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On the role of strain in blue copper proteins

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Abstract

Theoretical investigations of the structure and function of the blue copper proteins are described. We have studied the optimum vacuum geometry of oxidised and reduced copper sites, the relative stability of trigonal and tetragonal Cu(II) structures, the relation between the structure and electronic spectra, the reorganisation energy, and reduction potentials. Our calculations give no support to the suggestion that strain plays a significant role in the function of these proteins; on the contrary, our results show that the structures encountered in the proteins are close to their optimal vacuum geometries (within 7 kJ/mole). We stress the importance of defining what is meant by strain and to quantify strain energies or forces in order to make strain hypotheses testable.

Key words: blue copper proteins, quantum chemical calculations, entatic state theory, induced-rack theory, protein strain

Introduction

The blue copper proteins are a group of electron transfer proteins characterised by a number of unusual properties, e.g. a bright blue colour, a narrow hyperfine splitting in the electronic spin resonance (ESR) spectra, and high reduction potentials [1-3]. Moreover, crystal structures of the oxidised form of these proteins show a structure distinct from what is normally observed for small inorganic complexes: The copper ion is bound to the protein in an approximate trigonal plane formed by a cysteine (Cys) thiolate group and two histidine (His) nitrogen atoms. The coordination sphere in most blue copper sites is completed by one or two axial ligands, typically a methionine (Met) thioether group, but sometimes also a back-bone carbonyl oxygen atom (in the azurins) or instead an amide oxygen atom from the side chain of glutamine (in the stellacyanins) [1–4]. Such a geometry is similar to what can be expected for Cu(I) complexes, and reduced blue copper proteins have copper coordination geometries that are very close to those of the oxidised proteins [2,3,5]. Naturally, this is a functional advantage for an electron transfer protein; if the two oxidation states of the copper centre have similar structures, the reorganisation energy will be low, and the rate of electron transfer will be high [6].

These unusual properties of the oxidised form of the blue copper proteins have traditionally been explained by protein strain: It has been suggested that the rigid protein forces the Cu(II) ion to bind in a geometry more similar to the one preferred by Cu(I). This is the essence of the entatic state and the induced-rack hypotheses for the blue copper proteins (in their original formulation), which actually were suggested before any structural information was available for the proteins [7–10]. The suggestions have later been extended into general hypotheses for metalloproteins [11,12].

However, this suggestion has recently been challenged [13–14]. In particular, we have shown by quantum chemical calculations that the cupric geometry in the blue copper proteins is very close to the optimal vacuum structure of a Cu(II) ion with the same ligands [14]. Why are then the properties of the blue copper proteins so unusual, if not by protein strain? During the last five years, we have investigated this question using theoretical methods. In this paper, we describe our results and relate them to the strain hypotheses in their original [7-12] and modified formulations [15–16]. The paper is a commentary on the two accompanying articles by Gray, Malmström, and Williams [17] and Larsson [18], discussing the influence of the protein on the properties of the blue copper proteins.

The optimal geometry of the blue copper coordination sphere

We have optimised the geometry of Cu(imidazole)$_2$(SCH$_3$)(S(CH$_3$)$_2$)$^+$ as a realistic model of the oxidised prototypical Cu(His)$_2$CysMet blue copper centre (e.g. in plastocyanin) using the density functional B3LYP method [14]. The results in Fig. 1 and Table 1 show that the optimised geometry is virtually identical to the one observed experimentally in the blue copper proteins. Almost all bond lengths and bond angles around the copper ion are within the range observed in crystal structures, and most of them are close to the average values for the proteins. Only two small, but significant, differences can be observed, a slightly too long Cu–S$_{Cys}$ bond and a slightly too short Cu–S$_{Met}$ bond. These differences can be fully explained by the dynamics of the system, which gives an average Cu–S$_{Met}$ bond length at least 10 pm longer than the quantum chemical optimum (due to the flat potential of this bond) [19], and to deficiencies in the theoretical method (the more accurate CASPT2 method [20], i.e. second-order multiconfigurational perturbation theory, gives a 7 pm shorter Cu–S$_{Cys}$ bond and a 7 pm longer C–S$_{Met}$ bond [14]). Equally convincing results have been obtained for the optimal structure of Cu(imidazole)$_2$(SCH$_3$)(OCCH$_3$NH$_2$)$^+$, a model of the ligand sphere of oxidised stellacyanin [21], as can be seen in Table 1. It should be noted that no information from the crystal structure has been used to obtain these structures; they are entirely an effect of the chemical preferences of the copper ion and its four ligands. Thus, the cupric structure in the oxidised blue copper proteins is neither unnatural nor strained.

For the corresponding model of the reduced blue copper site, Cu(imidazole)$_2$(SCH$_3$)(S(CH$_3$)$_2$), the optimal vacuum structure is more tetrahedral than in the proteins and has a short Cu–S$_{Met}$ bond (237 pm, see Table 1) [14]. However, the potential surface of the Cu–S$_{Met}$ bond is extremely flat. If the length of this bond is fixed at the crystal value (290 pm) and the complex is reoptimised, a structure is obtained that is virtually identical to the crystal structure of reduced plastocyanin. This structure is only 4 kJ/mole less stable than the optimal tetrahedral structure, which is within the error limits of the method [14]. Moreover, dynamic effects, solvation, and improvements of the theoretical method tend to diminish this difference [19,22]. Therefore, we cannot decide whether the reduced structure is slightly distorted by the protein or not, but it is clear that the energy needed to distort the Cu–S$_{Met}$ bond length is extremely small.

Trigonal and tetragonal Cu(II) structures

Why does a Cu(II) ion assume a trigonal structure with the ligands in the blue copper proteins, whereas most inorganic cupric complexes are tetragonal (square-planar, square pyramidal, or distorted octahedral) [17,23]? We have faced this question by optimising the geometry of a number of models of the type $Cu^{II}(NH_3)_3X$, where X is SH^-, SeH^-, OH^-, Cl^-, NH_2^-, and some other ligands related to the cystine thiolate group [24]. The results show that all complexes may assume two types of structures, both reflecting the Jahn-Teller instability of the tetrahedral Cu(II) complex. This instability can be lifted either by a D_{2d} distortion, leading to a tetragonal structure, or by a C_{2v} distortion, leading to a trigonal structure. The tetragonal structure is stabilised by four favourable interactions between the singly occupied Cu 3*d* orbital and *p* orbitals on the four (equatorial) ligands, as is shown in Fig. 2a . This gives rise to the well-known square-planar Cu(II) complexes.

If one of the ligands instead has the ability to form a strong bond with the copper ion, however, a trigonal structure can be stabilised. Fig. 2b shows that in such a structure, two of the ligands still form bonds to the copper ion, whereas a *p* orbital of the third ligand overlaps with two lobes of the Cu(II) ion, thereby occupying two positions in a square coordination plane, giving rise to a trigonal planar geometry. The fourth ligand cannot overlap with the singly occupied orbital, and therefore has to become a weakly bound axial ligand, explaining the long bond to the methionine ligand in the blue proteins. Thus, this long bond is a result of the electronic structure of the complex, rather than a cause of the trigonal structure, imposed by the protein [25].

For small and hard X ligands, such as NH_3 and OH^-, the tetragonal $Cu^{II}(NH_3)_3X$ structure is most stable (by 30–70 kJ/mole) and the trigonal structure is only a transition state [24]. For large, soft, and polarisable ligands, such as SH^- and SeH^-, on the other hand, the two types of structures have approximately the same stability (within 15 kJ/mole). Interestingly, the tetragonal structure is most stable for $Cu(NH_3)_3(SH)^+$, whereas the trigonal structure is more stable for $Cu(NH_3)_2(SH)(SH_2)^+$, showing that the methionine ligand is also important for the structure of the blue copper proteins. Therefore, it is unlikely that a trigonal cupric structure is retained in denatured blue copper proteins [15] as the site is then open to water molecules that will stabilise a tetragonal structure. This also explains why no trigonal cupric structures are encountered in the Cambridge data base [17]; there simply is no complex with the appropriate set of ligands, $CuN_2S^-S^0$ [26,27]. Recently, a trigonal planar Cu(II) structure was reported, with one thiolate ligand and two nitrogen donors from a $NH(CH)_3NH^-$ derivative [28]. The trigonal structure of this complex is reproduced by our calculations, and it is caused by the presence of only three ligands, of which the thiolate forms a favourable bond, the negative charge of the nitrogen ligand stabilises a low coordination number, and the bulky side groups prohibit the approach of other ligands and provide weak axial interactions.

For soft, negatively charged ligands, much charge is transferred from the ligand to the copper ion, so that the actual charge on the copper ion becomes closer to +1 than to +2. Therefore, such complexes are strongly distorted towards a tetrahedron (the normal geometry of a Cu(I) complex). This effect is most conspicuous for the tetragonal complexes, which are far from planar (e.g. $Cu(NH_3)_3(SH)$ in Fig. 2a). It has turned out that a group of blue copper proteins, the so called rhombic type 1 proteins, actually have a tetragonal, rather than a trigonal, structure. This gives an explanation to the structural and spectroscopic differences between these proteins and the normal axial type 1 proteins, which share the same copper ligand sphere [24,29]. For example, tetragonal models have a longer $Cu–S_{Cys}$ bond and a shorter $Cu–S_{Met}$ than trigonal models, as is illustrated in Table 1. Differences in the angles subtended at the copper ion accompany these differences [24,29]. The two types of structures have almost the same energy (within about 7 kJ/mole) and which structure is most stable depends on the models used for the ligands. At present it is not possible to decide if the most stable structure of the typical blue copper ligand sphere is trigonal or tetragonal [19,24,29].

By free energy perturbations, we have studied why some proteins stabilise the trigonal structure, whereas other stabilise the tetragonal structure, although the ligand sphere is the same [19]. The results indicate that plastocyanin prefers the bond lengths and electrostatics of the trigonal structure, whereas nitrite reductase favours the angles in the tetragonal structure, both by 10–20 kJ/mole. Interestingly, the length of the $Cu–S_{Met}$ bond has a very small influence on the relative stability of the two conformations, contrary to the suggestions of Solomon and coworkers [25,30]. However, the most important implication of our results is that with the typical blue-copper ligands, the tetragonal Jahn–Teller distortion may at worst give rise to the structure found in nitrite

reductase, i.e. a fully functional site with reduction potentials and reorganisation energies similar to those of the trigonal blue copper proteins [31,32]. Thus, with these ligands there is no need for protein strain.

The relation between the structure and electronic spectra of blue copper proteins

A combination of spectroscopic measurements and theoretical calculations has shown that the blue colour of the axial type 1 proteins, such as plastocyanin and azurin, is caused by a transition from the Cu 3*d*–S_{Cys} 3*p* bonding orbital to the corresponding antibonding orbital [33–35]. We have refined the theoretical calculations, using a more accurate method (CASPT2) and taking into account the modulating effect from the protein matrix [36]. Moreover, both our and Solomon's groups have studied the spectra of rhombic type 1 proteins (nitrite reductase, cucumber basic protein, and pseudoazurin) and showed that they have a tetragonal ground state, in which the singly occupied orbital is a mixture of and interactions between Cu and S_{Cys} [29,37]. In fact, there is a close correlation between the spectrum and the structure of the copper site, for example described by the angle between the S_{Cys}–Cu–S_{Met} and N–Cu–N planes, denoted (is 0° in an ideal square-planar structure and 90° in a trigonal structure). This is succinctly illustrated in Fig. 3, which shows how the geometry and the electronic spectrum vary for $Cu(NH_3)_2(SH)(SH_2)^+$ optimised at different angles. It can be seen that the Cu–S_{Met} bond length decreases, the Cu–S_{Cys} bond length increases, and the intensity ratio between the two main features in the electronic spectrum ($_{460}/_{600}$) increases as the angle decreases from 90°. Axial type 1 copper proteins have = 80–90°, whereas rhombic type 1 proteins have = 60–75°.

However, the correlation is not restricted to these two types of proteins. By site-directed mutagenesis, copper sites involving cysteine have been constructed that have even larger $_{460}/_{600}$ ratios (and therefore a yellow colour). These have been termed type 1.5 and type 2 copper proteins [38]. Fig. 3 shows that they can be predicted to arise when the angle is even smaller. Thus, type 1.5 characteristics are obtained when = 40–65°, whereas type 2 properties are observed for complexes with = 0–30°, i.e. for almost ideal square–planar Cu–cysteinate complexes. Detailed calculations on more realistic models of the type 1.5 and 2 copper sites have confirmed this suggestion [29,39].

This has led us to propose [29] that axial type 1 proteins have a trigonal structure with a bond between Cu and S_{Cys}. The other three types of copper proteins have instead a tetragonal structure with mainly bonds to the four copper ligands. They differ in the flattening of the geometry, for example described by the angle. Rhombic type 1 proteins, which are most distorted towards a tetrahedron, arise when one of the ligands is large and polarisable, forming a weak bond. If all ligands bind strongly, but still are rather soft (e.g. cysteine and histidine), type 1.5 sites arise, whereas with harder ligands (e.g. water) and preferably with two axial ligands, the strongly flattened type 2 copper sites are found. It is notable that all sites are formed naturally, following the preferences of the copper ion and its ligands, and not by any protein strain.

The only protein that does not fit into this view is stellacyanin. It has rhombic spectral characteristics, but the angle (84°) shows that the structure is trigonal. This is also confirmed by our calculations: the optimised trigonal structure of $Cu(imidazole)_2(SCH_3)(OCCH_3NH_2)^+$ is closely similar to the crystal structure of cucumber stellacyanin (c.f. Table 1) [21]. The high $_{460}/_{600}$ ratio of this protein is instead caused by the axial ligand. In the stellacyanins, the axial ligand is a glutamine side-chain amide group, which binds much stronger to the copper ion (221–227 pm) than the normal axial methionine ligand (265–330 pm). This strong axial interaction leads to a significant amount of Cu–S_{Cys} interactions (about 18%) in the ground-state singly occupied orbital, which explains the high $_{460}/_{600}$ ratio [21]. Recently, Solomon and coworkers have suggested that the copper site in stellacyanin avoids a tetragonal distortion by protein strain [40]. However, our geometry optimisations clearly show that structures closely similar to the crystal structure of stellacyanin can be obtained without any protein strain (c.f. Table 1) [21]. This again illustrates that the Jahn–Teller instability of tetrahedral Cu(II) sites can be lifted not only by a tetragonal distortion, but also by a trigonal distortion (like the plastocyanin models).

Reorganisation energies

According to the semiclassical Marcus theory, the rate of electron transfer depends on the reduction potential, the electronic coupling matrix element, and the reorganisation energy. Of these,

the inner-sphere reorganisation energy falls out by being a property of only the copper site, and can therefore be expected to be modulated by the protein during evolution. We have estimated inner-sphere reorganisation energies for a number of models with relevance to the blue copper proteins by calculating the energy difference (again by the density functional B3LYP method) of the reduced complex at the optimum geometry of reduced and the oxidised complex or vice versa [31]. For our best model of plastocyanin, $Cu(imidazole)_2(SCH_3)(S(CH_3)_2)^+$, we obtain an inner-sphere reorganisation energy of 62 kJ/mole. This value can be combined with a theoretical estimate of the outer-sphere reorganisation energy for the physiological docking complex between plastocyanin and cytochrome *f*, 42 kJ/mole [41], to get a very approximate total self-exchange reorganisation energy for plastocyanin of 100 kJ/mole (the outer-sphere reorganisation energy is probably not additive [31]). This energy is slightly lower than the experimentally measured reorganisation energy for plastocyanin (120 kJ/mole) [42]. The reorganisation energy of azurin, which is the best studied blue copper protein [43–47], is slightly lower (about 80 kJ/mole), but it is likely that azurin, with its bipyramidal copper site, has a lower reorganisation energy than the pyramidal site in plastocyanin [31].

Thus, the inner-sphere reorganisation energy of our blue copper models in vacuum is similar to the one in the proteins. This indicates that the proteins do not alter the reorganisation energy to any significant degree, i.e. that protein strain is not important for the low reorganisation energies of the blue copper proteins. On the contrary, our results show that an important mechanism used by the blue copper site to reduce the reorganisation energy is the *flexible* bond to the methionine ligand, which can change its geometry at virtually no cost (less than 5 kJ/mole) [19,31]. This mechanism is actually the *antithesis* of the strain hypotheses, which suggest that a low reorganisation energy is obtained by the rigid protein obstructing any change in geometry.

Our calculations also give further indications how the proteins have reached a low reorganisation energy. The low coordination number of the copper ion in the proteins is unfavourable for the reorganisation energy, but necessary since Cu(I) normally does not bind more than four ligands. Instead, a low reorganisation energy is partly attained by the use of soft ligands with small force constants (histidine and methionine), and partly by the use of polarisable ligands that give rise to structures that are similar in the two oxidation states (cysteine and methionine). Interestingly, realistic models of the rhombic type 1 proteins nitrite reductase and stellacyanin have larger inner-sphere reorganisation energies than the plastocyanin model, 78 and 90 kJ/mole.

Reduction potentials and the revised rack theory

Recently, Malmström and Gray have shown that the reduction potential of denatured azurin is higher than for the native protein [15,45,48]. This provides another argument against a strained conformation of the oxidised form of the azurin, since it shows that the reduced copper site gains more from unfolding than the oxidised site, especially as unfolding would increase the solvent accessibility of the site, thereby favouring Cu(II) and lowering the reduction potential. Moreover, it shows that the overall effect of the folding of the protein is a *lowering* of the reduction potential [15], i.e. the effect of the so-called “rack” is opposite to the one it was originally suggested to explain, viz. the *high* reduction potentials of the blue copper proteins. This has led Malmström to present a revised rack theory based on the suggestion by Solomon and co-workers that the entatic nature of the blue copper sites only involves the protein imposing the $Cu(I)–S_{Met}$ bond length [25,30]. Malmström et al. propose that this modulation of the $Cu–S_{Met}$ bond length is used by the protein to determine the reduction potential of the site together with the solvent accessibility [15–17]. We have examined this suggestion by several types of calculations.

First, we have used free energy perturbations to estimate the maximum strain energy plastocyanin or nitrite reductase can mobilise to resist a certain copper geometry [19]. These calculations show that the proteins are quite indifferent to the $Cu–S_{Met}$ bond length. It costs less than 5 kJ/mole to change the length of this bond between the values observed in different crystal structures or in optimised vacuum models. This energy is at least a factor of two too low to explain the observed differences in the $Cu–S_{Met}$ bond length [19]. Therefore, the protein cannot change the electronic structure of the copper site by imposing a certain $Cu–S_{Met}$ bond length, contrary to what has repeatedly been suggested [13,25,37,40,49]. However, replacement of the axial (or any other) ligand by another ligand will, of course, affect the relative stability between the trigonal and tetragonal structures as our calculations showed [24]. In particular, it is obvious that if the fourth ligand in a tetragonal structure is removed, the trigonal structure will be stablised, as was observed in a nitrite reductase mutant [49,50].

Second, quantum chemical calculations of the potential energy surface of the Cu–S_{Met} bond have shown that it costs less than 10 kJ/mole to change the Cu–S_{Met} bond length by 100 pm around its optimum value both in the oxidised and reduced states, a range larger than the natural variation in this bond [14,19]. This shows that even if the proteins could constrain this bond, it would affect the electronic part of the reduction potential by less than 10 kJ/mole, or 100 mV, i.e. much less than the variation found among the blue copper proteins (180 –770 mV [17]). Moreover, a constrained Cu(I)–S_{Met} bond would destabilise the reduced state and therefore *decrease* the reduction potential, contrary to the suggestion of a raised potential by Solomon [25] and the fact that the blue copper proteins are characterised by high reduction potentials. Thus, the Cu–S_{Met} bond length varies strongly among the blue copper proteins because this bond is more flexible than the other Cu–ligand bonds, but this very flexibility makes it useless for any significant role in the function of the blue copper proteins.

However, there are other contributions to the reduction potential than the electronic part, most importantly the solvation energy of the active site caused by the surrounding protein and solvent. We have therefore studied the reduction potential of the blue copper proteins using various methods to include the solvation effects [32]. The results in Fig. 4 show how the reduction potential varies when the Cu–S_{Met} bond is constrained in the reduced, oxidised, or both states. It should be noted that these calculations involve full optimisations of all other geometric parameters, so the effect of any change in the Cu–S_{Cys} bond length is included in these results. Evidently, the effect of the Cu–S_{Met} bond is small, less than 50 mV. As expected, it leads to a reduction of the potential (compared to the unconstrained state) if the Cu(I) state is constrained (the reduced structure is destabilised), an increase if the oxidised state is constrained, and a varying effect if both states are constrained to the same value. This is also in accordance with mutation studies of the axial methionine ligand in azurin [51], showing that most substitutions give only modest changes (less than 60 mV). The largest effects are found for mutations to hydrophobic residues, which increase the reduction potential by up to 140 mV (both electronic and solvation effects), and also mutations that change the structure of the copper site, e.g. the Met121Glu mutant that has a 110 mV lower reduction potential and a much more flattened site [52].

Furthermore, we have tested the suggestion [16,17] that the axial carbonyl oxygen ligand also influences the reduction potential (a short bond as in azurin gives a low reduction potential, whereas a longer bond as in plastocyanin and rusticyanin gives a high reduction potential). Again, our results show that the potential energy surface for this bond is too soft to account for the variation in reduction potential among the blue copper proteins, and that the solvation effects from this ligand are also small (the total effect is less than 140 mV) [32]. Instead, the large variation in the reduction potential of the various blue copper proteins seems to arise mainly from the solvent exposure of the copper site and the orientation of polar groups around the copper site [53-55]. Recent calculations on the dimeric Cu_A site in cytochrome *c* oxidase give similar results [56]: the vacuum geometry of the site is closely similar to the crystal structure and the potential surfaces of the Cu–Cu, Cu–S_{Met}, and Cu–O bonds are all very flat, suggesting that they have little influence on the structure and reduction potential of the site. This conclusion disagrees with earlier descriptions [49,57,58].

Protein strain

The suggestion that proteins use mechanical strain for their function is an old but still viable hypothesis [e.g. 11,12,59–61]. The most classical example of a protein for which strain has been suggested to play a functional role is lysozyme [62]. It was originally suggested that this protein forces its substrate to bind in an unfavourable conformation, viz. a conformation similar to the transition state. However, theoretical calculations by Levitt and Warshel convincingly showed that strain has a negligible influence on the rate of this enzyme; instead, the catalytic power is gained from favourable electrostatic interactions in the transition state [63]. This and other cases have led several biophysical chemists to argue strongly against strain as an important factor in enzyme catalysis [63–65].

To make strain hypotheses testable, it is vital to define what is meant by strain. Warshel has defined strain as distortions caused by covalent interactions (bond, angles, and dihedrals) and possibly also the repulsive part of the Van der Waals interaction [63]. This is close to the intuitive conception of mechanical strain and we fully agree with this definition. Unfortunately, it is hard to unambiguously distinguish between the various energy terms in experiments and most calculations, except in classical simulations.

Therefore, our quantum chemical estimates include terms that strictly are not strain. For example, we have estimated strain as the change in geometry of the metal coordination sphere when it is moved from vacuum to a protein. However, this includes in addition to covalent strain also all other effects from the protein, including electrostatic interactions and solvation effects. Thus, it overestimates the effect of strain.

Unfortunately, the concept strain in the entatic state and the induced rack theories is not clearly defined. The original publications emphasised the rigid protein and the strained cupric conformation, i.e. mechanical strain in the meaning of Warshel. However, lately they have started to embrace virtually any modifying effect of the protein. For example, Gray, Malmström and Williams in their commentary include exclusion of water as a “constraining factor” [17]. Without an unambiguous definition, it is impossible to test the strain hypotheses. Moreover, if any modifying effect of the protein is included in the concept, all proteins are strained or entatic by definition, but at the same time such a hypothesis would lose its predictive value. We prefer to quantify the contribution of various well-defined protein effects (including strain) to the unusual properties of the blue copper proteins, rather than lumping them together as constraining factors.

It must be recognised that any metal or any other molecule necessarily acquires slightly different properties when bound to a protein. This is an effect of the trivial fact that a protein is different from vacuum or solution (it has another effective dielectric constant and presents specific electrostatic interactions). Such changes have been studied for a number of protein–ligand complexes, and Liljefors et al. have argued that the energies involved are less than 13 kJ/mole if the reference state is the ligand in solution [66]. If the reference state instead is vacuum, appreciably larger energies are observed. We have, for example, calculated energies associated with the change in geometry of a metal coordination sphere when inserted from vacuum into a protein to 30–60 kJ/mole for the catalytic and structural zinc ions in alcohol dehydrogenase [67–70]. We suppose that the entatic state and induced rack hypotheses are intended to deal with systems where the strain is larger than normal and have a functional significance. Therefore, we consider distortions smaller than 13 kJ/mole insignificant.

We have shown that the cupric structure of the blue copper proteins is not strained (in Warshel’s sense) to any significant degree [14,21,29], especially if the dynamics at ambient temperatures and the dielectric surroundings of the copper site are considered [19,32,22][1]. The electronic structure explains why the proteins with a cysteine ligand have structures close to a tetrahedron, whereas inorganic complexes are mostly tetragonal [24]. Furthermore, we and other groups have shown that the unusual spectroscopic properties and the high reduction potential of the blue copper proteins are a natural consequence of the covalent nature of the interaction between copper and the cysteine thiolate group [21,24,25,29,33–37,40,53,54,55,73]. Similarly, we have

1Gray et al. in their article claim that their data show convincingly that the protein fixes the geometry of the copper site [17]. We have pointed out [14], however, that metal-substituted blue copper proteins shows a variation in the metal–ligand bond lengths of up to 102 pm, which neither is very small nor gives any indication of a rigid site. Moreover, the changes reflect the softness of the metal (c.f. our detailed investigation of Co-azurin [71]), showing that the geometry of the site is determined by the metal rather than the protein. Similarly, mutation studies of the copper ligands in azurin have provided strong experimental evidence for a flexible copper site [72]. As to the copper-free structures, there are several alternative reasons why it is favourable to have a pre-formed metal site in the protein [14], as is discussed by Larsson in his commentary [18].

shown that the low reorganisation energy is also intrinsic to the blue copper site [31]. Thus, strain is not needed to explain any of the unusual properties of the blue copper proteins and there is no indication that mechanical strain has any functional value for the proteins.

However, this does not mean that the protein is unimportant for the function of the blue copper proteins. On the contrary, we fully agree with Gray, Malmström and Williams [17] that the blue copper proteins, like all other proteins, have evolved to optimise their particular biochemical function. Thus, the protein provides the proper ligands to the copper site and protects it from unwanted ligands, most prominently water molecules that would stabilise a flattened tetragonal structure with a high reorganisation energy. Second, the protein modifies the dielectric properties of the surroundings of the copper site, thereby reducing the outer-sphere reorganisation energy and modulating the reduction potential of the copper site. Third, the protein offers a proper path or matrix for electron transfer and the docking sites for the donor and acceptor proteins [18,49].

Clearly, the blue copper proteins also modulate the geometry of the copper site. The rhombic type 1 proteins stabilise a tetragonal structure, whereas the axial type 1 proteins stabilise the trigonal structure of the same copper coordination sphere. However, the energy needed for such a stabilisation, less than 7 kJ/mole [29], is less than the typical distortion energies occurring in all proteins due to the subtle mismatch between the protein and the ligand sphere [66-70]. Furthermore, the forces leading to such a stabilisation include electrostatics and other factors usually not defined as mechanical strain, and the functional value of this stabilisation is unclear since both types of sites are present in proteins with a similar function. In addition, it is possible that the proteins may modify the length of the Cu(I)–S_{Met} bond. In this case, however, the energies involved are so low (less than 4 kJ/mole [14,19,22]) that we cannot decide whether this distortion is real or only reflects the uncertainty of the calculations. Moreover, we have provided strong evidence against any functional value of such a distortion [31,32].

In conclusion, we have provided a series of investigations where we address the function and properties of the blue copper proteins. We have emphasised the importance of defining the hypotheses (What is strain? In what respects is the copper site strained? What is the functional value of the strain?), and we have discussed strain in quantitative terms (How large is the energy or force?), since any molecule necessarily becomes slightly distorted when bound to a protein. We have in no case found any indication of a functional role for strain in the blue copper proteins. Thus, strain hypotheses for the blue copper proteins seem to be a case for Ockham’s razor.

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Table 1. Comparison of the geometry of optimised models and crystal structures of blue copper proteins [14,19,29]. Ax is the axial ligand and the angle between the S_{Cys}–Cu–Ax and N–Cu–N planes.

Model	Distance to Cu (pm)			Angle subtended at Cu (°)				
	S_{Cys}	N	Ax	N–N	S_{Cys}–N	S_{Cys}–Ax	N–Ax	
$Cu(imidazole)_2(SCH_3)(S(CH_3)_2)^{+a}$	218	204	267	103	120-122	116	94-95	90
Plastocyanin oxidised	207-221	189-222	278-291	96-104	112-144	102-110	85-108	77-89
$Cu(imidazole)_2(SCH_3)(S(CH_3)_2)$	232	214-215	237	109	105-108	115	107-113	89
$Cu(imidazole)_2(SCH_3)(S(CH_3)_2)^{b}$	227	205-210	290	119	112-120	99	100-101	88
Plastocyanin reduced	211-217	203-239	287-291	91-118	110-141	99-114	83-110	74-80
$Cu(imidazole)_2(SH)(S(CH_3)_2)^{+c}$	223	205-206	242	100	97-141	103	95-126	62
Nitrite reductase oxidised	208-223	193-222	246-270	96-102	98-140	103-109	84-138	56-65
$Cu(imidazole)_2(SCH_3)(OCCH_3NH_2)^{+a}$	217	202-206	224	103	122-125	113	92-95	88
Stellacyanin oxidised	211-218	191-206	221-227	97-105	116-141	101-107	87-102	82-86

[a] Trigonal structure
[b] The Cu–S_{Met} bond length was constrained to 290 pm.
[c] Tetragonal structure

Figure Legends

Figure 1. A comparison of the optimised structure of $Cu(imidazole)_2(SCH_3)(S(CH_3)_2)^+$ [14] and the crystal structure of plastocyanin (shaded) [4].

Figure 2. The singly occupied orbitals of the tetragonal (a) and trigonal (b) $Cu(SH)(NH_3)_3^+$ complex [39].

Figure 3. The variation of the $Cu–S_{Cys}$ and $Cu–S_{Met}$ bond lengths and the quotient of the oscillator strengths of the peaks around 460 and 600 nm as a function of the dihedral angle [29].

Figure 4. Calculated reduction potentials of the $Cu(imidazole)_2(SCH_3)(S(CH_3)_2)^{0/+}$ complex as a function of the $Cu–S_{Met}$ bond. Three curves are given. In the first (squares), the $Cu–S_{Met}$ bond in the reduced complex constrained to the indicated distances. In the second (diamonds), the $Cu–S_{Met}$ bond distance has been constrained in the oxidised complex. In the last (circles), the $Cu–S_{Met}$ distance has been constrained to the same value in both complexes. The energy scale has been selected so that the unconstrained case has a reduction potential of 0 mV for all lines. The calculations involve full quantum chemical geometry optimisations at each $Cu–S_{Met}$ distance, and solvation effects (in water) are estimated from the charge distribution using the MEAD program [74] (numerical solution of the Poison–Boltzmann equation). The calculations differ from those of Solomon and co-workers [13] in that geometry optimisations have been performed and a more accurate method for the solvation energies has been used.