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The influence of axial ligands on the reduction potential of blue copper proteins

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Abstract

The reduction potentials of blue copper sites vary between 180 and about 1000 mV. It has been suggested that the reason for this variation is that the proteins constrain the distance between the copper ion and its axial ligands to different values. We have tested this suggestion by performing density functional B3LYP calculations on realistic models of the blue copper proteins, including solvent effects by the polarisable continuum method. Constraining the Cu–S_{Met} bond length to values between 245 and 310 pm (the range encountered in crystal structures) change the reduction potential by less than 70 mV. Similarly, we have studied five typical blue copper proteins spanning the whole range of reduction potentials: stellacyanin, plastocyanin, azurin, rusticyanin, and ceruloplasmin. These studies included the methionine (or glutamine) ligand as well as the back-bone carbonyl oxygen group that is a ligand in azurin and is found at larger distances in the other proteins. The active-site models of these proteins show a variation in the reduction potential of about 140 mV, i.e. only a minor part of the range observed experimentally (800 mV). Consequently, we can conclude that the axial ligands have a small influence on the reduction potentials of the blue copper proteins. Instead, the large variation in the reduction potentials seems to arise mainly from variations in the solvent accessibility of the copper site and in the orientation of protein dipoles around the copper site.

Key words: Blue copper protein; Entatic state theory; Induced rack theory; Quantum chemical calculations; Reduction potential

Introduction

Blue copper proteins constitute a class of electron transfer proteins that differs from most small inorganic copper complexes in a number of properties. For example, they typically exhibit an intense blue colour, an electron spin resonance spectrum with narrow hyperfine splittings, and a high reduction potential [1, 2]. Furthermore, crystal structures of the blue copper proteins show an unusual trigonal cupric geometry [3, 4, 5]. The copper ion is coordinated by three strong ligands forming an approximate trigonal plane: one cysteine thiolate ion and two histidine nitrogen atoms. In addition, an axial ligand binds to the copper ion at a longer distance. In most proteins, the axial ligand is a methionine thioether group, but in stellacyanin and related proteins it is instead a glutamine amide group. In azurin both methionine and a back-bone amide oxygen atom are axial ligands.

Already in the 1960's it was suggested that the anomalous properties of the blue copper proteins are caused by an unusual cupric coordination geometry. More precisely, the entatic state and the induced-rack hypotheses, in their original formulations, propose that the protein forces the Cu(II) ion to bind in a geometry similar to the one preferred by Cu(I), i.e. tetrahedral [6, 7]. Such a strained geometry of the oxidised copper site would decrease the change in coordination geometry at reduction, thereby increasing the rate of electron transfer. Moreover, it implies a destabilisation of the oxidised state which would explain the high reduction potential of the blue copper proteins.

However, these suggestions have recently been challenged [8, 9, 10]. In particular, we have shown by quantum chemical geometry optimisations that an isolated Cu(II) ion with the same ligands as in the proteins assumes a geometry that is closely similar to the one observed experimentally [10]. Further investigations have shown that the trigonal structure as well as the spectroscopic features can be traced back to the copper–cysteine interaction, rather than to strain [11, 12, 13, 14]. In the typical (axial) blue copper proteins, copper and the cysteine thiolate group form a highly covalent π bond where a S_{Cys} $3p$ orbital overlaps with two lobes of the singly occupied Cu $3d$ orbital. Thus, S_{Cys}

formally occupies two positions in a square coordination. The other two lobes of the Cu $3d$ orbital form σ bonds with the two histidine ligands, whereas additional ligands have to overlap with doubly occupied Cu $3d$ orbitals and therefore become axial ligands at longer distances above and below the trigonal plane [14].

However, in another type of blue copper proteins, the rhombic type 1 proteins, the electronic structure is more similar to the one found in small inorganic copper complexes, i.e. the copper ion forms σ bonds to four ligands in a tetragonal arrangement, including methionine and the cysteine (strictly speaking, the bond to the latter group is a mixture of σ and π interactions) [14, 15, 16]. Nevertheless, these structures are also equilibrium structures, even if they are much more tetrahedral than most small inorganic copper complexes. This is because the cysteine ligand forms a strongly covalent bond to the copper ion, in which much charge is transferred from the S_{Cys} group to the Cu(II) ion [14, 16]. Consequently, both the trigonal and tetragonal copper sites are quite close to a tetrahedron and therefore also quite similar to the reduced complexes. This, in combination with the fact that the histidine and especially the methionine ligand form soft and flexible bonds to the copper ion, gives a low reorganisation energy and therefore a high rate of electron transfer [17]. Thus, the appropriate choice of ligands in the blue copper site gives a well-adapted electron transfer site without any need of protein strain.

The reduction potential is central for the function of electron-transfer proteins, since it determines the driving force of their reaction and therefore must be poised between the reduction potentials of the donor and acceptor species. For example, plastocyanin has a reduction potential around 370 mV [18], which falls adequately between the reduction potentials of cytochrome f (340 mV) and P700 in photosystem I (490 mV) [19]. In order to attain this, the protein often has to modulate the reduction potential of the redox-active group. This is very evident for the blue copper proteins, which show reduction potentials ranging from 184 mV for stellacyanin [18] (i.e. almost the same as for copper in aqueous solution, 150 mV [20]) to about 1000 mV for the type 1 copper site in domain 2 of ceruloplasmin [21, 22]. The latter two copper sites are untypical in that stellacyanin

has a glutamine amide oxygen group as the axial ligand (instead of methionine), whereas the ceruloplasmin centre does not have any axial ligand at all (a leucine group replaces the normal methionine ligand). However, blue copper proteins with the typical ligand sphere, $CuHis_2CysMet$, have reduction potentials that span a range from 260 mV (amicyanin, pseudoazurin, and nitrite reductase) to 680 mV (rusticyanin) [18, 19], although they share the same active-site ligands.

Recently, Solomon and coworkers have provided evidence indicating that it is only the Cu(I)–S_{Met} bond that is constrained by the blue copper proteins [8]. A normal Cu(I)–S_{Met} bond length is about 230 pm, whereas in the blue copper proteins, the observed bond length is around 290 pm. Such an elongation can be predicted to significantly reduce the charge donation of the ligand to the copper ion, which would increase the reduction potential. In fact, density functional Xα calculations indicate that the reduction potential would increase by more than 1000 mV by this elongation of the Cu–S_{Met} bond [8].

Malmström et al. have extended this hypothesis to include also other axial ligands [23, 24]. They point out that stellacyanin, with a very low reduction potential, has the strongest axial ligation among the studied blue copper proteins, a glutamine amide group at a distance of 220 pm [25]. Azurin has two axial ligands (methionine and a back-bone carbonyl group) at distances around 310 pm and a larger reduction potential, 285–310 mV [18, 19]. In plastocyanin, the Cu–O distance has increased to about 390 pm, and the reduction potential has increased to 380 mV. In rusticyanin, the Cu–O distance is even longer, 590 pm, and the carbonyl oxygen does not point against the copper site. This is correlated with a high reduction potential, 680 mV. Finally, in fungal laccase and ceruloplasmin, which have the highest known reduction potentials, 750–1000 mV [19], the methionine ligand is replaced by a leucine group, so that the copper site is three-coordinate. Thus, they propose that the protein fold dictates the reduction potential of the copper site by varying the strength of the axial ligation [23, 24].

We have in several papers investigated the geometry of the blue copper sites and the coordination of axial ligands [10, 14, 16, 26, 27]. These studies have invariably shown

that the geometry of the copper sites found in the blue copper proteins are close to those observed in optimised structures of realistic model systems in vacuum. Moreover, neither the protein nor the copper site seems to be sensitive to the length of the Cu–S_{Met} bond; it costs less than 5 kJ/mole (corresponding to 50 mV) to change it over the range observed in crystal structures of blue copper proteins [27]. This gives a quite different picture of the interaction between the copper ion and the axial ligand than the one described by Solomon or Malmström. Therefore, we here present a detailed investigation of the influence of the axial ligands (both the methionine and the back-bone carbonyl oxygen) on the redox potential of the blue copper proteins, obtained by accurate density functional methods, including solvation effects by the self-consistent reaction field approach [28].

Methods and details of calculations

Two series of calculations have been performed. First, we have investigated how much the reduction potential can be altered in blue copper proteins with the typical ligands $CuHis_2CysMet$ if the Cu–S_{Met} bond is enforced to differ from the equilibrium value. To this end, we have used the $Cu(Im)_2(SCH_3)(S(CH_3)_2)^{0/+}$ model, constraining the Cu–S_{Met} distance to seven values between 230 and 310 pm, while the rest of the geometry has been either fully optimised or has been kept fixed at the equilibrium geometry.

Second, to quantify the effect of also the back-bone carbonyl group and other axial ligands on the reduction potential, we have done calculations where the Cu–O and Cu–S_{Met} distances have been constrained to experimental values and the remaining degrees of freedom has been relaxed. For this purpose, we have studied the following series of blue copper proteins: stellacyanin, azurin, plastocyanin, ceruloplasmin, and rusticyanin. Stellacyanin was modelled by $Cu(Im)(Im(CH_2)_2NHCOCH_3)(SCH_3)(CH_3CONH_2))^{0/+}$, ceruloplasmin by $Cu(Im)(Im(CH_2)_2NHCOCH_3)(SCH_3)^{0/+}$, whereas the other proteins were modelled by $Cu(Im)(Im(CH_2)_2NHCOCH_3)(SCH_3)(S(CH_3)_2)^{0/+}$, c.f. Figure 1. Thus, the $S(CH_3)_2$ and CH_3CONH_2 were used as models for the methionine and glutamine residues, respectively, whereas the back-bone amide group was modelled by $CH_3CONH(CH_2)_2Im$, a

model that includes the link to the neighbouring histidine ligand in the same way as in the proteins. The values used for the constrained Cu–S_{Met} and Cu–O distances were taken from crystal data and they were allowed to differ for the reduced and oxidised forms, as is shown in Table 1. **Fig 1**

For each structure, the geometries of both the oxidised and reduced forms of the complexes were optimised with the hybrid density functional method B3LYP using the quantum chemical software Turbomole [29]. In all calculations, we used for copper the double-ζ basis set of Schäfer et. al. [30] (62111111/33111/311), enhanced with diffuse p, d, and f functions with exponents 0.174, 0.132, and 0.39. For the other atoms, the 6-31G* basis sets were employed [31]. Only the pure five d and seven f-type functions were used. Experience have shown that geometries obtained with the B3LYP approach do not change much when the basis sets are increased beyond this level [10].

When calculating redox potentials, solvation effects are almost as important as electronic effects. Therefore, the solvation energy of the copper complexes were estimated by the polarised continuum method (PCM) [32]. In this method, the molecule is placed in a cavity formed by overlapping atom-centered spheres surrounded by a dielectric medium. The induced polarisation of the surroundings is represented by point charges distributed on the surface of the cavity and the field of these charges in their turn affects the wavefunction. Thus, solvation effects are included in the wavefunction in a self-consistent manner. In addition to this electrostatic term, the PCM method includes three terms that affect only the solute energy and not the wavefunction. These represent the free energy of forming a cavity in the solvent (the cavitation energy), the dispersion solute–solvent energy, and the exchange solute–solvent energy, respectively [33, 34, 35]. We have decided to not include these terms in the reported energies. The cavity term is rather large and vary erratically with the Cu–S_{Met} bond distance depending on how far a solvent molecule may come into the clefts between the various ligands. This behaviour is not relevant for the proteins since the active site is buried from the solvent. Inclusion of this term does not change the conclusions of this paper (that the reduction potential does not vary much when the

interaction with the axial ligands is varied), but it would make the potential curves harder to interpret. The dispersion and exchange terms are insignificant, changing the reduction potentials by less than 7 mV for all considered complexes.

We have used the conductor PCM method, CPCM [28], as implemented in the Gaussian 98 software [36] combined with the B3LYP method. For the solvent, we have used parameters corresponding to water, e.g. a dielectric constant of 78.39. This most likely overestimates the solvent effects, which is quite appropriate since we want to obtain an upper limit for the influence of solvent effects on the reduction potential (the vacuum calculations, where the dielectric constant is unity, provides the lower limit).

For the atom-centred spheres in the PCM model, we have employed the default values for the radii, i.e. those obtained according to the united atom model for Hartree-Fock (UAHF) strategy, where the radii depends on the hybridisation and substituents on each of the atoms. The hydrogen atoms are included in the radii of the heavier atoms, to make the cavity surface smoother. This approach has been thoroughly tested and has been shown to give good results compared to experiments [37]. In order to get a better description of the cavity surface and charges induced by the solute, a smaller than default area of each surface element has been used (TSARE=0.4 $Å^2$).

Initial test calculations showed that the solvent changes the energy and charge distribution of the system, whereas the effect on the geometry is rather limited for all bond distances and angles (less than 5 pm or 5°), except for the Cu–S_{Met} bond. Since the latter bond is constrained in all calculations, we can use vacuum geometries without any reoptimisation in the solvent. Therefore, the reduction potentials were calculated as a simple difference of single-point energy calculations in solvent, performed on the vacuum equilibrium geometries of the reduced and oxidised complexes. The calculations were run on IBM SP2, CRAY C90, and SGI Origin 2000 or Octane workstations.

Results and discussion

The influence of the methionine ligand on the reduction potential

We have previously shown by quantum chemical calculations that it costs less than 10 kJ/mole to change the Cu–S_{Met} bond length 100 pm around the optimal value in both the oxidised and reduced states, i.e. a range appreciably wider than the natural variation in this bond among the various blue copper proteins [10, 27]. This indicates that even if the proteins could constrain this bond, it would affect the electronic part of the reduction potential by less than 100 mV. This is a tenth of the contribution obtained by Solomon and coworkers [8] and much smaller than the variation found among the blue copper proteins. However, these results were obtained on a small model, $Cu(Im)_2(SCH_3)(S(CH_3)_2)^{0/+}$, in vacuum. It is conceivable that solvation may change the energies. Therefore, we have recalculated these curves on a more realistic model, taking account of solvation effects self consistently in the quantum chemical calculation using the CPCM method.

Figure 2 shows the potential energy curves both in vacuum and in solution for $Cu(Im)_2(SCH_3)(S(CH_3)_2)^{0/+}$ where the Cu–S_{Met} bond length has been constrained to different values between 230 and 310 pm and the rest of the geometry has been optimised (at shorter distances, the energy increases sharply, e.g. to 15–30 kJ/mole at 210 pm for all the four cases in Figure 2). It can be seen that all potential curves are quite flat; it costs less than 9 and 7 kJ/mole to vary the Cu–S_{Met} distance by 80 pm for the reduced and oxidised complexes in solution, respectively (4 and 2 kJ/mole in vacuum). Some details of the potential curves need to be commented. **Fig 2**

The Cu(I)–S_{Met} potential in vacuum has a minimum at 236 pm. However, the curve in Figure 2 indicates that there is another minimum at a large distance, where the methionine is in the second coordination sphere of the copper ion. This reflects the weak interaction between methionine and copper, and the fact that the complex contains good hydrogen-bond donors, which compete with the copper ion for the methionine ligand. In the protein, the first-sphere coordination can be stabilised by hydrogen-bonds and non-

polar interactions with the surrounding groups, which are not present in the small model. Therefore, we have concentrated only on the first-sphere coordination of the methionine model.

Interestingly, the first-sphere minimum disappears in solvent. This may explain why the Cu(I)–S_{Met} distance seems to be longer in crystal structures than in structures optimised in vacuum [10]. In the protein, the effective dielectric constant around the copper site is probably lower that in these calculations (which use the dielectric constant of water), and there are other interactions that may stabilise the first-sphere coordination of the methionine ligand.

The oxidised model shows also two minima in vacuum. Here, the global minimum (corresponding to the structure found in the proteins) is at 266 pm, and a second minimum is found around 230 pm. This reflects a change in the electronic structure of the complex; when the Cu(II)–S_{Met} distance is shortened, the methionine ligand becomes an equatorial ligand and one of the histidines is forced to be axial [16]. Below 230 pm, the energy increases again. In solution, this local minimum at a short Cu(II)–S_{Met} distance disappears. Moreover, as for the reduced complex, the optimal Cu(II)–S_{Met} distance in solution is longer than in vacuum, about 290 pm.

According to Solomon's hypothesis, the long Cu–S_{Met} bond length is enforced by the protein in order to modulate the reduction potential. However, it is not clear to what distance it is constrained in the two oxidation states. Therefore, we have tested the hypothesis by studying three limiting cases. First, we constrained the Cu–S_{Met} bond only in the oxidised state. This gives the curve marked with squares in Figure 3. Here, the reduction potential varies by less than 70 mV, reflecting the flat nature of the oxidised potential energy surface. As expected, the reduction potential increases relative to the unconstrained state, since the oxidised structure is destabilised (constrained). **Fig 3**

Similarly, the curve with diamonds in Figure 3 shows the reduction potential when the Cu–S_{Met} bond length in the reduced complex has been constrained (but not in the oxidised

complex). Since this curve reflects the potential energy surface of the reduced complex the effect is slightly larger, 100 mV.

However, it is more natural to assume that the rigid protein would constrain both states of the copper, leading to the same bond length for both the reduced and oxidised structures. This situation is indicated by circles in Figure 3 and the curve shows that such constraints may affect the reduction potential by less than 50 mV. It is notable that at short distances (230–270 pm), the curve is flat, reflecting that the potential curves of the reduced and oxidised complexes in solution are nearly parallel (c.f. Figure 2). However, at longer distances, the reduction potential increases since the reduced complex prefers a lower coordination number more than the oxidised complex.

Strictly speaking, it is unlikely that the Cu–S_{Met} distances should be exactly the same in the oxidised and reduced structures, unless the constraining force from the protein is much larger than the counteracting force of the copper complex (which is not the case according to other calculations [27]). Instead, the observed Cu–S_{Met} distance is the position where these two opposing forces are of equal magnitude. Since neither the force constant of the Cu–S_{Met} bond nor the optimum bond distance in vacuum are the same in the oxidised and reduced copper complex, the constrained bond length should not be exactly the same, but it is hard to speculate about their exact values. It is notable, however, that the optimum vacuum Cu–S_{Met} bond length of Cu(I) models complexes is shorter than for the Cu(II) models [10], whereas the force constant is larger [27] (c.f. also Figure 2). Therefore, a constrained Cu(I)–S_{Met} bond should be *shorter* than a constrained Cu(II)–S_{Met} bond. Yet, the opposite is observed in most crystal structures [19], thereby providing a further strong argument against the suggestion that the Cu–S_{Met} bond should be constrained by the proteins.

In conclusion, the results in Figure 3 show that the blue copper proteins can modulate the reduction potential by at most 100 mV by constraining the Cu–S_{Met} bond length. Clearly, this is far too small to explain the high reduction potentials of these proteins compared to copper in aqueous solution (150 mV [20]), nor the large variation among

them (260–680 mV for proteins with this ligand sphere [18]). It is notable that for this comparison, the investigated range of the Cu–S_{Met} bond is too wide; native blue copper proteins have Cu–S_{Met} bond lengths ranging between 247 and 310 pm [19]. According to Figure 3, such a range corresponds to a variation in the reduction potential of less than 70 mV. Thus, a control of the Cu–S_{Met} bond length can at most be used to a limited fine-tuning of the reduction potential of the blue copper proteins.

All these calculations have been performed using water as the solvent around the copper site. This does not mean that we suggest that water is a good model of the protein or that the copper site should be solvent exposed. Instead, these calculations are intended to give an upper limit of the effects of solvent on the calculated reduction potentials. Water, with a dielectric constant of about 80, is the most polar solvent available in biological systems. The lower limit of the solvent effects is given by the corresponding results in vacuum, where the solvent effects are absent (the dielectric constant is unity). The Cu–S_{Met} potential surfaces in vacuum have already been described in Figure 2. Figure 4 shows the corresponding vacuum reduction potentials (for the cases where the reduced, oxidised, or both structures have been constrained). As expected, these curves show a smaller variation of the reduction potential than in the solvent, less than 45 mV. Thus, we can conclude that the possible variation of the reduction potential caused by constraints in the Cu–S_{Met} bond length is less than 70 mV, irrespectively of the actual solvent effects caused by the protein and the surrounding water solution. It should be noted that by this procedure of calculating the upper and lower bounds of the solvent effects, we obtain results that are applicable for any blue copper protein (i.e. the results are not restricted to a particular protein) and we can also use a more accurate method than is available if the full detail of the protein should be treated [38, 39]. **Fig 4**

Interestingly, Solomon and coworkers have, on the basis of similar calculations, suggested that the Cu–S_{Met} may change the reduction potential by more than 1000 mV [8]. There are many reasons why these results differ so strongly from ours. First, they calculate the electronic effects with another, appreciably less accurate, density functional technique, the

Xα scattered wave method. Second, they include the solvation by the simplest possible method, the Born model, where the entire complex is considered as a point charge in a sphere. Clearly, the PCM method, where solvation effects are calculated self-consistently, is more accurate. Third, and probably most importantly, they do not relax the geometry of any of the complexes; instead, the geometry is taken from the crystal structure of a small pseudo-tetrahedral model complex with little relevance for the blue copper proteins. If the geometries are not optimised, all calculated energies will be too high and the compensating contraction of the other Cu–ligand bond lengths (e.g. the Cu–S_{Cys} bond) is neglected. In our calculations all such effects are treated. Therefore, our results are more reliable than those of Solomon and coworkers.

It may be argued, however, that our calculations underestimate the effect of the axial ligand, since we relax the geometry of the rest of the complex. It is conceivable that the protein site is so rigid that it restricts the change in the geometry of the copper ion and its ligands. We have tested this possibility by performing a series of calculations in which the reduction potential is calculated for complexes with different Cu–S_{Met} bond lengths, keeping the rest of the geometry fixed at the equilibrium geometry. This tests the limiting case of a totally rigid protein. The resulting reduction potentials are shown in Figure 5, which again shows three curves representing the cases where the reduced, oxidised, or both states have been constrained, respectively. Fixing the geometry of the rest of the complex quite strongly affects the potential curves of the Cu–S_{Met} bond. However, the reduction potential (i.e. the difference of the potential curves) is not changed so much, and in particular, the range of variation of the potential is not significantly changed, except for the shortest bond length (230 pm), where the energy of the oxidised complex increases strongly. Therefore, for the natural range of Cu–S_{Met} bond lengths (245–310 pm), the effect on the reduction potential is not larger than for the calculations with optimised geometries, i.e. less than 70 mV. Thus, the difference between our and Solomon's results do not arise from the fact that we let the structure relax as the Cu–S_{Met} bond length changes, but rather from that they use a hypothetical structure quite far

from the equilibrium geometry. **Fig 5**

Finally, it should also be noted that Solomon and coworkers have only provided evidence that the Cu(I)–S_{Met} bond is elongated by the protein [8]; no evidence for a similar constraint for the oxidised bond seems to be available. However, if only the reduced structure is constrained and not the oxidised one, then the reduced structure is destabilised by the constraint, and therefore the reduction potential must *decrease*, contrary to the suggestion of a raised potential by Solomon et al. [12, 40] and the fact that the blue copper proteins are characterised by high reduction potentials. This can be seen in Figures 3–5 where the reduction potential is always negative if only the reduced complex is constrained.

Influence of the other axial ligands on the redox potential

As was discussed in the introduction, Malmström and coworkers have recently suggested that the blue copper proteins determine the reduction potential by modulating the distance between the copper ion and the axial ligands, both the methionine (or glutamine in stellacyanin) ligand and the back-bone carbonyl group, which is a ligand in azurin and is found at larger distances in the other proteins [23, 24]. More precisely, they suggest that the reduction potential of the copper site without any axial ligands is about 800 mV when it is enclosed in a medium with a low dielectric constant (the protein). Lower potentials are obtained by introducing axial ligands and varying their binding strength. This variation is said to be under firm control by the folding of protein, i.e. it is determined by protein strain.

In the previous section we have shown that the natural variation in the Cu–S_{Met} bond length cannot change the reduction potential by more than 70 mV. In this section we examine if the back-bone amide group has a larger effect on the reduction potential. We also examine the effect of removing the methionine ligand or replacing it by a glutamine group at a short distance. This has been done by a series of calculations on models mimicking the active site of five blue copper proteins with widely different reduction potentials: stellacyanin, azurin, plastocyanin, rusticyanin, and ceruloplasmin (the type

1 copper site in domain 2, missing the methionine ligand). These complexes include realistic models of both the methionine or glutamine ligand and the back-bone amide group. For each complex the Cu–O and Cu–S_{Met} (Cu–O_{Gln} in stellacyanin) distances have been constrained to the values found in crystal structures (different values for the two oxidation states if data is available), as is listed in Table 1, and the remaining degrees of freedom have been relaxed. The optimised structures of the oxidised form of the azurin, stellacyanin, and ceruloplasmin models are shown in Figure 1.

The calculated reduction potentials are also shown in Table 1. Somewhat unexpectedly, the calculated reduction potentials cover only a range of about 140 mV, whereas the experimental range is over 800 mV. For the three proteins modelled by the same complex (plastocyanin, azurin, and rusticyanin), the calculated reduction potential parallels the experimental ones. In fact, for plastocyanin and azurin, the change in the axial ligation seems to account for the most of the difference in reduction potential (48 out of 70 mV). However, this does not imply that these proteins have selected their reduction potentials by protein strain; it is more likely that it is a simple effect of the fact that plastocyanin presents only one axial ligand to the copper ion, whereas azurin presents two. For the difference between plastocyanin (or azurin) and rusticyanin, axial ligation seems to be less important; it accounts for only a third of the difference (95 out of 305 mV). Clearly, other factors are more important for this change in reduction potential, most prominently changes in the solvation (water accessibility) and the orientation of dipoles around the metal site [38, 39, 41]. **Tab 1**

For the other two proteins (stellacyanin and ceruloplasmin), the difference between the calculated and experimental reduction potentials is larger, even if the general trends are correct; the stellacyanin model has a reduction potential between those of the plastocyanin and azurin models (13 mV higher than azurin and 35 mV lower than plastocyanin), whereas the ceruloplasmin model has a reduction potential slightly lower than the one of rusticyanin (30 mV lower than rusticyanin and 95 mV higher than plastocyanin). However, these results are in good agreement with reduction potentials obtained for mutants

of the axial methionine ligand in azurin. They have shown that if the methionine ligand is replaced by a hydrophobic, non-bonding, amino acid (e.g. Ile, Leu, or Val) the reduction potential increases by 112–138 mV [42]. This is in excellent agreement with our result that the ceruloplasmin model has a 113 mV higher reduction potential than the azurin model.

Similarly, a mutation of the methionine ligand to a glutamine, which yields a protein with spectroscopic and geometric features (e.g. a short Cu–O_{Gln} bond of about 225 pm) very similar to those of stellacyanin, has a reduction potential that is only 23 mV lower than the wild-type protein [43]. This is only a forth of the observed difference between azurin and stellacyanin (100–120 mV). In this case, our calculations do not fully reproduce the experiment, since they predict an increase of the reduction potential of stellacyanin compared to azurin by 13 mV. However, in energy terms, the difference is not very large, only 4 kJ/mole, which is well within the error limits of our method, especially considering that different models have been used for the two complexes, which reduces the cancelation of errors.

In fact, we have calculated the reduction potentials for all the models in Table 1 also by another solvation method, the self-consistent isodensity polarised continuum model (SCI-PCM) [44]. This method calculates the size of the cavity directly from the wavefunction so that no radii has to be defined, but it is more expensive and it has been shown to give slightly worse solvation energies than the CPCM model [37]. Interestingly, this method gives almost identical results (the relative reduction potentials are the same within 3 mV) for all complexes in Table 1, except for the stellacyanin model, where it predicts an *decrease* by 47 mV compared to azurin (corresponding to 280 mV in Table 1). This is 24 mV more than what is observed experimentally, and it shows that this figure is quite uncertain. Still, it is clear that the change in reduction potential is quite limited.

Thus, already from mutation studies, it can be concluded that the influence of the axial ligands on the reduction potentials of the blue copper proteins is rather limited, less than 160 mV. Only if the methionine ligand is replaced by a strong ligand, e.g. a glutamate

group, can the reduction potential be significantly decreased, by up to 109 mV [45]. However, such a mutation leads to an appreciably more flattened site [45] and introduces a strong negatively charged oxygen ligand, effects that are well-known to favour the oxidised state [20]. Moreover, no such sites are known in nature, so they cannot explain the natural variation of the reduction potentials of the blue copper proteins. Our results are in good agreement with these studies, showing a variation in the reduction potential of about 140 mV when the axial ligation is varied.

Concluding remarks

The goal of this investigation was to test the suggestions of Solomon and Malmström, that the axial ligands of the copper sites in the blue copper proteins are crucial for their reduction potentials. Our results clearly show that this is not the case. Variations in the Cu–S_{Met} bond length over the range encountered in crystal structures affect the reduction potential by less than 70 mV. If also the back-bone amide ligand is considered and the methionine ligand is allowed to be removed or replaced, variations of up to 140 mV in the reduction potential are observed. This is only a minor part of the observed variation in the reduction potential of the blue copper proteins, about 800 mV. The rest of the variation is probably caused by solvation effects induced by the protein dipoles and the surrounding solvent, as detailed calculations on plastocyanin, rusticyanin, and pseudoazurin indicate [38, 39]. Of course, another important effect of the axial ligands is to prohibit other potential ligands, e.g. water, to bind to the copper site. Such a binding would strongly affect the reduction potential.

It should also be noted that we have not found any evidence for a significant control of the binding strength of the axial ligands by protein strain. Quantum chemical optimisations of complexes with the same ligands as in the active sites of blue copper proteins have yielded geometries that are virtually identical to the crystal structures for plastocyanin, nitrite reductase, and stellacyanin [10, 16, 26]. Admittedly, there are small differences between the optimised and experimental lengths of the Cu(II)–S_{Met} (e.g. about 10 pm for

plastocyanin), but classical simulations have shown that these disappears if the dynamics of the system at ambient temperatures are taken into account [27]. Thus, there does not seem to be any strain at all in the oxidised structures.

However, for the reduced models, the discrepancy is larger. For example, the Cu(I)–S_{Met} distance in reduced plastocyanin is about 290 pm, whereas our optimised models give a distance around 240 pm [10]. Dynamic effects elongate also this distance, but only by 8–9 pm [27]. However, a structure with the Cu–S_{Met} distance constrained to the crystal value is only 4 kJ/mole less stable than the optimum structure. This small energy is within the error limits of our theoretical method, and it shows that the bond is so floppy that it will vary with any changes in the dielectric properties in its surroundings. For example, the results in Figure 2 show that solvation effects tend to elongate this bond, and this may be the main reason for the long bond in the crystals.

In conclusion, we see no effect of the protein on the oxidised Cu(II)–S_{Met} bond, whereas the Cu(I)–S_{Met} bond may be somewhat elongated, probably by solvation effects. This is in qualitative agreement with Solomon's original suggestion that only the reduced structure is changed by the protein [8]. However, quantitatively, our results differ strongly from their interpretation that the elongation of the Cu(I)–S_{Met} bond should be crucial for the structure and reduction potentials of the blue copper proteins. We show that in energy terms, the change is very small (less than 4 kJ/mole), and that it can have only a very small influence on the reduction potentials. Moreover, our results give no support to the later suggestions by Solomon and Malmström that the Cu–S_{Met} bond should be changed by the protein also in the oxidised state [23, 24, 40]. For these sites, the long Cu–S_{Met} bond as well as the short Cu–S_{Cys} bond naturally follow from the electronic structure of the system (a π bond between Cu and S_{Cys}).

For the optimal distance between the copper ion and the axial back-bone amide ligand in azurin, we do not have any conclusive results yet, but our preliminary investigations indicate that the case is similar to reduced Cu–S_{Met} bond, i.e. that the Cu–O bond is very floppy and that the crystal structure is within a few kJ/mole from the optimum vacuum

structure [46]. However, the results in Table 1 show that the concerted influence of both axial ligands is less than 140 mV on the reduction potential. This is similar to what is found by mutation studies. Moreover, it should be noted that the largest effects are obtained when the methionine ligand is removed or replaced by glutamine. These effects are caused by the protein presenting a different set of ligands to the copper ion and they are clearly not due to protein strain. This is illustrated by geometry optimisations of stellacyanin models in vacuum, which reproduces the crystal geometry well, including the short Cu–O_{Gln} distances [26], and also by the fact that our calculations of the reduction potential of a model without any methionine ligand reproduce the change in the reduction potential of methionine-to-leucine mutants well [42]. Thus, our studies have once again shown that the structure and properties of the blue copper proteins are determined mainly by the copper ion and its ligands, and not by protein strain.

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Figure legends

Figure 1. The optimised structures of the oxidised form of the azurin (a), stellacyanin (b), and ceruloplasmin (c) models.

Figure 2. Potential energy curves in vacuum and solution for the $Cu(Im)_2(SCH_3)(S(CH_3)_2)^{0/+}$ complex with the Cu–S_{Met} bond constrained to different distances.

Figure 3. The calculated reduction potential for the $Cu(Im)_2(SCH_3)(S(CH_3)_2)^{0/+}$ model in water solution with different constraints in the Cu–S_{Met} bond length, as discussed in the text, but the rest of the geometry relaxed. The energy scale has been selected so that the unconstrained case has a reduction potential of 0 mV for all curves.

Figure 4. The calculated reduction potential for the $Cu(Im)_2(SCH_3)(S(CH_3)_2)^{0/+}$ model in vacuum with different constraints in the Cu–S_{Met} bond length, as discussed in the text, but the rest of the geometry relaxed. The energy scale has been selected so that the unconstrained case has a reduction potential of 0 mV for all curves.

Figure 5. The calculated reduction potential for the $Cu(Im)_2(SCH_3)(S(CH_3)_2)^{0/+}$ model in water solution with different constraints in the Cu–S_{Met} bond length, as discussed in the text, and the rest of the structure fixed at the equilibrium geometry. The energy scale has been selected so that the unconstrained case has a reduction potential of 0 mV for all curves.

Table 1: A comparison of the calculated and experimental reduction potentials for five protein models. The lengths of the constrained distances are also given.

	E_0 (mV)		$Cu–S_{Met}$ distance		Cu–O distance (pm)		
Protein	calc.[a]	exp.[b]	Reduced	Oxidised	Reduced	Oxidised	References
Stellacyanin[c]	340	184	269	225	340	347	[43]
Azurin	327	305	321	312	325	316	[47, 48]
Plastocyanin	375	375	287	282	400	389	[4, 5]
Rusticyanin	470	680	290	288	596	585	[49, 41]
Ceruloplasmin	440	$\geq$1000			372	372	[21]

[a] The reduction potentials have been normalised so that the one of plastocyanin reproduces the experimental value.

[b] [18, 22]

[c] For stellacyanin, the close ligand is O_{Gln}, not S_{Met}.