials almost as high as those of the unfolded proteins (Table 1). Thus, there must be small changes only in the structure of the metal sites in these intermediate forms of the proteins, which, however, have a large effect on the potential. It has been suggested [46] that one or more hydrogen bonds, stabilizing the cavity surrounding the metal site, are broken by GuHCl and that this may cause relaxation of the hydrogen-bond network, promoting dissociation of an axial ligand.

With both azurin and the Cu<sub>A</sub> domain, the high-potential intermediates are formed only when starting with the oxidized proteins. The reduced intermediate must, however, exist transiently, since the cyclic voltammetry (CV) scans on the oxidized intermediate are reversible. This is probably related to a lower stability of the metal sites in the reduced proteins. The oxidized intermediates can retain their native structure even if GuH<sup>+</sup> may relax axial-ligand interactions, because of the inherent stabilities of the active site structures [35,36]. Upon reduction of the oxidized intermediate forms, on the other hand, the reduced sites will lack such stabilities [36], and axial ligands may dissociate on GuH<sup>+</sup>-induced perturbation of the site structure.

#### 5. Effects of protein folding on the redox sites of blue copper proteins

The protein fold controls the thermodynamics and kinetics of electron transfer in redox proteins, as will here be discussed with blue copper proteins as examples. The finding that oxidized azurin is more stable than the reduced protein [15] contradicts

the long entertained idea that the ligand arrangement of  $\text{Cu}^{2+}$  is strained by the protein fold (see Section 2.1). Instead, as has already been discussed (Section 4), calculations have shown that the trigonal core formed by the Cys S and His N atoms (Fig. 3) is a stable ligand arrangement in an oxidized blue site. There must, however, be additional structural effects of the protein fold to explain how the reduction potentials of blue copper proteins can be tuned to range from about 0.2 V (stellacyanin) to about 0.8 V (fungal laccase).

We have recently suggested [44,46] that a key feature in the tuning of the reduction potentials of blue copper proteins is the strengths of the axial bonds, as illustrated in Fig. 6. Unfolded azurin as well as an unfolded  $\text{Cu}_A$  domain have potentials around 0.5 V, which can be ascribed to the strong electron-donating tendency of Cys S and the high affinity for the strong ligands (S and N). The unfolded site is exposed to  $\text{H}_2\text{O}$ , which stabilizes  $\text{Cu}^{2+}$  and prevents the potential from going even higher. Thus, the first effect of the protein fold is hydrophobic encapsulation, which increases the potential by exclusion of  $\text{H}_2\text{O}$  and enclosing the metal ion in a medium of low dielectric constant. This is undoubtedly a general tuning mechanism in metalloproteins, and its magnitude can be estimated from data for heme proteins, whose unfolded forms have reduction potentials close to that of free heme in aqueous solution. In cytochrome *c* the potential is raised 0.35 V compared with free heme on folding [41], whereas the up-tuning is smaller in the folding of cytochrome  $b_{562}$  [42], whose site is more exposed, and smallest in the folding of myoglobin, whose site is available to the solvent [49].

If the up-tuning of the potential by hydrophobic encapsulation is the same in blue copper proteins as in cytochrome *c*, this would bring the potential to that of

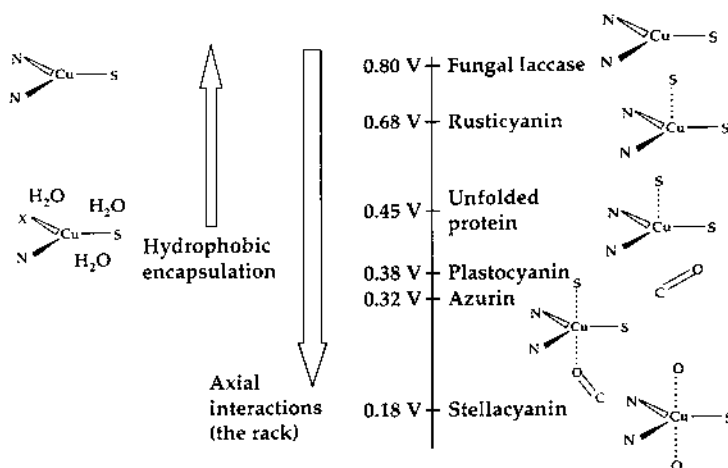


Fig. 6. The effect of the protein fold on the reduction potentials of blue copper proteins. In the unfolded proteins, Cu is still coordinated to Cys and at least one His, and it is surrounded by  $\text{H}_2\text{O}$  molecules, which results in an intermediate potential. Exclusion of  $\text{H}_2\text{O}$  by hydrophobic encapsulation raises the potential to 0.8 V. In proteins with lower reduction potentials, these are down-tuned by varying strength of axial ligation, as illustrated in the figure.

fungal laccase (Fig. 6). This is in agreement with the finding that in this protein the metal is in a hydrophobic cavity devoid of potential axial ligands [50]. In the proteins with lower potentials, the protein fold decreases the potential to different extent by varying the strength of axial ligation. This is strongest in stellacyanin, which has the lowest potential. In this protein the Met ligand found in most blue proteins is replaced by a glutamine, an O atom of which forms a strong bond with  $\text{Cu}^{2+}$ . Azurin has a higher potential because of the weak Met S bond, and the potential is raised further in plastocyanin, in which the bond to a carbonyl O has been weakened. Finally, rusticyanin has a potential only about 0.1 V below that of fungal laccase, which is ascribed to further removal of the carbonyl O atom and a very hydrophobic environment surrounding the metal site [51].

Finally, it should be mentioned that the different stabilities of oxidized and reduced redox metalloproteins make possible the triggering of protein folding by electron-transfer, as has been demonstrated with cytochrome *c* [41], cytochrome  $b_{562}$  [42] and myoglobin [43]. It is possible with a number of redox proteins to find conditions in which the protein is completely unfolded in one redox state and completely folded in the other, so that the removal or addition of an electron triggers a folding process. We have recently shown [48] that oxidation of reduced, unfolded  $\text{Cu}_A$  in 7.5 M GuHCl induces folding of this protein. Since the redox reaction may also be triggered photochemically by the use of laser flashes to initiate the electron-transfer event, this can bring studies of folding kinetics down to short time scales ( $\mu\text{s}$ ), as has been shown with heme proteins [41–43], whereas such investigations were earlier largely limited to the stopped-flow time scale (ms).

## 6. Concluding remarks

In this review we have tried to illustrate how the structure of the metal site in metalloproteins is determined by the protein fold, with a blue (azurin) and a purple (*Thermus*  $\text{Cu}_A$  domain) copper protein as examples. These proteins are redox metalloproteins, and the active-site structure created by the protein fold tunes their reduction potentials. The tuning mechanism involves hydrophobic encapsulation of the metal site followed by a strict control of ligand interactions, but undoubtedly other effects, such as electrostatic interactions, are also important. The proposed tuning mechanism is not limited to copper proteins but may also apply to other metalloproteins, including non-redox proteins.

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