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Structure, strain, and reorganisation energy of blue-copper models in the protein

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Abstract

The copper coordination geometry in the blue copper proteins plastocyanin, nitrite reductase, cucumber basic protein, and azurin has been studied by combined density functional (B3LYP) and molecular mechanical methods. Compared to quantum chemical vacuum calculations, a significant improvement of the geometry is seen (towards the experimental structures), not only for the dihedral angles of the ligands, but also for the bond length and angles around the copper ion. The flexible Cu-S_{Met} bond is well reproduced in the oxidised structures, whereas it is too long in some of the reduced complexes (too short in vacuum). The change in the geometry compared to the vacuum state costs 33-66 kJ/mole. If the covalent bonds between the ligands and the protein are broken, this energy decreases by ~25 kJ/mole, which is an estimate of the covalent strain. This is similar to what is found for other proteins, so the blue copper proteins are not more strained than other metalloproteins. The inner-sphere self-exchange reorganisation energy of all four proteins are ~30 kJ/mole. This is 30-50 kJ/mole lower than in vacuum. The decrease is caused by dielectric and electrostatic effects in the protein, especially the hydrogen bond(s) to the cysteine copper ligand, and not by covalent strain.

Keywords

blue copper proteins; entatic state theory; protein strain; QM/MM method; reorganisation energy

Introduction

The blue (or type 1) copper proteins are a group of proteins that in their oxidised state exhibit a number of unusual properties, viz. a bright blue colour, a narrow hyperfine splitting in the electron spin resonance spectra, and high reduction potentials [1-3]. These macroscopic properties are accompanied by an extraordinary cupric coordination geometry: 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 an axial ligand, typically a methionine (Met) thioether group. The azurins, however, are characterised by an additional fifth ligand, a backbone carbonyl group at a large distance (~300 pm; it is found at a distance of >380 pm in the other proteins) [1-4]. In addition, they have unusually long Cu-S_{Cys} and Cu-S_{Met} distances (~220 and 310 pm, respectively).

Also the four-coordinate blue copper proteins with a methionine ligand show a variation in the structural and spectroscopic properties [1-5]. The axial type 1 copper proteins, such as plastocyanin, have a short Cu-S_{Cys} bond (~210 pm) and a long Cu-S_{Met} bond (~290 pm). On the other hand, the rhombic type 1 copper proteins, e.g. nitrite reductase, cucumber basic protein, and pseudoazurin, have a longer Cu-S_{Cys} bond (~215 pm) and a shorter Cu-S_{Met} bond (~260 pm). There seems to be a continuous transition between the rhombic and axial copper proteins, which can be described by the angle between the N_{His}-Cu-N_{His} and the S_{Cys}-Cu-S_{Met} planes, denoted in the following. It is 77-89° for plastocyanin, 70-75° for pseudoazurin and cucumber basic protein, and 56-65° for nitrite reductase. This transition in the geometry is reflected in the electronic structure of the copper site. In the trigonal structures, the ground-state orbital involves a pure Cu-S_{Cys} bond, whereas the tetragonal structures involve an increasing character of the interaction between copper and S_{Cys} [6,7].

The blue copper proteins serve as electron-transfer agents. Their distorted trigonal geometry is intermediate between the tetrahedral coordination preferred by Cu(I) and the

tetragonal geometry of most Cu(II) complexes. As a result, the change in geometry upon reduction of Cu(II) to Cu(I) is small [2,3,8], which gives a small reorganisation energy and allows for a high rate of electron transfer [9]. It has been suggested (the entatic state and induced-rack theories) that the protein forms a rigid structure, which forces the Cu(II) ion into a coordination geometry more similar to that preferred by Cu(I) [10-13]. This would explain both the high rate of electron transfer and the unusual properties of the oxidised copper site.

However, these hypotheses have recently been challenged [14,15]. In particular, we have shown that the optimum geometry of $Cu(imidazole)_2(SCH_3)(S(CH_3)_2)^+$, calculated by quantum chemical methods, is very close to the crystal structure [15]. It has also been shown that most of the differing properties of the blue copper proteins can be explained by the interaction between the copper ion and its ligands, rather than by protein strain [6,7,16-28]. Yet, there are small differences between the optimised models and the experimental structures. For example, the calculated Cu-S_{Cys} distances are somewhat too long and the Cu-S_{Met} distances too short. These differences are especially pronounced for the reduced models, where the optimum vacuum structure is tetrahedral with a Cu-S_{Met} bond of 237 pm (compared to 290 pm in the protein) [15]. Even if the discrepancy is small in energy terms (less than 4 kJ/mole), it has been taken as an evidence that the Cu(I)-S_{Met} bond is strained by the protein [14,29,30].

However, the discrepancy may also arise from factors not included in the vacuum calculations, e.g. hydrogen bonds and electrostatic interactions between the protein and the metal site. The best way to study such interactions is to directly compare vacuum geometries and energies with those observed in proteins. Unfortunately, available crystal structures are not accurate enough to make such a comparison meaningful. Another method is to optimise the copper model inside the protein, using combined quantum chemical and molecular mechanical methods [31-35]. Such methods have the advantage of involving the same systematic errors in vacuum and in the protein. Therefore, they will cancel in the comparison. In this paper, we present such calculations for four blue copper proteins: plastocyanin, nitrite reductase, cucumber basic protein, and azurin. We study the change in geometry caused by the

protein and discuss it quantitatively in relation to the strain hypotheses. In addition, we estimate the inner-sphere reorganisation energy of the copper site in the protein and show that it is decreased by electrostatic interactions in the proteins.

Methods

The updated ComQum method

Combined quantum chemical and molecular mechanical geometry optimisations were performed using the program ComQum [33]. This program has been updated to combine the quantum chemical software Turbomole [36] with the classical simulation software Amber [37], yielding the ComQum-00 version. Below, we describe this method shortly concentrating on changes made relative to the original version [33].

The total system (protein and solvent) is divided into four subsystems. The central system 1 is optimised by quantum chemical methods. System 2 includes all atoms of all amino acids (or solvent molecules) within a radius r_2 of any atom in the quantum system. It is optimised by classical methods. Similarly, system 3 comprises all atoms of all amino acids within a radius r_3 of system 2. It is included in all calculations, but is kept fixed at the crystal geometry. System 4, finally contains the rest of the total system and is ignored in the calculations.

In the quantum chemical calculations, system 1 is represented by a wave function, whereas systems 2 and 3 are modelled by an array of point charges, one for each atom, taken from the libraries of the classical program. In the classical force and energy calculations, systems 1-3 are represented by the standard force field of the classical software, but without any electrostatic interactions (which are already treated by quantum mechanics). Special action is taken when there is a bond between the classical and quantum chemical systems (a junction) [33]. The quantum chemical system is truncated by hydrogen atoms at the junctions, the positions of which are linearly related to the corresponding heavy (typically carbon) atoms in the full system.

The total energy is calculated as:

$$E_{tot} = E_{QC} + E_{MM123} - E_{MM1} \quad (1)$$

Here, E_{QC} is the quantum chemical energy of system 1 with a H junction atom, including all the electrostatic interactions. Similarly, E_{MM1} is the classical energy of system 1, still with a H junction atom, but without any electrostatic interactions. Finally, E_{MM123} is the classical energy of systems 1-3 with C junction atoms and no electrostatics. The philosophy behind this energy is that the total energy should involve as much quantum chemistry as possible and that terms from the H junctions shall cancel out, so that the total energy represents a system with a C junction. This approach is closely similar to the one used in the Oniom method [35]. The calculated forces are the gradient of this energy. Due to the different junction atoms in system 1 and systems 1-3, the gradients have to be corrected using the chain rule [38].

Within each cycle of the quantum chemical geometry optimisation, the geometry of system 2 is optimised by a full molecular mechanics optimisation. Such an optimisation step takes about the same time as one wave function calculation. In these calculations, systems 1-3 are represented by standard parameters from the classical force field. Electrostatic interactions are included, using standard charges for systems 2 and 3, but for system 1, we employ charges fitted to the quantum chemical electrostatic potential (ESP charges) using the Merz-Kollman method [39]. In order to save time and make the charges more stable (the ESP charges are sensitive to the orientation of the molecule, the fitting density, and numerical instabilities of the fitting procedure [40]), ESP charges were only calculated at the first geometry using a high density of potential points (~2000/atom). They were then related to the Mulliken charges at the same geometry by an additive constant and in the subsequent steps, ESP charges were estimated from the Mulliken charges using these constants. Since the geometry does not change much, such a procedure works well and did not lead to errors in the charges larger than 0.05 e (similar to the uncertainty of the Merz-Kollman method [40]). The charges of the junction atoms were adapted so that the total charge of the residue was the same as in the quantum calculations (except for an integer constant if the residue is charged outside the

quantum system). This way, charge transfer is allowed between the residues in the quantum system and the charge of the junction atom is changed from what is typical for a hydrogen atom to that of a carbon atom.

In the present implementation the quantum chemical program package Turbomole [36] has been combined with the molecular dynamics software Amber [37]. The interface consists of five small procedures moving information (coordinates, energies, forces, and charges) between the two programs, a program constructing all input files, and a shell script driving the geometry optimisation. No changes have been made to the code of Turbomole or Amber, except for a few rows that writes out the classical forces in Amber.

Differences between the original [33] and the new implementation of ComQum are the following:

1. System 4 is ignored (no integer charges are included, since much larger systems 2-3 are used).
2. System 3 is included in all calculations of molecular mechanics forces and energies.
3. The charges of system 1 are determined slightly differently (as described above).
4. Hydrogen atoms bound to the junction carbon atoms do not move together with the junction atom (i.e. they are treated as the other classical atoms).
5. Forces on the junction atoms are corrected for the different junction atoms [38].
6. The Amber software (instead of Mumod) is used for the classical calculations.
7. Point charges are represented by the keyword point_charges instead of nuclei without any basis functions (which allows a larger number of atoms to be included and simplifies the programs).
8. Offset forces are not used.

Details of the calculations

The quantum chemical calculations were performed with the density functional method B3LYP (unrestricted formalism for Cu^{II}), as implemented in the Turbomole software [36,41].

For copper, we used the double- basis set of Schäfer et al. (62111111/33111/311) [42], enhanced *p*, *d*, and *f* functions with exponents 0.174, 0.132, and 0.39. For the other atoms, we have employed the 6-31G* basis set [43]. This basis has been shown to give almost converged geometries and energies [15,44]. The full geometry of system 1 was optimised until the change in energy and the coordinates were below 0.26 kJ/mole and 0.53 pm, respectively. Then, system 2 was fixed and the optimisation was continued until the changes were below 2.6 J/mole and 0.053 pm. In some cases, system 2 was kept fixed all the time and the tighter thresholds were used.

The potential function of Amber contains a harmonic potential for bond stretches and angle bending, a truncated trigonometric series (n=1-3) for the dihedral angles, a Coulombic term for the electrostatic interactions, and a 6-12 Lennard-Jones potential for the Van der Waals interactions. The force field does not contain any specific terms for hydrogen bonds. The interactions between the copper ion and its ligands were treated purely quantum mechanically; in the molecular mechanical gradients the copper terms cancel out and in the classical optimisation of system 2 the copper ion interacts only by a non-bonded potential (no bonds were defined around the copper ion). The Lennard-Jones parameters of copper were 117 pm and 4.77 kJ/mole [45].

ComQum calculations were run with plastocyanin (both reduced and oxidised structures), nitrite reductase, cucumber basic protein, and azurin. The coordinates were taken from the Brookhaven data bank files 1plc, 5pcy, 2cbp, 1nic, and 4azu [46-50]. The proteins were protonated and equilibrated together with a water cap (2.4-2.8 nm), as described before [7,24,51]. For plastocyanin and cucumber basic protein, the whole protein was included in the calculations, whereas for nitrite reductase and azurin (which are a trimer and a tetramer, respectively), all amino acids more than 2.4 nm from the copper ion (in subunit A and C, respectively) were ignored. The number of amino acids, water molecules, and atoms in each protein are shown in Table 1.

System 1 consisted of $Cu(Im)_2(SCH_3)(S(CH_3)_2)^+$ (Im = imidazole) with junctions at the C atom of Met and His and at C of Cys. For azurin we used instead the Cu(Im) $(ImCH_2CH_2NHCOCH_3)(SCH_3)(S(CH_3)_2)^{0/+}$ model with additional junctions at the backbone C and N atoms of Glu-41 and His-42, respectively. It includes the fifth carbonyl ligand and its backbone connection with one of the imidazole ligands. In system 2, all amino acids within 1.6 nm of any atom in system 1 were included, 2860-4460 atoms. System 3 included the rest of the proteins and the solvent cap.

In order to decide how much of the change between models optimised in vacuum and with COMQUM is caused by covalent strain, we did some calculations where the bond between the ligand and the protein backbone was broken. This was done by keeping the quantum system intact, removing the H atoms of the His and Met ligands, and representing the backbone of the ligand residues by a Gly residue without any side-chain atom. No junction atoms were defined and the C and H atoms (all atoms for the Cys ligand) of the backbone residue had no charge or Van der Waals parameters (otherwise they would be too close to the quantum atoms; we did not want to break the backbone).

Inner-sphere reorganisation energies were estimated in the same way as in our vacuum studies of the blue copper proteins [25]. The reorganisation energy for the oxidised complex ($_{ox}$) was calculated as the difference in energy of the Cu(II) system at its optimal geometry and at the optimal geometry of the reduced complex. Likewise the reorganisation energy of the reduced complex ($_{red}$) was calculated as the energy of the Cu(I) system at its optimal geometry minus the energy of the Cu(I) complex calculated at the geometry optimal for the Cu(II) complex. The total inner-sphere reorganisation energy for a self-exchange reaction ($_i$) is the sum of $_{ox}$ and $_{red}$. In the protein, this energy was calculated from the energies of the isolated quantum system only. For calculations with a fixed protein, we could have included also the electrostatic interaction with the protein in the reorganisation energies. This change the individual values of $_{ox}$ and $_{red}$, but not the sum $_i$ by more than a few kJ/mole. However,

for the flexible proteins, this procedure would include a significant amount of the outer-sphere contribution in the reorganisation energy and it would give unstable energies (see below). All calculations were run on SGI Origin 2000 or Octane workstations.

Results and discussion

Geometries

The results of the ComQum calculations are shown in Table 2, together with data obtained by quantum chemical calculations in vacuum [15,24] and the result of crystallographic investigations of the proteins. Five protein structures were used in the ComQum calculations: oxidised plastocyanin, cucumber basic protein, nitrite reductase, and azurin, and reduced plastocyanin [46-50]. For all proteins, we performed one calculation in which the protein was kept fixed (i.e. system 2 was not relaxed with Amber), and one in which the protein was relaxed. In some cases, we also did a calculation where the connection between the metal ligand and the backbone of the protein was removed, as is described in the Methods section. All proteins were studied with the copper ion in both oxidation states.

The four calculations with an oxidised copper site in plastocyanin gave very similar structures, even if they differ in which crystal structure has been used. They are clearly different from those obtained in vacuum, as can be seen in Figure 1. In particular, the Cu-S_{Cys} and Cu-N distances are 1-5 and ~6 pm shorter than in vacuum. This makes the structures more similar to the crystal structures. The S_{Cys}-Cu-S_{Met}, S_{Met}-Cu-N, and angles are also more similar to what is found in the crystal structures. The Cu-S_{Met} bond length is close to the crystal value for the structures based on the oxidised crystal structure (286-290 pm), but it is slightly too long in those based on the reduced crystal (303-310 pm).

The results are similar for four of the structures of the reduced complex in plastocyanin. There is only small structural differences between these four ComQum optimisations. The Cu-S_{Cys} and Cu-N distances are 12 and ~10 pm shorter than in vacuum, i.e. a larger change than for the oxidised complexes. This makes the structures more similar to the crystal structures,

even if the Cu-S_{Cys} bond is still slightly too long (~6 pm, caused by deficiencies in the B3LYP method [15,44]).

Yet, the most conspicuous difference between the ComQum and vacuum structures is the large increase in the Cu-S_{Met} bond length. In the structures optimised with ComQum, this bond is 310-339 pm, whereas it is 237 pm in the optimum vacuum structure and ~290 pm in the crystal structures. Thus, the general effect of the protein is to elongate this bond. However, ComQum overestimates this effect somewhat. This is due to the flexibility of the bond [15,28,52], combined with problems in the classical force field. Apparently, the molecular mechanics part of the calculations is not accurate enough to describe the fine-tuned (involving energies less than 5 kJ/mole) interplay between methionine group and the copper ion on one hand and the surrounding enzyme on the other hand.

Interestingly, if the connection between the protein and the metal ligands is removed, the Cu-S_{Met} bond becomes very large, 375 pm (this is the only geometric parameter that shows any clear effect of this change). This shows that the long Cu-S_{Met} bond is not an effect of covalent strain; on the contrary, strain actually seems to *shorten* the bond. The Cu-S_{Met} bond is longer in the protein than in vacuum probably because of the decrease in the bond lengths of the Cu-S_{Cys} and Cu-N bonds, which in its turn is caused by hydrogen bonds to the protein.

However, in one structure (the one with a fixed oxidised protein) the Cu-S_{Met} bond length is only 245 pm, i.e. only 8 pm longer than in vacuum. This leads to a small increase in the Cu-S_{Cys} and Cu-N bond lengths, and larger changes in the angles of the complex. Apparently, there are two stable configurations of this ligand with similar energies, which may explain why ComQum has problems to describe it correctly.

Next, the oxidised copper site in nitrite reductase was studied to see if we could obtain a tetragonal structure for this site, although such a structure cannot be found in vacuum with the same quantum system (it is only a transition state; the vacuum structure in Table 2 is obtained with a complex without the methyl group on the cysteine model). The results in Table 2 shows that this is actually possible. The angles in the ComQum structures are 48-61°, which clearly

show that the structures are tetragonal. The ComQum structures also have two large S_{Met}-Cu-N and S_{Cys}-Cu-N angles, which are much smaller in the trigonal plastocyanin structures. Again, ComQum shortens the Cu-S_{Cys} and Cu-N bonds and elongate the Cu-S_{Met} bond compared to vacuum geometries. However, for nitrite reductase, this change disappears when the connection between the protein and the metal site is removed. The latter structure is similar to the vacuum structure, except for a larger variation in the S_{Met}-Cu-N angles and a smaller angle (even smaller than in any crystal structure).

The reduced structures of nitrite reductase are quite similar to the reduced plastocyanin structure. However, they retain some of the differences between oxidised nitrite reductase and plastocyanin, although the reduced structure of the two enzymes in principle should be the same. Thus, the nitrite reductase structures have a 2-5 pm longer Cu-S_{Cys} bond and a much shorter Cu-S_{Met} bonds (253-272 pm, compared to 310-375 pm). Moreover, one of the S_{Cys}-Cu-N angles is ~10° smaller than that in plastocyanin and one of the S_{met}-Cu-N angles is ~20° larger than normal. The angle is also unusually small.

We have also studied the structure of the oxidised cucumber basic protein. This protein was included, because its structure is intermediate between trigonal and tetragonal, as is flagged by the angle of 70°, and it was interesting to see if ComQum could reproduce also this structure. Quite satisfactorily, the calculated structures are intermediate, with angles of 68-69°. Most distances and angles around the copper ion are also quite close to the crystal values, as can be seen in Table 2. As for the nitrite reductase structure, the geometry became quite similar to the tetragonal vacuum structure if the covalent interactions were removed (except for the S-Cu-S angle).

One of the structures of the reduced cucumber basic protein (with a fixed protein) is similar to the corresponding nitrite reductase structure. However, in a few aspects, e.g. the and S_{Met}-Cu-N angles, it is intermediate between the plastocyanin and nitrite reductase structures (as

the oxidised structure). The Cu-S_{Met} bond is short, 250 pm, i.e. only 13 pm longer than in vacuum.

The other structure (with a flexible protein) is more different. In particular, it has a long Cu-N bond, 258 pm, indicating that it is partly dissociated. In fact, we could also obtain a fully dissociated structure, as can be seen in Table 2. It has a Cu-N bond length of 380 pm, but it was less stable than the partly dissociated structure. This shows that Cu(I) has a problem to stabilise a high coordination number, giving a delicate balance between first- and second-sphere interactions, which the molecular mechanics calculations have problems to describe (in combination with that B3LYP tend to give too weak Cu(I) bonds).

Finally, we performed similar calculations on azurin. This is a hard test case, because it is virtually impossible to get the correct bond lengths to the axial ligands of this complex in vacuum [51]; the vacuum structures included in Table 2 were obtained with both these bonds constrained to their crystal values. In the ComQum calculations, these bond lengths are reasonably reproduced, especially the Cu-O bond. The reason for this is most likely that in the folded protein, the backbone has a smaller mobility than the side chains. The oxidised complex is quite similar to the crystal as well as the vacuum structure, except for a too long Cu-S_{Met} bond with a free enzyme. For the reduced complex, the ComQum calculations show a tendency to contract the Cu-N and Cu-S_{Cys} bonds. However, when the protein is allowed to relax, one of the imidazole groups partly dissociate from the copper ion (265 pm). This strongly distorts the structure away from the crystal (where both imidazole groups bind).

Very recently, a paper was published, in which crystallographic and EXAFS data are combined to yield accurate structures of both the reduced and oxidised copper site in azurin II from Alcaligenes xylosoxidans [53] (i.e. a different protein than used in the calculations). This is said to give structures with an accuracy of about 2 and 5 pm for the equatorial and axial bond lengths, respectively, i.e. appreciably more accurate than for normal crystal structures. These data are included in Table 2, and a comparison with our calculations show a good agreement, especially for except for the angles around the copper ion. Naturally, there are

appreciable differences for some of the axial distances, but it is also notable that the Cu-N distances are 7-13 pm too long in the calculations. Most importantly, however, the change in the bond lengths to the equatorial ligands upon reduction is very well reproduced, viz. 4-6 pm compared to 5-7 pm for the Cu-N bonds and 5-6 pm compared to 7 pm for the Cu-S bond. This gives a strong credibility to our calculated reorganisation energies.

In conclusion, COMQUM give structures that are significantly more similar to experimental structures than vacuum calculations. This is expected for the orientation of the histidine rings and the dihedral angles of all the methyl groups (c.f. Figure 1), since they are determined by low-energy modes which can easily be determined by the steric bulk of the protein and the fold of the backbone. However, the bond lengths and angles around the copper ion are also improved. The reason for this is partly the inclusion of hydrogen bonds and the dielectric surrounding of the protein site, partly the covalent interactions of the protein ligands. Interestingly, the structure of the plastocyanin site seems to mainly be determined by the former factors, whereas the nitrite reductase site seems to be determined more by covalent interactions. This is in excellent agreement with our free energy calculations of these two proteins [52], which indicated that plastocyanin preferred the trigonal structure by electrostatic interactions, whereas nitrite reductase favoured the angles in the tetragonal structure.

The success of COMQUM to predict geometries indicate that it could be used to predict the structure of mutants (especially mutants in or close to the active site) and metal substituted blue copper proteins. This has been done successfully for the interpretation of perturbed angular correlation of -rays experiments on Cd-substituted alcohol dehydrogenase [54].

Strain energies

The COMQUM calculations also give us an opportunity to directly estimate strain energies in the proteins. We can calculate the energy of the isolated quantum system at the vacuum and COMQUM geometries. The difference between these two energies (E_{str}) is a measure of the

energy cost to change the geometry of the metal site from the optimum vacuum structure to the one found in the protein. From Table 2, it can be seen that E_{str} ranges from 33 to 66 kJ/mole in the studied models. This is similar to what was found for the catalytic and structural zinc ions in alcohol dehydrogenase, 30-60 kJ/mole [33,54-56]. Rubredoxins have slightly lower E_{str} energies, 14-37 kJ/mole, whereas [2Fe-2S] ferredoxins have higher values, 43-110 kJ/mole [57]. Thus, there is no indication that the blue copper proteins have higher E_{str} energies than what is typical for other proteins.

It is notable that calculations with a fixed protein give higher strain energies than those with a relaxed protein (2-6 kJ/mole). This is quite natural because some of the strain energy may be caused by the uncertainties in the crystal structure, the protein force field, and the quantum chemical calculations. Moreover, the native protein (e.g. reduced plastocyanin for the Cu(I) system and oxidised plastocyanin for the Cu(II) system) gives slightly lower strain energies (~2 kJ/mole) than if the other protein structure was used, showing that the protein relaxation cannot fully reproduce the changes in the protein during oxidation or reduction.

The most conspicuous difference, however, is between the reduced and oxidised complexes. E_{str} is appreciably larger for the reduced sites (45-66 kJ/mole) than for the oxidised sites (33-45 kJ/mole). The reason for this is most likely that the reduced site is more flexible (i.e. it has lower force constants) than the oxidised site. Direct calculations of the force constants of the Cu-ligand interactions of the blue copper models have shown that those of Cu(I) are lower than those of Cu(II) [25,52]. If it is assumed that the energy function of the protein is the same in the two cases and that equilibrium is assumed when the forces of the protein and the copper site are equal, a simple harmonic model shows that strain energies of the Cu(I) and Cu(II) sites should be inversely related to their force constants. This is in qualitative agreement with the observed values.

Finally, it can be noted that the two rhombic proteins (nitrite reductase and cucumber basic protein) have slightly larger E_{str} energy than plastocyanin in both oxidation states. This

indicates that the rhombic proteins are more strained than the axial ones, which may be taken as an evidence that the intrinsically stable state of the blue-copper site is the trigonal structure. This conclusion is in accordance with our latest estimates [44], but the energy difference is still smaller than the error limit of the method (2 kJ/mole).

The E_{str} values can be interpreted as some sort of strain energies. However, they contain all differences between the protein and vacuum, i.e. many terms than are not commonly considered as 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 [58]. 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 quantum chemical calculations. However, a step in this way is to remove the connection between the protein and the metal ligands. This way, all changes caused by covalent interactions are removed. As we saw above, this led to only small changes for plastocyanin, but more appreciable changes for nitrite reductase and cucumber basic protein. From Table 2, it can be seen that the removal of covalent interactions decrease E_{str} strongly, to 16-21 kJ/mole. The difference, 21-29 kJ/mole is close to the strain energy in the sense of Warshel [58]. This energy is lower than what was found for alcohol dehydrogenase (33 kJ/mole; only one structure) [56]. Again this shows that the blue copper proteins are not more strained than other proteins.

Reorganisation energies

Finally, we have also calculated reorganisation energies from the ComQum data. The inner-sphere reorganisation energy was calculated for the isolated quantum system and the results are collected in Table 3. If possible, we used the same crystal structure and optimisation protocol for both oxidation states. If one of the oxidation states was not available, we used instead the average of the available structures.

First, it can be seen that the $_{red}$ values (15-29 kJ/mole) are larger than the $_{ox}$ values (0-19 kJ/mole). This is because the Cu-S_{Cys} and Cu-N bond lengths are shorter in the COMQUM calculations than in vacuum. This makes the COMQUM Cu(I) structures similar to the vacuum Cu(II) structures, whereas the COMQUM Cu(II) structures becomes less similar to the vacuum Cu(I) structures. The fact that one $_{ox}$ value is negative only shows that the COMQUM Cu(I) structure is more similar to the vacuum Cu(II) structure than the COMQUM Cu(II) structure. If the electrostatics are included in the calculated $_{red}$ and $_{ox}$ values, they become more similar in size (e.g. 14 and 16 kJ/mole for reduced plastocyanin with a fixed enzyme).

Second, with two exceptions, the total inner-sphere reorganisation energies are amazingly stable and quite low, 27-38 kJ/mole. This shows that the reorganisation energy is appreciably lower in the proteins than in vacuum (61-84 kJ/mole [25]). Moreover, they seem to be the same in the three proteins, whereas in vacuum, the trigonal structure (found in plastocyanin) has an appreciably lower reorganisation energy than the tetragonal (found in nitrite reductase) or the azurin structure.

The two structures that do not fit into this view are cucumber basic protein and azurin, both with a flexible protein. They have appreciably higher reorganisation energies, 57-114 kJ/mole. As can be seen in Table 2, the reason for these large values are that the reduced structures in these two complexes have a lowered coordination number, due to a partly dissociated histidine ligands (Cu-N distances of 258-265 pm). Apparently, the reduced complexes are rather insensitive to this change, whereas it strongly disfavours the oxidised structure, leading to a high reorganisation energy. Since the crystal structures of reduced azurin do not show any such dissociation, this is an artefact of the optimisation, or at least only a minor species. Consequently, the high estimate of $_{i}$ can safely be ignored. Most importantly, however, these results show that the histidine ligand must not dissociate during electron transfer, because this would lead to a very high reorganisation energy.

Thus, we see that the reorganisation energy of the blue copper sites are smaller in the proteins than in vacuum by at least 30 kJ/mole. Most of this decrease seems to come from electrostatic effects, especially hydrogen bonds. In fact, a model with two hydrogen bonds to S_{Cys} (by water molecules) has an reorganisation energy of only 33 kJ/mole, compared to 61 kJ/mole without them.

The effect of covalent interactions (strain) can be seen from the three calculations without the connection between the ligands and the protein. These have reorganisation energies that are lower (by 3-12 kJ/mole) than with the connection. Thus, the effect of covalent strain is small and it actually *increases* the reorganisation energy and therefore counteract the electrostatic effects of the protein. Results in the same direction have been obtained experimentally on the azurin His117Gly mutant, reconstituted by imidazole, which has a three times as high rate of electron transfer as the native protein [59].

For plastocyanin, the main contribution to the decrease in the reorganisation energy seems to be the decrease in the change in bond lengths of the three strong copper ligands (especially the Cu-S_{Cys} interaction) upon oxidation. All three distances decrease in the COMQUM calculations, but more for the reduced complex than for the oxidised one. Thus, the Cu-S_{Cys} bond decrease by 14 pm in vacuum, but only 4-6 pm in the COMQUM calculations. For the Cu-N bonds, the decrease in the protein is 1-6 pm compared to 10-11 pm in vacuum. In fact, the total inner-sphere reorganisation energy is closely related to sum of the squares of the changes in these three distances, as could be expected from a harmonic model [25].

For the other three proteins, this relation is less clear, indicating that changes in the angles are also involved. This is in accordance with our observation that the angles in the reduced nitrite reductase structure were distorted towards those of the oxidised structure. For azurin, the decrease in reorganisation energy seems to be the result of a general improvement of all geometric parameters, as can be seen in Figure 2.

We have also tried to calculate *total* reorganisation energies (i.e. both the inner-sphere and outer-sphere components) from the COMQUM data. This was done by considering all energy

terms (quantum chemical as well as classical, including the effect of the altered charge distribution of the quantum system on the protein) in the calculation. However, this led to a very large variation in the results and in several cases negative reorganisation energies. The reason for this is the local minima problem. We use a single minimised structure, for the calculation of the reorganisation energy. However, differences in the hydrogen-bond structure or the positions of the water molecules strongly affect the estimates, and it is impossible to ensure that we have found the global minimum. Moreover, the reorganisation energies should be free energies, so they should reflect the dynamics of the system, especially for the solvent molecules. Therefore, direct estimates of the outer-sphere reorganisation energy from the ComQum data are not reliable. Instead, they could be estimated by free energy perturbation techniques using molecular dynamics restrained to the ComQum structures, as has recently been suggested [60].

Finally, it should be noted that the low inner-sphere reorganisation energies in the protein (~30 kJ/mole) fit well with available experimental estimates of the reorganisation energy of blue copper proteins. By series of ingenious experiments, Gray and coworkers have estimated the total self-exchange reorganisation energy of azurin to around 80 kJ/mole [61]. The outer-sphere reorganisation energy has not been determined for this protein, but estimates of this contribution in similar proteins have given results in the range 33-54 kJ/mole [62,63]. Thus, the reorganisation energies obtained with ComQum seem to be reasonable estimates. However, accurate calculations of both components of the reorganisation energy in an experimentally characterised donor-acceptor complex would give a more detailed and accurate comparison. Work in this direction has been initiated at our department.

Concluding remarks

The present calculations show that combined quantum chemical and molecular mechanical geometry optimisations provide a proper method to estimate the structure of an active site inside a protein. With such methods, we include electrostatic and dielectric effects as well as

covalent interactions present in the protein. In this respect, they are more general than methods which only constrain certain geometric parameters or positions of atoms to the values encountered in proteins [64].

We have seen that the ComQum calculations improve the geometries of the copper sites compared to vacuum calculations. The effect is largest for the dihedral angles of the ligands (c.f. Figure 1), but the bond lengths and angles around the copper ion are also significantly improved (c.f. Table 2). It should not be forgotten, however, that already the vacuum structures catch most of the geometric features of the copper sites (c.f. Figure 2), showing that they are not more strained than any other metal site in a protein.

In two aspects, the ComQum calculations fail to reproduce crystallographic data. The first is the tendency of one of the histidine ligands of Cu(I) to dissociate. The second is the long Cu(I)-S_{Met} distances in some of the calculations. As discussed above, this is an effect of the weak interaction of the Cu(I) ion with its ligands and that the geometry is determined by a fine-tuned competition between first- and second-sphere interactions. It is not too unexpected that ComQum cannot properly describe all such interactions involving energies of a only few kJ/mole. However, it is interesting to note the large variation in the Cu-S_{Met} bond allowed by plastocyanin: 286-375 pm with a flexible protein, and 245-339 pm with the protein fixed. This indicates that the protein in fact may be designed to allow an appreciable variation and dynamics in this bond.

A vacuum calculation can never reproduce all dihedral angles of a protein metal site. In vacuum, they are determined by the weak interactions (mostly CH···S hydrogen bonds) that can arise within the small model system. In the protein, these dihedrals are determined by the fold of the protein (the direction of the protein backbone and steric interactions with other groups in the protein). Therefore, it is necessary to include the protein in the optimisations to get the dihedrals right. As can be seen in Figure 1, the change in geometry is appreciable.

In energy terms, the effect of changing the dihedrals is limited. However, there are many such changes, and therefore they can sum up to significant energies. Moreover, it should

remembered that the copper complex involve three strong hydrogen-bond partners (S_{Cys} and the N-H atoms of the two imidazole rings). These atoms form strong hydrogen bonds with polar groups in the protein. For example, in plastocyanin, S_{Cys} forms a hydrogen bond to O in Asn-38, whereas H ^{2} of His-37 and 87 forms hydrogen bonds to O of Ala-33 and a crystal water molecule, respectively. Each such hydrogen bond involve energies of 15-30 kJ/mole, and therefore can distort the structure significantly. With this in mind, it is not too surprising that the strain energies obtained from the COMQUM calculations are still rather large, 33-66 kJ/mole if we compare to the vacuum state, and 21-29 kJ/mole, if only covalent interactions (strain in the normal meaning [58]) are considered. Most importantly, however, the strain energies are similar in size to what was observed for the catalytic and structural zinc ions in alcohol dehydrogenase and for iron-sulphur proteins [33,54-57]. Thus, there is no evidence that the blue copper proteins should be more strained than any other metalloprotein.

Finally, the COMQUM calculations allows us to calculate inner-sphere reorganisation energy in the proteins. The results show that the blue copper proteins reduce the reorganisation energies by 30-50 kJ/mole compared to vacuum. Again, this is mainly an effect of the dielectric milieu around the copper ion (especially the hydrogen bonds to S_{Cys}) which reduce the difference in the Cu-S_{cys} and Cu-N bond lengths during reduction. A similar effect was seen in iron-sulphur proteins [57]. Clearly, this reduced reorganisation energies is most important for the reaction rate, and therefore the function, of the blue copper proteins.

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Figure legends

Figure 1. A comparison between the crystal structure of oxidised plastocyanin (light grey) [46] and the structures of $Cu(Im)_2(SCH_3)(S(CH_3)_2)$ optimised in vacuum (dark grey) [15] or with COMQUM in the oxidised plastocyanin structure (with a flexible protein and connections between the protein and the metal ligands).

Figure 2. A comparison of the change in geometry upon reduction of azurin calculated (A) in vacuum (Cu-O and Cu-S_{Met} distances fixed to the crystal values, c.f. Table 2) and (B) with COMQUM (fixed enzyme). The oxidised structures are shaded.

Table 1. The number of amino acids, water molecules, and atoms in the simulated proteins.

Protein	PDB file	Amino acids	Crystal water	Cap water	Atoms	System 2
Oxidised plastocyanin	1plc	100	109	1162	5256	3058
Reduced plastocyanin	5pcy	100	44	1227	5256	3058
Cucumber basic protein	2cbp	97	121	1153	5263	2860
Nitrite reductase	1nic	263	98	1434	8531	4270
Azurin	4azu	283	138	1411	8823	4460

Table 2. The geometry and E_{str} of the copper sites in plastocyanin, cucumber basic protein, nitrite reductase, and azurin optimised by COMQUM.

System[a]				E_{str}	Distance to Cu (pm)			Angle around Cu (°)				
Cu	Protein	Fix	Con	kJ/mole	S_{Cys}	N	S_{Met}[b]	N-N	S_{Cys}-N	S-S	S_{Met}-N	(°)
I	Vacuum				232	214-215	237	109	105-108	115	107-113	89
	Pc red	Yes	Yes	50.6	219	204-207	339	104	122-129	105	85-102	79
		No	Yes	44.7	221	203-208	339	103	120-134	104	78-101	76
		No	No	21.3	222	203-212	375	103	120-136	105	72-103	71
	Pc ox	Yes	Yes	58.2	222	208-214	245	91	113	119	99-117	83
		No	Yes	46.8	220	205-208	310	101	124-126	107	87-103	81
	Crystal				211-217	203-239	287-291	91-118	110-141	99-114	83-110	74-80
II	Vacuum				218	204	267	103	120-122	116	94-95	90
	Pc red	Yes	Yes	36.1	215	199-200	303	105	121-127	102	85-108	76
		No	Yes	34.7	217	199	310	106	122-127	100	84-108	75
	Pc ox	Yes	Yes	36.0	213	197-198	286	101	124	105	88-109	78
		No	Yes	32.6	214	197-198	290	103	123-125	105	86-106	78
	Crystal				207-221	189-222	278-291	96-104	112-144	102-110	85-108	77-89
I	Nir	Yes	Yes	58.6	222	207-216	253	94	107-125	108	90-134	65
		No	Yes	53.8	224	205-217	272	98	110-134	105	87-123	66
II[c]	Vacuum				223	205-206	242	100	97-141	103	95-126	62
	Nir	Yes	Yes	40.7	217	201-203	263	98	106-132	103	88-133	61
		No	Yes	40.6	219	200-203	262	100	104-135	105	87-129	61
			No	19.7	224	203-205	241	97	91-146	105	89-141	48
	Crystal				208-223	193-222	246-270	96-102	98-140	103-109	84-138	56-65
I	CBP	Yes	Yes	51	223	207-211	250	96	111-126	115	87-119	71
		No	Yes	66.4	223	202-258	244	92	117	114	86-122	72
		No	Yes	54.7	213	190-421	380					
II[d]	CBP	Yes	Yes	33.8	217	199-202	261	100	110-134	109	86-118	68
		No	Yes	44.9	217	199-210	273	100	118-128	104	84-118	69
			No	15.5	222	201-203	245	100	101-140	112	88-116	68
	Crystal				216	193-195	261	99	110-138	111	83-112	70
I	Vacuum				223	207-213	321,325	103	117-140	98	87-90	89
	Azurin	Yes	Yes	60.6	219	203-208	322,317	102	123-131	115	80-90	84
		No	Yes	47.2	215	194-265	294,333	95	98-166	95	74-86	75
	Crystal				222-231	205-217	321-325 319-325	100-108	119-132	109	77-94	
	EXAFS				219	191-201	335,298	106	120-133	105	74-89	
II	Vacuum				217	202	312,316	103	124-132	107	82-91	84
	Azurin	Yes	Yes	39.2	213	198-202	327,293	104	123-132	111	76-91	81
		No	Yes	33.8	214	199-204	368,282	104	121-135	111	69-91	78
	Crystal				212-227	197-212	287-326 275-316	98-108	116-138	105-111	69-98	75-87
	EXAFS				212	186-194	339,282	106	121-133	105	74-88	

[a] The system is defined by the oxidation state of the copper ion (Cu), the protein (Pc red = reduced plastocyanin, Pc ox = oxidised plastocyanin, CBP = cucumber basic protein, Nir = nitrite reductase, Vacuum = quantum chemical optimisation in vacuum [15,24], Crystal = range observed in the available crystal structures in the Brookhaven protein data bank), EXAFS = combined crystal exafs structures [53], whether the protein has been

kept fixed or not during the ComQum optimisation (Fix), and whether there was any connection between the metal ligands and the protein backbone or not (Con).

[b] For azurin, the second figure is the Cu-O bond length.

[c] This is the tetragonal structure, which in vacuum is obtained with the $Cu(Im)_2(SH)(S(CH_3)_2)^+$ model.

[d] This is a structure intermediate between trigonal and tetragonal that cannot be found in vacuum.

Table 3. The reorganisation energies of several types of blue copper proteins calculated with ComQum and in vacuum.

System[a]			Reorganisation energy (kJ/mole)		
Protein	Fix	Con	λ_{red}	λ_{ox}	λ_{i}
Vacuum trigonal [25]			32.7	28.8	61.5
Reduced Plastocyanin	Yes	Yes	23.4	3.7	27.1
	No	Yes	25.7	0.4	29.7
	No	No		-2.5[b]	
Oxidised Plastocyanin	Yes	Yes	18.9	19	37.9
	No	Yes	26	5.1	31.1
Vacuum tetragonal [25]			37.7	39.8	77.5
Nitrite reductase	Yes	Yes	21.9	9.2	31.1
	No	Yes	28.9	5.2	34.1
	No	No	23.3[b]		
Cucumber basic protein	Yes	Yes	18.4	8.2	26.6
	No	Yes	15.4	41.5	57
	No	No	3.4[b]		
Vacuum azurin			40.7	43.1	83.8
Azurin	Yes	Yes	15	13.1	28.1
	No	Yes	23.1	91.3	114.4

[a] The system is defined by the protein, whether system 2 has been kept fixed or not during the ComQum optimisation (Fix), and whether there was any connection between the metal ligands and the protein backbone or not (Con).

[b] Calculated relative to the average value of the other sites