The cupric geometry of blue copper proteins is not strained

Ryde, Ulf; Olsson, Mats H M; Pierloot, Kristine; Roos, Björn O.

Published in:
Journal of Molecular Biology

DOI:
10.1006/jmbi.1996.0484

1996

Document Version:
Peer reviewed version (aka post-print)

The cupric geometry of blue copper proteins is not strained

Ulf Ryde[1*], Mats H. M. Olsson[1], Kristine Pierloot[2], and Björn O. Roos[1]

[1] Department of Theoretical Chemistry, Lund University, Chemical Centre, P.O.B 124, S-221 00 Lund, Sweden

[2] Department of Chemistry, University of Leuven, Celestijnenlaan 200F, B-3001 Heverlee, Leuven, Belgium

[*] Author to whom correspondence should be addressed. Tel: 46-46-2224502, Fax: 46-46-2224543, E-mail: teoulf@garm.teokem.lu.se

Subject area: Proteins, nucleic acids, and other biologically important macromolecules

Keywords: B3LYP method; blue copper protein; entatic state theory; induced-rack theory; protein strain; quantum chemical geometry optimisation

Summary

The geometry of several realistic models of the metal coordination sphere in the blue copper proteins has been optimised using high level quantum chemical methods. The results show that the optimal vacuum structure of the Cu(II) models is virtually identical to the crystal structure of oxidised blue copper proteins. For the reduced forms, the optimised structure seems to be more tetrahedral than the one found in the proteins, but the energy difference between the two geometries is less than 5 kJ/mole, i.e. within the error limits of the method. Thus, the results raise strong doubt against hypotheses (entatic state and the induced-rack theory) suggesting that blue copper proteins force the oxidised metal coordination sphere into a structure similar to that preferred by Cu(I) in order to minimise the reorganisation energy of the electron transfer reaction. Instead, a small reorganisation energy seems to be reached by an appropriate choice of metal ligands. In particular, the cysteine thiolate ligand appears to be crucial, changing the preferred geometry of the oxidised complexes from square-planar to a more trigonal geometry.

1. Introduction

Blue copper proteins (type 1) have attracted chemists' interest during the last 40 years by their intense blue colour, distinctive electron paramagnetic resonance spectra, and unusually high reduction potentials (Sykes, 1990, Adman, 1991). These unusual macroscopic characteristics are accompanied by an unprecedented cupric geometry: a copper ion bound in a distorted trigonal plane formed by a cysteine thiolate group at an unusually short distance (207-220 pm) and two histidine nitrogen atoms at normal distances (190-210 pm). In addition, a methionine sulphur atom, and in some proteins also a backbone amide oxygen, binds in an axial position at a large distance (260-330 pm; Colman et al., 1978, Guss & Freeman, 1983, Norris, 1986, Adman 1991, Guss et al., 1992).

In essence, such a geometry is intermediate between the one preferred by Cu(I) (tetrahedral) and Cu(II) (tetragonal; Cotton & Wilkinson, 1988). Consequently, the geometry changes only slightly when the copper ion is reduced at normal pH: the copper-ligand distances increase by 0-15 pm, while the angles hardly change at all, see **Fig. 1 and Table 1** (Guss & Freeman, 1983, Shepard et al., 1990). From an evolutionary viewpoint, this is most appropriate because the blue copper proteins are electron transport proteins; a small change in geometry gives a small reorganisation energy, and thus a high rate of electron transfer (Marcus & Sutin, 1985).

These extraordinary properties, alien from inorganic copper chemistry, early led to the proposal that the blue copper proteins force the Cu(II) ion into a geometry more similar to the one preferred by Cu(I) (Williams, 1963, Malmström, 1965). These ideas were later extended into general hypotheses for metal proteins: the entatic state theory (Vallee & Williams, 1968, Fraústo da Silvia & Williams, 1991, Williams, 1995) and the induced-rack theory (Gray & Malmström, 1984, Malmström, 1994), suggesting that proteins may provide pre-formed sites that strain the metal into a catalytically poised state.

The fact that the copper-free forms of the blue copper proteins are very similar to the metal-loaded forms (Nar et al., 1992b, Shepard et al., 1993, Garrett, et al., 1984), indicating that the chelating site is present before the metal is bound, have been taken as a strong evidence for the hypotheses. Moreover, the idea of a rigid coordination sphere is supported by the similarity between the structure of Hg-substituted and native plastocyanin (Church et al. 1986).

Yet, several recent crystal structures show that blue copper proteins may accept quite extensive changes in the geometry around the metal ion. For example, Zn^{2+} and Ni^{2+}-substituted azurin have longer Cu-S bonds (Cys: 228-248 pm and Met: 334-341 pm) and a much shorter Cu-O bond (234-235 pm) than the native protein (Nar et al., 1992a, Sjölin et al., 1993, Tsai, et al., 1994), and one form of copper-free azurin has a water molecule in the metal site leading to appreciable changes (up to 220 pm) in the local structure (Nar et al., 1992b). Furthermore, if the coordinating methionine residue in azurin is mutated to glutamine, qualitatively different coordination geometries are observed (Romero et al., 1993): in the oxidised form the copper ion is four-coordinate with the O^{ε} atom of glutamine at 227 pm distance, while in the reduced form the copper ion becomes almost two-coordinated with the cysteine sulphur and a histidine nitrogen at 193-211 pm distance and the O^{ε} atom and the other histidine nitrogen at about 270 pm distance.

Theoretical evidence also disfavours the hypotheses. Solomon and co-workers have estimated the forces on the metal ligands resulting from the oxidation of a model of reduced plastocyanin and shown that these forces are consistent with the structure in the oxidised protein (Guckert et al., 1995). Moreover, Levitt (1974), Fersht (1985), and Warshel (1991) have argued strongly against strain as an important factor in protein function.

Quantum chemistry provides a suitable means to study the influence of a protein on the geometry of a metal coordination sphere. By optimising a model of the metal complex quantum mechanically in vacuum, the actual coordination preferences of the metal ion with the same ligands as in the protein can be established, and by comparing this optimal structure with the geometry in the protein, the strain induced by the protein onto the metal coordination sphere can

be quantified and partitioned into contributions from covalent bonds, electrostatics, etc. We here present an extensive series of accurate geometry optimisations of models of the copper site in blue copper proteins. The results provide clear evidence that the preferred structure of the models is closely similar to the metal geometry in the proteins, and thus call for a revaluation of the strain hypotheses.

2. Methods

The copper coordination sphere in the blue proteins was modelled by $[CuX_2YZ]^{(0-1)+}$, where X = NH_3 or imidazole, Y = SH^- or CH_3S^-, and Z = H_2S or $(CH_3)_2S$. The full geometry of the models was optimised until the maximum and the root-mean-squared gradients were below 223 and 149 kJ/mole/pm, and the maximum and root-mean-squared changes in geometry were below 0.16 pm and 0.11 pm. Several starting structures were tested to reduce the risk of being trapped in local minima and all chemical feasible conformations of the complexes were considered.

The geometry optimisations were performed by the hybrid density functional method B3LYP† (Barone, 1994), which has been shown to give as good or better geometries and energies as correlated ab initio methods for first row transition metal complexes (Ricca & Bauschlicher, 1994, 1995, Holthausen et al., 1995). Three other density functional methods, as well as the ab initio Hartree-Fock method, were also tested, namely the local density approximation (LDA), the Becke gradient-corrected method (B-VWN), and the so called BLYP method (Barone, 1994). They all gave inferior and sometimes even qualitatively different geometries. Møller-Plesset second order perturbation theory (MP2) gave similar geometries (within 4 pm and 4°) but to an appreciably larger cost.

After an extensive calibration procedure, we decided to use the standard fine grid in the Mulliken software (Rice, et al., 1995), combined with the double-ζ copper basis (62111111/33111/311) of Schäfer et al. (1992), enhanced with diffuse *p*, *d*, and *f* functions with exponents 0.174, 0.132, and 0.39 (called DZpdf), and the 6-31G* basis sets for the other atoms (Hehre et al., 1986). Only the pure 5 *d* and 7 *f* functions were used. Cu(II) systems were treated within the unrestricted formalism. All geometry optimisations were performed with the Mulliken quantum chemistry software, version 2.25b (Rice, et al., 1995).

For accurate energy calculations, ab initio second order multi-configurational perturbation theory on a complete active space self-consistent field wave function was performed (CASPT2, Andersson et al., 1992). The active space consisted of five Cu 3*d* orbitals, five correlating Cu 4*d* orbitals, and for Cu(II) an additional S 3*p* orbital (10 or 11 active electrons, respectively). The core orbitals on all atoms were kept frozen (including 3*s* and 3*p* on copper) and the small atomic natural orbital (ANO) basis sets by Pierloot et al. (1995) were used, contracted in the following way: (7*s*3*p*)/[2*s*1*p*] for H, (10*s*6*p*3*d*)/[3*s*2*p*1*d*] for C and N, (13*s*10*p*4*d*)/[4*s*3*p*2*d*] for S, and (17*s*12*p*9*d*4*f*)/[6*s*4*p*3*d*2*f*] for Cu. These calculations were performed with the quantum chemistry software MOLCAS 3.0 (Andersson et al., 1994). All calculations were run on IBM RISC RS/6000 workstations.

3. Results and Discussion

(a) The choice of ligand models

The typical copper coordination sphere in blue copper proteins consists of two histidine, one cysteine and a more distant methionine ligand (the back-bone amide ligand present in some blue copper proteins was not considered in this study). There are several ways to model these ligands, and eight combinations were tested: histidine was modelled either as ammonia or imidazole, cysteine as SH^- and SCH_3^-, and methionine as SH_2 and $S(CH_3)_2$.

As can be seen in **Table 2**, if ammonia is replaced by the more realistic imidazole ligand, the Cu-N distances decrease 5-9 pm, while the Cu-S_{Met} distance (S_{Met} is the sulphur atom of the methionine model) increases 4-17 pm. For Cu(II) the Cu-S_{Cys} bond length (S_{Cys} is the sulphur ion of the cysteine model) and the angles around the copper ion hardly change at all, while for Cu(I) the Cu-S distance and the angles change 1-3 pm and 3-25°. When SH^- is replaced by SCH_3^-, only small differences are observed (less than 3 pm and 5°), except for the Cu-S_{Met} distance and the S_{Met} -Cu-S_{Cys} angle that may change by up to 6 pm and 11° (**Table 3**). Somewhat unexpectedly, if SH_2 is replaced by $S(CH_3)_2$, larger shifts are observed. As can be seen in **Table 4**, for Cu(II) the Cu-S_{Met} distance decreases about 30 pm, while the Cu-N and Cu-S_{Cys} distances increase slightly (1-3 pm). As a result, the angles involving S_{Met} increases (3-8°) and the other angles decrease (1-4°). For Cu(I) the differences in the distances are smaller, but those in the angles are larger.

In conclusion, $S(CH_3)_2$ is necessary to get a reliable result, and ideally Cu(imidazole)$_2$(CH_3S)($S(CH_3)_2$) should be used to model the blue copper sites. It should be noted also, that all the small ligand models (NH_3, SH^-, and SH_2) contain polar hydrogens that may give rise to strong internal hydrogen bonds that distort the structure. This is particularly important in the Cu(I) complexes with their weaker Cu-ligand interaction. Therefore, all results reported refer to the Cu(imidazole)$_2$(CH_3S)($S(CH_3)_2$) model, except for CASPT2 energy calculations, where the smaller Cu(NH_3)$_2$(SH)($S(CH_3)_2$) model was used.

In most copper models, a plane of symmetry is possible. It turns out, however, that if the imidazole rings are tilted with respect to each other, thereby destroying the symmetry, a slightly more stable conformation is obtained (5-10 kJ/mole), which also is more similar to the one found in the proteins. Therefore, if not otherwise stated, all structures with imidazole were optimised without symmetry.

(b) The optimal geometry of Cu(II) complexes

In **Fig. 2** the optimised geometry of [Cu(imidazole)$_2$(SCH_3)($S(CH_3)_2$)]$^+$ is shown and compared to the structure of oxidised plastocyanin. The overall structure is strikingly similar to the crystal structure of the blue copper proteins: the copper ion is bound in a distorted trigonal geometry with three ligands at short distances (imidazole: 204 pm, cysteine 218 pm) and with methionine as a weaker axial ligand at 267 pm distance. The copper ion is 46 pm out of the N_2S_C plane.

This result is rather unexpected because most small inorganic Cu(II) complexes assume a tetragonal geometry (square-planar, square-pyramidal, or distorted octahedral). For comparison, we therefore optimised a square-planar structure of [Cu(NH_3)$_2$(SH)($S(CH_3)_2$)]$^+$. It turned out to be 13.3 kJ/mole less stable than the corresponding trigonal structure. It also had rather different copper bond distances: 4 pm shorter Cu-N bonds, a 9 pm shorter Cu-S_{Met} bond, and a 16 pm longer Cu-S_{Cys} bond. Thus, the preferred geometry of a Cu^{2+} ion with ligands mimicking those in the blue copper proteins is *not* square-planar as is usually assumed, but instead a geometry closely similar to the one actually found in the proteins.

In **Table 5**, the copper-ligand bond lengths and angles of the structure in Fig. 2 are compared with the experimental structures of blue copper proteins. The bond lengths and angles of the optimised structure reproduce the crystal structures excellently; all parameters are well within the range encountered in different blue copper protein crystals and they differ only slightly from the experimental averages (less than 15 pm and 5°).

A characteristic feature of the crystal structures of the blue copper proteins is that the angles involving the two histidine ligands are distinctly different. Thus, the two S_{Cys}-Cu-N angles are 111° and 130° and the two S_{Met}-Cu-N angles are 91° and 105° (average for the 18 structures in Table 1). As can be seen in Table 5, the optimised model does not give such a large difference for these angles (2° and 1°, respectively). Taking this divergence into account, the maximum difference in angles between the optimised and the experimental structures increases to 10°, but the angles of the optimised model are still well within the ranges found experimentally. The physiological significance of these differences in the angles is not clear, but in energy terms, the effect is small, less than 5 kJ/mole.

Two other small but possibly significant differences between the optimised and experimental structures can be seen in Table 5: the Cu-S_{Cys} bond is slightly too long (6 pm), and the Cu-S_{Met} bond is too short (15 pm). This is at least partly due to deficiency in the theoretical method. If each distance is optimised with the CASPT2 method, keeping the rest of the geometry fixed, the Cu-S_{Cys} distance decreases 7.1 pm and the Cu-S_{Met} distance increases 6.5 pm.

Furthermore, the Cu-S_{Met} potential surface is unusually flat; the quadratic force constant is only 0.0060 kJ/mole/pm^2 which is 4-11 times weaker than normal metal ligand bonds (Ryde, 1995). **Fig. 3** shows the B3LYP and CASPT2 energy of $[Cu(NH_3)_2(SH)(S(CH_3)_2)]^+$ as a function of the Cu-S_{Met} distance (optimised at each point with the B3LYP method). The curves are very similar, but the CASPT2 curve is shifted 7 pm to larger bond lengths and it is also a bit steeper. It can be seen that the 8 pm difference between the CASPT2 corrected optimised Cu-S_{Met} bond length and the experimental one corresponds to less than 0.5 kJ/mole in energy. Moreover, the Cu-S_{Met} bond length may change 50 pm around the minimum at a cost of less than 5 kJ/mole. Consequently, the Cu-S_{Met} bond length should be extremely sensitive to the electrostatic properties of the surrounding protein matrix (and also to the theoretical treatment). This explains why the length of the Cu-S_{Met} bond in blue copper proteins from diverse sources varies so widely, ranging from 262 pm in cucumber basic protein to 310 pm in parsley plastocyanin (Sykes, 1990, Adman, 1991).

Naturally, the blue copper models may assume a large number of different conformations. In fact, the structure in Fig. 2 does not show the global minimum, but the conformation that is most similar to the one found in the crystal (called the *crystal* conformation below). If the C_{Cys}-S_{Cys}-Cu-S_{Met} dihedral angle of this structure is changed from 0° to 180°, i.e. if the side chain of the cysteine residue is rotated so that it points away from the methionine sulphur atom, a less crowded conformation is obtained that, in vacuum, is 5.9 kJ/mole more stable than the one in Fig. 2. It is stabilised by weak hydrogen bonds from the methionine CH_3 groups to the cysteine sulphur ion. In the crystal conformation, however, the sulphur ion is exposed to the surroundings and in the protein better hydrogen bond donators are available (e.g. the back-bone amid H of Asn-38 in plastocyanin). Such hydrogen bonds in the protein certainly more than compensate for the increased crowding in the crystal conformation (a typical NH-S^- bond gives about 30 kJ/mole). Moreover, as can be seen in Table 5, the metal-ligand bond lengths and angles of the two conformations do not differ very much, except for the 17° larger S_{Cys}-Cu-S_{Met} angle in the crystal conformation.

There are some further structures with other values of the C_{Cys}-S_{Cys}-Cu-S_{Met} and C_M-S_{Met}-Cu-S_{Cys} dihedral angles. They are all quasi three-coordinate with the methionine ligand 330-350 pm from the copper ion and are less stable than the conformation in Fig. 2. Therefore, they are probably of less interest in the present context.

In conclusion, the estimated structure of $[Cu(imidazole)_2(SCH_3)(S(CH_3)_2)]^+$, corrected for the errors in the B3LYP method, is virtually identical to the average experimental structure. The bond lengths differ by less than 8 pm and the angles by less than 10°. Thus, the blue copper proteins do not strain the oxidised metal geometry significantly.

(c) The optimal geometry of Cu(I) complexes

Fig. 4 shows the optimal structure of $Cu(imidazole)_2(SCH_3)(S(CH_3)_2)$. As can be seen in the figure and from the copper ligand bond distances and angles in **Table 6**, it is tetrahedral (bond angles 105-124°), with nearly equal Cu-S_{Cys} and Cu-S_{Met} bond lengths (232 and 237 pm) and with the Cu ion 81 pm out of the N_2S_C plane. Although all geometric parameters except the Cu-S_{Met} bond length are within the range encountered in different protein crystals, the structure clearly differs from the experimental one.

Since the major difference between the optimised and the experimental structure is the Cu-S_{Met} bond length, we next optimised $Cu(imidazole)_2(SCH_3)(S(CH_3)_2)$ with the Cu-S_{Met} bond constrained to the average experimental value, 290 pm. This resulted in a structure that is much more similar to the one found in the proteins, see **Fig. 5**. Although the general geometric features of this structure closely resemble the experimental ones, details of the fit are less accurate than for the oxidised complexes (e.g. the tilt of the imidazole rings and the dihedral angles of the side chains of the cysteine and methionine models). This can be attributed to the weak CH-S hydrogen bonds from the methionine CH_3 group and both imidazole rings to S_{Cys} (290-347 pm) and from the side chain of the cysteine model to S_{Met} (335 pm). Apparently, the lower charge on the copper ion makes the copper-ligand interaction weaker, so that the structure is to a large extent determined by CH-S interactions. This also gives rise to a very flat potential surface of the molecule. In fact, despite the extensive geometry differences between the structures in Figs. 4 and 5, the energy difference is only 4.3 kJ/mole, i.e. within the error limits of the method. This means that the geometry can be strongly affected by the surrounding protein, where more and better hydrogen donators are available.

As can be seen in Table 6, the copper bond lengths and angles of the structure in Fig. 5 are rather similar to those in crystals of reduced blue copper proteins. All geometric parameters are within the experimental range, and most are near the experimental averages. As for the oxidised systems, the Cu-S_{Cys} bond length seems to be slightly too long (9 pm), and as an effect the Cu-N bonds are too short. This can again be partly attributed to the B3LYP method; the optimal Cu-S_{Cys} bond length estimated with the CASPT2 method is 3.4 pm shorter. The distance between the Cu ion and the N_2S_C plane is 36 pm.

Fig. 6 shows the B3LYP and CASPT2 energy of $Cu(NH_3)_2(SH)(S(CH_3)_2)$ as a function of the Cu-S_{Met} bond length (optimised with B3LYP in each step), i.e. as the geometry change from tetrahedral to trigonal. The curves are rather similar, but the CASPT2 curve is steeper and displaced 5 pm to shorter distances. Again, the Cu-S_{Met} bond is rather soft, although twice as stiff as the Cu^{2+}-S_{Met} bond (the harmonic force constant is about 0.013 kJ/mole/pm^2). The experimental range of the Cu-S_{Met} bond, 270-315 pm, corresponds to less than 10 kJ/mole in energy.

In analogy with the oxidised complexes, there exists several other conformations of the reduced model system. Again, the conformations in Fig. 5 and 6 are not most stable, but instead a conformation with the dihedral angle C_{Cys} -S_{Cys} -Cu-S_{Met} =180° (the side chain of the cysteine residue points away from the methionine sulphur atom). The energy difference between the two conformations is of the same magnitude as for the oxidised complexes, 5.1 kJ/mole, which easily can be reversed by hydrogen bonds in the protein. Moreover, as the curves in Fig. 6 indicate, there also exists a three-coordinate structure. It has the thioether ligand in the second coordination sphere of the copper ion at a very large distance, 665 pm. This is because the

thioether molecule makes only week CH hydrogen bonds to the copper complex; in practice it is dissociated. The three-coordinate structure is in fact 1.5 kJ/mole more stable than the four-coordinate structures, but hydrogen bonds in the protein probably stabilise the four-coordinate structures.

In conclusion, the potential surface of the reduced models is very flat. With the B3LYP method, the optimal structure seems to be more tetrahedral than in the crystals. However, the geometry may be changed to reproduce the crystal geometry by increasing the Cu-S_{Met} bond length 40 pm, to a cost in energy that is within the error limits of the method.

(d) Concluding remarks

The present results lend no support to the suggestion that the blue copper proteins force the metal coordination sphere into an unnatural (Cu(I)-like) geometry as frequently has been assumed before (Vallee & Williams, 1968, Fraústo da Silvia & Williams, 1991, Williams, 1995, Gray & Malmström, 1984, Malmström, 1994). On the contrary, they show that the optimal vacuum structure of a realistic model of the active site of the oxidised blue copper proteins (i.e. the structure preferred by the metal and its ligands) closely resembles the crystal structure of the proteins.

This conclusion is in accord with available experimental data. Recent progress in crystallography and site directed mutagenesis of blue copper proteins have shown an increasing number of variations of the metal coordination geometry. The structures of the Zn^{2+}-substituted azurin and the M121Q mutant of azurin are particularly informative in this aspect, showing qualitatively different coordination geometries compared to the native protein (Nar et al., 1992a, Sjölin et al., 1993, Romero et al., 1993, Tsai et al., 1994).

The fact that the metal geometry in Hg^{2+}-substituted plastocyanin is similar to the native structure has been taken as an evidence for protein strain (Church et al. 1986). However, if this structure is compared with those of Ni^{2+}-, Zn^{2+}- and Cd^{2+}-substituted azurin, it becomes clear that it is the chemical properties of the metal ion and the ligands that determine the local geometry and not the protein. The latter three proteins have a much shorter bond to the back-bone amide oxygen than the native protein (235, 232, and 276 pm) and a slightly longer bond to the methionine sulphur (333, 344, and 323 pm; Nar et al., 1992a, Shepard et al. 1993, Sjölin et al., 1993, Blackwell et al., 1994, Tsai, et al., 1995). Furthermore, these bond lengths clearly reflect the difference in softness of the three metals. In this perspective, the similarity between the Hg^{2+}-substituted and native plastocyanin seems to be only fortuitous.

Similarly, the observation that the copper-free form of the proteins resembles the metal loaded forms must not necessarily be taken as an evidence for a strained metal geometry. Instead, this may be a means to facilitate metal bonding. If the metal chelating site was not present before the metal is bond, logically metal binding would be harder. Moreover, the copper-free structure is stabilised by several favourable hydrogen bonds (Nar et al., 1992b, Shepard et al., 1993, Garrett, et al., 1984).

The unusual macroscopic characteristics of the oxidised blue copper proteins were the initial inspiration for the strain hypotheses. Yet, recent progress in bio-inorganic chemistry, has led to the synthesis of small inorganic models that reproduce most of the properties of the blue copper proteins (Bouwman et al., 1990, Kitajima, 1992, Kitajima et al., 1992). It now seems likely that the peculiarities of the blue proteins mainly are an effect of the copper-thiolate interaction, which has been hard to study in small complexes due to the oxidising property of Cu(II).

Our results show that the protein does not change the oxidised complexes into a Cu(I)-like structure. On the contrary, it seems that *reduced* complexes may be slightly distorted from the ideal quasi-tetrahedral structure into a more Cu(II)-like one. Yet, the potential surface is very flat and this change in geometry costs less than 5 kJ/mole in energy. This energy difference is so

small that it cannot be ruled out with the present methods that the optimal geometry of the reduced complexes in fact is the trigonal crystal conformation.

In conclusion, the results lead us to propose that the blue copper proteins have constructed a metal coordinating site that minimises the electron transfer reorganisation energy, not by straining the geometry, but by an appropriate choice of metal ligands, viz. ligands that are a compromise between those favoured by the Cu(II) and Cu(I) ions. In particular, the cysteine thiolate ligand seems to be crucial, changing the coordination preference of the Cu(II) ion from tetragonal to trigonal. Moreover, the methionine thioether ligand gives a very soft bond that easily may be manipulated by the electrostatic potential and the local dielectric environment around the metal and that can change much (e.g. during electron transfer) at a little expense of energy.

Naturally, the protein may have other functions in addition to providing appropriate ligands to the copper ion. For example, the protein forms a protected environment that prohibits the contact between two copper-thiolate units (thereby inhibiting the homeolysis to Cu(I) and a disulphide) and keeps potential ligands in the solution away from the copper ion. The latter function is probably important for the stabilisation of the trigonal structure, since water molecules can act as a strong equatorial ligand as well as weak axial ligand, in both cases stabilising a tetragonal geometry. This may explanation why many inorganic thiolate complexes do not show all blue-copper properties. To some extent the protected protein environment can be substituted by strained ligands, where the strain favours a trigonal structure relative to a tetragonal one; therefore, the most successful model systems involve sterically hindered ligands (Kitajima, et al., 1992). A protein matrix is more effective in this respect, however, which is illustrated by the number of excellent blue copper models that have been constructed in metal substituted and engineered proteins, e.g. insulin, alcohol dehydrogenase, and Cu_2Zn_2-superoxide dismutase (Kitajima, 1992).

It is also clear that the protein influences the geometry and other chemical properties of the copper complex. For example, the protein stabilises the crystal conformation of the S_{Met}-Cu-S_{Cys}-C_{Cys} dihedral angle in relation to the optimal conformation by a hydrogen bond to S_{Cys}. Probably, the protein also tunes the reduction potential, manipulates the soft Cu-S_{Met} bond, and perhaps changes the optimal Cu(I) structure from tetrahedral to the one encountered in the crystals. This is not a special property of the blue copper proteins, however, but instead an inevitable effect of any protein, presenting an ordered array of charges and dipoles and a dielectric milieu widely different from water solution.

It should be noted that all calculations have been performed in vacuum. This means that we compare the crystal structure of the blue copper proteins with the optimal structure in vacuum and define the latter as the unstrained state. There are other possible choices of reference states, most importantly a complex with the same ligands in water solution. However, such a state is less well-defined, since the number of ligands may change in water: For example, should water be allowed to become an axial ligand, or even an equatorial ligand? May a protein ligand be replaced by water? Should other strong ligands present in the cell be considered? Moreover, a change in the number of ligands around the copper ion is a process clearly different from strain (in the common mechanical sense). It is therefore important to separate these two mechanisms to change the structure of copper complexes, and to find out which is important in the blue copper proteins. Our results clearly show that strain in the restricted mechanical meaning of covalent interactions (Warshel, 1991) plays a minor role for the copper coordination geometry .

Acknowledgements

This investigation has been supported by grants from the Swedish Science Research Council (NFR), the Belgian National Science Foundation (NFWO) and the Belgian Government (DPWB).

References

Adman, E. T. (1991). Copper protein structures. Adv. Prot. Chem. 42, 145-197.

Andersson, K., Malmqvist, P.-Å. & Roos, B.O. (1992). Second-order perturbation theory with a complete active space self-consistent field reference function. J. Chem. Phys. 96, 1218.

Andersson, K., Blomberg, M. R. A., Fülscher, M. P., Kellö, V., Lindh, R., Malmqvist, P.-Å., Noga, J., Olsen, J., Roos, B. O., Sadlej, A. J., Siegbahn, P. E. M., Urban, M. & Widmark, P.-O. (1994). MOLCAS version 3. University of Lund, Sweden

Barone, V. (1994). Inclusion of Hartree-Fock exchange in the density functional approach. Benchmark computations for diatomic molecules containing H, B, C, N, O, and F atoms. Chem. Phys. Lett. 226, 392-398.

Blackwell, K. A. , Anderson, B. F. & Baker, E. N. Metal substitution in a blue-copper protein: the crystal structure of cadmium-azurin at 1.8 Å resolution. (1994). Acta Cryst. D50, 263-270.

Bouwman, E. , Driessen, W. L. & Reedijk, J. (1990). Model systems for type 1 copper proteins: Structures of copper coordination compounds with thioether and azole-containing ligands. Coord. Chem. Rev. 104, 143-172.

Church, W. B. , Guss, J. M., Potter, J. J. & Freeman, H. C. (1986). The crystal structure of mercury-substituted poplar plastocyanin at 1.99-Å resolution. J. Biol. Chem. 261, 234-237.

Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A. Ramshaw, J. A. M. & Venkatappa, M. P. (1978). X-ray crystal structure analysis of plastocyanin at 2.7 Å resolution. Nature 272, 319-324.

Cotton, F. A. & Wilkinson, G., (1988). In Advanced inorganic chemistry, Wiley, New York.

Fersht, A. (1985). In Enzyme Structure and Mechanisms, pp. 341-342, W. H. Freeman & Co, New York.

Fraústo da Silvia, J. J. R. & Williams, R. J. P. (1991). In The Biological Chemistry of the Elements, pp. 182-184, Clarendon, Oxford.

Frisch, M. J., Frisch, Æ. & Foresman, J. B. (1994) Gaussian 94 User's reference, p. 60, Gaussian, Inc., Pittsburgh.

Garrett, T. P. J., Guss, J. M., Rogers, S. J. & Freeman, H. C. (1984). The crystal structure of poplar apoplastocyanin at 1.8-Å resolution. J. Biol. Chem. 259, 2822-2825.

Gray, H. B. & Malmström, B. G. (1983). On the relationship between protein-forced ligand fields and the properties of blue-copper centers. Comments Inorg. Chem. 2, 203-209.

Guckert, J. A. , Lowery, M. D. & Solomon, E. I. (1995). Electronic Structure of the reduced blue copper active site: Contributions to reduction potentials and geometry. J. Am. Chem. Soc. 117, 2817-2844.

Guss, J. M. & Freeman, H. C. (1983). Structure of oxidized poplar plastocyanin at 1.6 Å resolution. J. Mol. Biol. 169, 521-563.

Guss, J. M., Harrowell, P. R., Murata, M., Norris, V. A. & Freeman, H. C. (1986). Crystal structure analyses of reduced (Cu^{I}) poplar plastocyanin at six pH values. J. Mol. Biol. 192, 361-387.

Guss, J. M., Bartunik, H. D. & Freeman, H. C. (1992). Accuracy and precision in protein crystal structure analysis: restrained least-squares refinement of the crystal structure of poplar plastocyanin at 1.33 angstroms resolution. Acta Cryst. B48, 790-807.

Hehre, W. J., Radom, L., Schleyer, P. v. R. & Pople, J. A. (1986). In Ab initio molecular orbital theory, Wiley-Interscience, New York.

Holthausen, M. C. , Mohr, M. & Koch, W. (1995). The performance of density functional/Hartree-Fock hybrid methods: the binding in cationic first-row transition metal methylene complexes. Chem. Phys. Lett. 240, 245-252.

Kalverda, A. P. , Wymenga, S. S. , Lommen, A. , van der Ven, F. J. M. , Hilbers, C. W. & Canters, G. W. (1994). Solution structure of the type 1 blue copper protein amicyanin from Thiobacillus versutus. J. Mol. Biol. 240, 358-371.

Kitajima, N. , Fujisawa, K. , Tanaka, M. & Moro-Oka, Y. (1992). X-ray structure of thiolatocopper(II) complexes bearing close spectroscopic similarities to blue copper proteins. J. Am. Chem. Soc. 114, 9232-9233.

Kitajima, N. (1992) Synthetic approach to the structure and function of copper proteins, Adv. Inorg. Chem, 39, 1-77.

Levitt, M. (1974). In Peptides, polypeptides and proteins, (Blout, E. R., Bovey, F. A., Goodman, M. & Lotan, N. eds.), p. 99-102, Wiley, New York.

Malmström, B. G. (1965). Two forms of copper in copper-containing oxidases. In Oxidases and related redox systems (King, T. E., Mason, H. S. & Morrison, M. eds.), vol. 1, pp. 207-216, Wiley, New York.

Malmström, B. G. (1994). Rack-induced bonding in blue-copper proteins. Eur. J. Biochem. 223, 711-718.

Marcus, R. A. & Sutin, N. (1985). Electron transfers in chemistry and biology. Biochim. Biophys. Acta, 811, 265-322.

Moore, J. M. , Case, D. A., Chazin, W. J. , Gippert, G. P. , Havel, T. F. , Powls, R. & Wright, P. E. (1988). Three-dimensional solution structure of plastocyanin from green alga Scenedesmus obliquus. Science 240, 314-317.

Nar, H., Huber, R. , Messerschmidt, A., Filippou, A. C. , Barth, M. , Jaquinod, M. , Kamp, M. V. D. & Canters, G. W. (1992a). Characterization and crystal structure of zinc azurin, a by-product of heterologous expression in Escherichia coli of Pseudomonas aeruginosa copper azurin. Eur. J. Biochem. 205, 1123-1129.

Nar, H., Messerschmidt, A., Huber, R., Kamp, M. V. D. & Canters, G. W. (1992b). Crystal structure of Pseudomonas aeruginosa apo-azurin at 1.85 Å resolution. FEBS Lett. 306, 119-124.

Norris, G. E., Anderson, B. F. & Baker, E. N. (1986). Blue copper proteins. The copper site in azurin from Alcaligenes denitrificans. J. Am. Chem. Soc. 108, 2784-2785.

Penfield, K. W., Gewirth, A. A. & Solomon, E. I. (1985). Electronic structure and bonding of the blue copper site in plastocyanin. J. Am. Chem. Soc. 107, 4519-4529.

Pierloot, K. , Dumez, B. , Widmark, P.- O. & Roos, B. O. (1995). Density matrix averaged atomic natural orbital (ANO) basis sets for correlated molecular wave functions. IV. Medium size basis sets for the atoms H-Kr. Theoret. Chim. Acta 90, 87-114.

Ricca, A. & Bauschlicher, C. W. (1994). Successive binding energies of $Fe(CO)_5^+$. J. Phys. Chem. 98, 12899-12903.

Ricca, A. & Bauschlicher, C. W. (1995). A comparison of density functional theory with ab initio approaches for systems involving first transition row metals. Theor. Chim. Acta 92, 123-131.

Rice, J. E., Horn, H., Lengsfiels, B. H., McLean, A. D., Carter, J. T., Replogle, E. S., Barnes, L. A., Maluendes, S. A., Lie, G. C., Gutwski, M., Rude, W. E., Sauer, S. P. A., Lindh, R., Andersson, K., Chevalier, T. S,. Widmark, P.-O., Bouzida, D., Pacansky, G., Singh, K., Gillan, C. J., Carnevali, P., Swope, W. C. & Liu, B. (1995). Mulliken™ Version 2.25b, internal release, IBM Corporation, Almaden, USA.

Romero, A. , Hoitink, C. W. G. , Nar, H., Huber, R., Messerschmidt, A. & Canters, G. W. (1993). X-ray analysis and spectroscopic characterization of M121Q azurin. J. Mol. Biol. 229, 1007-1021.

Romero, A. , Nar, H., Huber, R., Messerschmidt, A., Kalverda, A. P. , Canters, G. W., Durley, R. & Mathews, F. S. (1994). Crystal structure analysis and refinement at 2.15 Å resolution of amicyanin, a type I blue copper protein, from Thiobacillus versutus. J. Mol. Biol. 236, 1196-1211.

Ryde, U. (1995). Molecular dynamics simulations of alcohol dehydrogenase with a four- or five-coordinate catalytic zinc ion. Proteins: Struct. Funct. Genet. 21, 40-56.

Schäfer, A., Horn, H. & Ahlrichs, R. (1992). Fully optimized contracted Gaussian basis sets for atoms Li to Kr. J. Chem. Phys. 97, 2571-2577.

Shepard, W. E. B., Anderson, B. F. , Lewandoski, D. A. , Norris, G. E. & Baker, E. N. (1990). Copper coordination geometry in azurin undergoes minimal change on reduction of copper(II) to Copper(I). J. Am. Chem. Soc. 112, 7817-7819.

Shepard, W. E. B., Kingston, R. L. , Anderson, B. F. & Baker, E. N. (1993). Structure of apo-azurin from Alcaligens dentrificans at 1.8 Å resolution. Acta Cryst. D49, 331-343.

Sjölin, L., Tsai, L.- C. , Langer, V. , Pascher, T., Karlsson, B. G., Nordling, M. & Nar, H. (1993). Structure of Pseudomonas aeruginosa zinc-azurin mutant Asn47Asp at 2.4 Å resolution. Acta Cryst. D49, 449-457.

Sykes, A. G. (1990). Active-site properties of the blue copper proteins. Adv. Inorg. Chem. 36, 377-408.

Tsai, L.- C. , Sjölin, L., Langer, V. , Bonander, N. , Karlsson, B. G., Vänngård, T., Hammann, C. & Nar, H. (1994). Crystal structure of the azurin mutant nickel - Trp48Met from Pseudomonas aeruginosa at 2.2 Å resolution. Brookhaven Protein Data bank, file PDB1NZR.

Vallee, B. L. & Williams, R. J. P. (1968). Metalloenzymes: the entatic nature of their active sites. Proc. Nat. Acad. Sci. USA 59, 498-505.

Warshel, A. (1991). In Computer modelling of chemical reactions in enzymes and solutions, pp. 209-211, J Wiley & Sons, New York.

Williams, R. J. P. (1963). The selective interactions of metal ions and protein groups. In Molecular basis of enzyme action and inhibition (Desnuelle, P. A. E. ed.), pp. 133-149, Pergamon Press, Oxford.

Williams, R. J. P. (1995) Energised (entatic) states of groups and of secondary structures in proteins and metalloproteins. Eur. J. Biochem. 234, 363-381.

Table 1. The metal coordination geometry of different blue copper proteins. The data are compiled from all entries in the April 1995 version of the Brookhaven Protein Data bank and some additional references (Guss & Freeman, 1983, Moore et al., 1988, Shepard et al., 1990, Kitajima et al., 1992, Romero et al., 1994, Kalverda et al., 1994). *n* is the number of different structures considered (including separately refined subunits). Azurin is not included in the ranges and averages, since its geometry differs appreciably from the other due to the fifth ligand.

Protein	*n*	Distance to Cu (pm)			Angle subtended at Cu (°)			
		S_{Cys}	N	S_{Met}	N-N	S_{Cys} -N	S_{Cys} -S_{Met}	S_{Met} -N
Reduced								
Plastocyanin	3	211-217	203-239	287-292	92-118	110-141	99-114	83-110
Pseudoazurin	1	217	216-229	291	102	108-140	107	85-110
Amicyanin	1	230	210-220	290				
Azurin	2	222-231	205-217	321-325	101-104	119-132	109-111	77-94
All	5	211-240	203-239	270-315	92-119	108-141	99-114	83-110
Average		218	215	290	103	123	108	97
Oxidised								
Plastocyanin	8	202-221	189-222	278-310	96-104	112-144	102-110	85-108
Pseudoazurin	3	214-216	197-227	266-276	94-100	110-144	107-109	86-112
Amicyanin	4	212-215	195-215	284-289	104-133	74-135	109-138	83-120
CBP	1	213	190-199	262				
Ascorbate oxidase	2	203-213	205-212	283-290				
Azurin	14	212-229	196-213	288-321	98-108	116-138	105-111	84-124
All	18	202-221	189-227	262-310	94-133	74-144	102-138	83-120
Average		212	206	283	103	120	111	98

Table 2. Change in the optimised copper bond distances and angles when $(NH_3)_2$ is replaced by $(imidazole)_2$. cr. indicates that the structure is in the crystal conformation and not in the optimal one. S_{Cys} and S_{Met} are the sulphur atoms of the cysteine and methionine models. C_s symmetry.

Other ligands	Distance to Cu (pm)			Angle subtended at Cu (°)			
	S_{Cys}	N	S_{Met}	N-N	S_{Cys} -N	S_{Cys} -S_{Met}	N-S_{Met}
$Cu(SCH_3)(SH_2)$, cr.	-1	-9	+17	+5	+3	+3	-25
$Cu(SH)(S(CH_3)_2)$, cr.	-1	-6	+9	+3	+10	-5	-8
$Cu(SCH_3)(S(CH_3)_2)$	+3	-7	+5	+5	+2	-2	-4
$Cu(SCH_3)(S(CH_3)_2)$, cr.	-2	-6	+7	+2	+12	-4	-9
$[Cu(SH)(SH_2)]^+$	0	-5	+5	+1	0	-3	+1
$[Cu(SH)(SH_2)]^+$, cr.	0	-5	+7	+3	0	-2	0
$[Cu(SCH_3)(SH_2)]^+$	0	-5	+6	+1	+1	-3	+1
$[Cu(SCH_3)(SH_2)]^+$, cr.	0	-5	+9	+4	0	-1	-1
$[Cu(SH)(S(CH_3)_2)]^+$	+1	-5	+4	+1	0	-3	+1
$[Cu(SH)(S(CH_3)_2)]^+$, cr.	+1	-6	+4	+2	0	-2	-1
$[Cu(SCH_3)(S(CH_3)_2)]^+$	0	-6	+4	0	+1	-3	+1
$[Cu(SCH_3)(S(CH_3)_2)]^+$, cr.	0	-6	+4	+2	0	-2	-1

Table 3. Change in the optimised copper bond distances and angles when SH^- is replaced by SCH_3^-. cr. indicates that the structure is in the crystal conformation and not in the optimal one. S_{Cys} and S_{Met} are the sulphur atoms of the cysteine and methionine models. C_s symmetry.

Other ligands	Distance to Cu (pm)			Angle subtended at Cu (°)			
	S_{Cys}	N	S_{Met}	N-N	S_{Cys} -N	S_{Cys} -S_{Met}	N-S_{Met}
$Cu(NH_3)_2$	-1	+1		-2	+1		
$Cu(NH_3)_2(SH_2)$, cr.	0	0	-1	-1	-1	+2	0
$Cu(NH_3)_2(S(CH_3)_2)$	-3	0	+6	0	+5	-4	-3
$Cu(NH_3)_2(S(CH_3)_2)$, cr.	-1	+2	-2	-3	-3	+10	-1
$Cu(imidazole)_2(S(CH_3)_2)$, cr.	-2	+2	-4	-4	-1	+11	-2
$[Cu(NH_3)_2]^+$	0	+1		+2	-1		
$[Cu(NH_3)_2(SH_2)]^+$	0	+1	+2	0	0	-3	0
$[Cu(NH_3)_2(SH_2)]^+$, cr.	0	+1	+4	+1	-1	+3	-1
$[Cu(NH_3)_2(S(CH_3)_2)]^+$	0	+2	+1	+1	0	-2	0
$[Cu(NH_3)_2(S(CH_3)_2)]^+$, cr.	0	+2	0	+1	-2	+7	-2
$[Cu(imidazole)_2(SH_2)]^+$	0	+1	+3	0	+1	-3	0
$[Cu(imidazole)_2(SH_2)]^+$, cr.	0	+1	+6	+2	-1	+4	-2
$[Cu(imidazole)_2(S(CH_3)_2)]^+$	-1	+1	+1	0	+1	-2	0
$[Cu(imidazole)_2(S(CH_3)_2)]^+$, cr.	-1	+2	0	+1	-2	+7	-2

Table 4. Change in the optimised copper bond distances and angles when SH_2 is replaced by $S(CH_3)_2$. cr. indicates that the structure is in the crystal conformation and not in the optimal one. S_{Cys} and S_{Met} are the sulphur atoms of the cysteine and methionine models. C_s symmetry.

Other ligands	Distance to Cu (pm)			Angle subtended at Cu (°)			
	S_{Cys}	N	S_{Met}	N-N	S_{Cys} -N	S_{Cys} -S_{Met}	N-S_{Met}
$Cu(NH_3)_2(SH)$, cr.	+2	-3	0	-1	0	+8	-13
$Cu(NH_3)_2(SCH_3)$, cr.	+1	0	-1	-3	-2	+16	-14
$Cu(imidazole)_2(SCH_3)$, cr.	0	+3	-11	-6	-3	+9	+2
$[Cu(NH_3)_2(SH)]^+$	+1	+1	-23	-2	-2	+4	+3
$[Cu(NH_3)_2(SH)]^+$, cr.	+1	+2	-26	-1	-3	+4	+5
$[Cu(NH_3)_2(SCH_3)]^+$	+1	+2	-24	-1	-2	+5	+3
$[Cu(NH_3)_2(SCH_3)]^+$, cr.	+1	+3	-30	-1	-4	+8	+4
$[Cu(imidazole)_2(SH)]^+$	+2	+1	-24	-2	-2	+4	+3
$[Cu(imidazole)_2(SH]^+)$, cr.	+2	+1	-29	-2	-3	+4	+4
$[Cu(imidazole)_2(SCH_3)]^+$	+1	+1	-26	-2	-2	+5	+3
$[Cu(imidazole)_2(SCH_3)]^+$, cr.	+1	+2	-35	-3	-4	+7	+4

Table 5. Comparison of the optimised structure of the crystal and optimal conformation of $[Cu(imidazole)_2(SCH_3)(S(CH_3)_2)]^+$ and the crystal structures of oxidised blue copper proteins.

Conformation	Distance to Cu (pm)			Angle subtended at Cu (°)			
	S_{Cys}	N	S_{Met}	N-N	S_{Cys} -N	S_{Cys} -S_{Met}	N-S_{Met}
crystal (Fig. 2)	218	204	267	103	120-122	116	94-95
optimal	218	204	264	103	125	99	99
experimental range	202-221	189-222	262-310	94-133	74-144	102-138	83-120
experimental average	212	206	283	103	120	111	98

Table 6. Comparison of the optimised structure of two structures of $Cu(imidazole)_2(SCH_3)(S(CH_3)_2)$ with the crystal structures of reduced blue copper proteins. In the fixed structure, the Cu-S_{Met} bond distance was constrained to 290 pm.

Structure	Distance to Cu (pm)			Angle subtended at Cu (°)			
	S_{Cys}	N	S_{Met}	N-N	S_{Cys} -N	S_{Cys} -S_{Met}	N-S_{Met}
tetrahedral (Fig. 4)	232	214-215	237	109	105-108	115	107-113
fixed (Fig. 5)	227	205-210	290	119	112-120	99	100-101
experimental range	211-240	203-239	270-315	92-119	108-141	99-114	83-110
experimental average	218	217	290	103	123	108	97

Legends to the figures

Figure 1. A comparison of the copper coordination sphere of the oxidised and reduced (shaded) blue copper protein plastocyanin. Atoms through C^{α} of the coordinating residues are shown. Data from Brookhaven Protein Data files PDB1PLC and PDB5PCY (Guss et al., 1986 & 1992).

Figure 2. The optimised structure of $[Cu(imidazole)_2(SCH_3)(S(CH_3)_2)]^+$ compared to the crystal structure of oxidised plastocyanin (shaded; Guss. et al., 1992).

Figure 3. The B3LYP (circles) and CASPT2 (squares) energy of $[Cu(NH_3)_2(SH)(S(CH_3)_2)]^+$ as a function of the Cu-S_{Met} distance (C_s symmetry). The geometry was optimised at each point with the B3LYP method. Note that the calculations were performed on a smaller system than those in Fig. 2 and Table 5, which explains the 8 pm shorter Cu-S_{Met} bond. The curves are shifted so that the minimum energy is 0 kJ/mole; the actual energy minima are -2629.912166 (B3LYP) and -2627.565024 H (CASPT2).

Figure 4. The optimised four-coordinate structure of $Cu(imidazole)_2(SCH_3)(S(CH_3)_2)$.

Figure 5. The optimised structure of $Cu(imidazole)_2(SCH_3)(S(CH_3)_2)$ with the Cu-S_{Met} bond length constrained to 290 pm, compared with the crystal structure of reduced plastocyanin (shaded; Guss. et al., 1986).

Figure 6. The B3LYP (circles) and CASPT2 (squares) energy of $Cu(SH)(NH_3)_2(S(CH_3)_2)$ as a function of the Cu-S_{Met} distance (C_s symmetry). The geometry was optimised at each point with the B3LYP method. Note that the calculations were performed on a smaller system than those in Fig. 5 and Table 6. The curves are shifted so that the minimum energy is 0 kJ/mole. The actual energy minima are -2630.118746 (B3LYP) and -2627.804323 H (CASPT2).

Footnotes

† Note that the implementation of the B3LYP method in the Mulliken chemical software differs slightly from the one defined in the Gaussian quantum chemistry software: Mulliken uses another fit of the Vosko-Wilk-Nusair correlation functional, namely the one recommended by the authors, called VNW5 in the Gaussian 94 manual (Frisch, et al., 1994).