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ORIGINAL ARTICLE

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Copper coordination in blue proteins

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Abstract The spectroscopic and electrochemical properties of blue copper proteins are strikingly different from those of inorganic copper complexes in aqueous solution. Over three decades ago this unusual behavior was ascribed to constrained coordination in the folded protein; consistent with this view, crystal structure determinations of blue proteins have demonstrated that the ligand positions are essentially unchanged on reduction as well as in the apoprotein. Blue copper reduction potentials are tuned to match the particular function of a given protein by exclusion of water from the metal site and strict control of the positions of axial ligands in the folded structure. Extensive experimental work has established that the reorganization energy of a prototypal protein, *Pseudomonas aeruginosa* azurin, is ~0.7 eV, a value that is much lower than those of inorganic copper complexes in aqueous solution. The lowered reorganization energy in the protein, which is attributable to constrained coordination, is critically important for function, since the driving forces for electron transfer often are low (~0.1 eV) between blue copper centers and distant (>10 Å) donors and acceptors.

H.B.G. and R.J.P.W. dedicate this paper to the memory of our colleague, Bo Malmström, who passed away on 9 February 2000. We and all others in biological inorganic chemistry will forever be indebted to Bo for his seminal contributions to our field.

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Introduction

In the period from 1950 to 1960 it was apparent that the properties of many metal and organic groups inside proteins were not those expected from common experience in chemistry. In this article we shall examine a particular example, that of copper in the blue proteins, so as to show how different views, sometimes apparently conflicting (see [1, 2] for reviews), can be brought to focus on underlying features of these unusual sites created by the nature of the proteins which contain them. We start by giving a description of the unusual character of this copper site while relating it to its functional role, before offering an explanation as to how the protein generates the special coordination environment. Throughout the paper we will keep in mind that the function of the copper is to facilitate electron transfer between reactants.

The blue copper proteins catalyze redox reactions in organisms ranging from bacteria to humans. Between 1950 and 1960, two properties of these proteins were discussed repeatedly at conferences and in publications [1–3]. One was the anomalously high reduction potential with respect to inorganic copper complexes in aqueous solution, and the second was the intense absorption at around 600 nm, giving rise to the blue color; in a survey paper, one of us attributed the high potentials to steric hindrance at the copper site, which would give a tetrahedral geometry, and it was suggested that the blue color was due to copper-ligand charge-transfer absorption [3]. (Later it was proposed that the ligand was a thiolate [4].) These features were assumed to generate good electron transfer properties, but this was not quantified. However, there was also uncertainty about the ground

state of copper in these proteins [3]. The uncertainty arose because the electron distribution in the ground state could have been either Cu^{2+}-L^- or Cu^+-$L^\bullet$. (At that time there was considerable debate as to how to describe oxidation states in highly covalent complexes [5].) New methods of study were clearly required. Moreover, there was the need to offer an explanation of how such an apparently unusual copper-thiolate complex arose, including mechanisms of its formation.

One method that could be used to determine the ground state was EPR spectroscopy; this method, then relatively novel in biochemical studies, was employed by Malmström and Vänngård [6] in 1960 to show that the blue copper proteins exhibited a most unusual ground-state EPR spectrum. The observation stimulated much heated discussion as to its causes. One possibility was that it was due to anomalously strong covalency, no matter what its source [6]; another was that it arose from a near tetrahedral geometry [3, 4]. The implication of the covalency was that the unpaired electron was spread over the ligands, giving obvious advantage in electron transfer reactions.

A little later, while discussing the unusual characteristics of Cu(II) in these proteins, one of us revived an earlier general hypothesis of Lumry and Eyring, not previously applied to single metal ion sites, that unusual properties could be induced by a "rack mechanism" [7]. This was a distinctly different approach to that in references [3] and [4], that the condition of the copper was due to a simple misfitting of copper to the stereochemistry imposed by the protein, since it is based on a mechanism for formation of the site. What was common to these views was that there were constraints at the copper site and that these constraints had functional value, which gave rise both to the tuning of the reduction potential and a lowering of the reorganization energy for electron transfer. The position with regard to both unusual organic and inorganic sites in proteins was brought together in a 1968 paper by Vallee and Williams [8] on the "entatic state".

In the 1970s, a much more detailed spectroscopic investigation of blue copper proteins established the charge-transfer nature of the blue color [9]. In addition, the discovery of ligand-field bands in the near-infrared region strongly indicated the geometry to be pseudo-tetrahedral [9]. Shortly thereafter, a crystal structure analysis of poplar plastocyanin confirmed that the blue site had an unexpected geometry, which we analyze below, and that the copper was bound by a thiolate and two histidine residues with a very distant methionine sulfur providing a possible fourth ligand [10]. Moreover, the protein was a β-sheet, which was believed to give a rigid construct that would fix the reduction potential and minimize the nuclear reorganization required for electron transfer. However, none of these studies investigated in a *quantitative* manner the presumed relationship of the structure and thermodynamic properties to the catalytic action.

Function

Although all blue copper proteins act as electron transfer agents, their functions vary according to the donor/acceptor reactions they mediate (Table 1) [11–16]. Several blue proteins mediate electron flow between substrates (donors) and dioxygen in the scheme:

$$\text{Substrate(donor)} \xrightarrow{e^-} \text{Cucenter} \xrightarrow{e^-} \text{Acceptor}(O_2) \quad (1)$$

Other examples involve electron transfer in photosynthetic chains. We can assess the functional value of a copper center in catalyzing electron transfer under different circumstances by looking at the factors that control the transfer rates, k_{ET} [17]. The key parameters are given in the semiclassical expression of Eq. 2:

$$k_{ET} = \frac{2\pi^2}{h\sqrt{\pi\lambda kT}} H_{AB}^2 \exp\left\{-\frac{(\Delta G^\circ + \lambda)^2}{4\lambda kT}\right\} \quad (2)$$

Here ΔG° is the standard free energy of the reaction (the difference in the reduction potentials of the sites), λ is the reorganization energy required for electron transfer, and H_{AB} is the electronic coupling matrix element. During the last 20 years, much work has been done to obtain experimental values for the variables in Eq. 2. It has been shown that the factor H_{AB}^2 is related roughly to the distance which the electron must travel between donor and acceptor through the protein [17–23]. Whatever the coupling mechanism, the role of the nuclear factor is clear. When the exponential term is unity ($\Delta G^\circ + \lambda = 0$), electron transfer

Table 1 Reduction potentials (mV vs. NHE) of blue copper proteins and their electron donors and acceptors

Protein	Potential (pH)	Donor	Potential (pH 7)	Acceptor	Potential (pH 7)
P. nigra plastocyanin	370 (7.5)	cyt *f*	340	$P700^+$	490
Thiobacillus ferrooxidans rusticyanin[a]	680 (1–3)	sulfatoiron(II) (pH 2.0)	≤650	cyt *a* (O_2)	(820)
human ceruloplasmin	490, 580 (5.5)	iron(II) complexes	<300[b]	type 3 Cu (O_2)	(820)
Rhus vernicifera laccase	394 (7.5)	phenols	<300[c]	type 3 Cu (O_2)	434 (820)
Polyporus versicolor laccase	785 (5.5)	phenols	<300[c]	type 3 Cu (O_2)	782 (820)
C. pepo medullosa ascorbate oxidase	344 (7.0)	ascorbate	295[d]	type 3 Cu (O_2)	344 (820)

[a] See [54]
[b] Ligation-dependent potentials
[c] Wide range of potentials
[d] E° for $Asc + H^+ + 2e^- \rightarrow HAsc^-$

Table 2 Coordination of Cu(II) in blue proteins

Protein	Coordination	PDB code	E ° (mV vs. NHE)
A. xylosoxidans azurin I (pH 8.0)[a,b]	5 [N_2S(OS)] trigonal bipyramidal (2.51 Å, 3.18 Å)	1RKR [57]	305 (pH 7.5)
A. xylosoxidans azurin II (pH 6.5)	5 [N_2S(OS)] trigonal bipyramidal (2.75 Å, 3.26 Å)	1ARN [58]	305 (pH 7.5)
P. aeruginosa azurin (pH 9.0)[a]	5 [N_2S(OS)] trigonal bipyramidal (2.93 Å, 3.13 Å)	5AZU [59]	293 (pH 8.0)
P. aeruginosa azurin (pH 5.5)[a]	5 [N_2S(OS)] trigonal bipyramidal (2.97 Å, 3.15 Å)	4AZU [59]	310
P. fluorescens azurin	5 [N_2S(OS)] trigonal bipyramidal (2.99 Å, 3.23 Å)	1JOI [60]	–
A. denitrificans azurin (pH 5.0)[a]	5 [N_2S(OS)] trigonal bipyramidal (3.13 Å, 3.11 Å)	2AZA [61]	285
P. putida azurin (pH~7)[a]	5 [N_2S(OS)] trigonal bipyramidal (3.14 Å, 3.01 Å)	1NWP [62]	295[c]
C. sativus stellacyanin	4 [N_2SO] distorted tetrahedral	1JER [63]	260
A. denitrificans (M121Q)azurin[a]	4 [N_2SO] distorted tetrahedral	1URI [64]	263
A. denitrificans (M121H)azurin (pH 3.5)[a,b,d]	4 [N_2SO] distorted tetrahedral	1A4C [65]	350
P. aeruginosa (M121E)azurin (pH 6.0)[a,b]	4 [N_2SO] distorted tetrahedral	1ETJ [66]	220 (pH 7.0)
cucumber basic protein	4 [(N_2S)(S)] trigonal pyramidal (2.60 Å)	2CBP [67]	317
A. xylosoxidans nitrite reductase	4 [(N_2S(S)] trigonal pyramidal (2.62 Å)	1BQ5 [68]	280 (pH 7.0)
A. xylosoxidans nitrite reductase[b]	4 [(N_2S(S)] trigonal pyramidal (2.63 Å)	1NDT [69]	260 (pH 7.0)
S. sp. PCC 6803 (A42D, D47P, A63L) plastocyanin[a,b]	4 [(N_2S(S)] trigonal pyramidal (2.64 Å)	1PCS [70]	325
M. extorquens pseudoazurin	4 [N_2S(S)] trigonal pyramidal (2.66 Å)	1PMY [71]	260 (pH 7.0)[e]
U. pertusa plastocyanin	4 [N_2S(S)] trigonal pyramidal (2.69 Å)	1IUZ [72]	363 (pH 7.0)
P. laminosum plastocyanin (pH 6.0)[a,b]	4 [N_2S(S)] trigonal pyramidal (~2.7 Å)	1BAW [73]	–
A. cycloclastes pseudoazurin	4 [N_2S(S)] trigonal pyramidal (2.70 Å)	1ZIA [74]	260 (pH 7.0)
A. faecalis pseudoazurin (pH 7)	4 [N_2S(S)] trigonal pyramidal (2.71 Å)	8PAZ [75]	269
P. aeruginosa (M121A)azurin (pH 5.1)[a,b]	4 [N_2S(O) trigonal pyramidal (2.74 Å)	2TSA [76]	373
S. pratensis plastocyanin[b]	4 [N_2S(S)] trigonal pyramidal (2.74 Å)	1BYO [77]	–
A. faecalis pseudoazurin (pH 6.8)	4 [N_2S(S)] trigonal pyramidal (2.76 Å)	1PAZ [78]	269
P. nigra plastocyanin (pH 6.0)	4 [N_2S(S)] trigonal pyramidal (2.82 Å)	1PLC [79]	370 (pH 7.5)
C. pepo medullosa ascorbate oxidase (pH 5.5)[a]	4 [N_2S(S)] trigonal pyramidal (2.86 Å)	1AOZ [80]	344
T. ferrooxidans rusticyanin (pH 4.6)	4 [N_2S(S) trigonal pyramidal (2.88 Å)	1RCY [81]	680
S. oleracea (G8D)plastocyanin (pH~4.4)	4 [N_2S(S)] trigonal pyramidal (2.88 Å)	1AG6 [82]	379
C. reinhardtii plastocyanin	4 [N_2S(S)] trigonal pyramidal (2.89 Å)	2PLT [83]	–
P. denitrificans amicyanin (pH 5–6)	4 [N_2S(S)] trigonal pyramidal (2.91 Å)	1AAC [84]	294
E. prolifera plastocyanin	4 [N_2S(S)] trigonal pyramidal (2.92 Å)	7PCY [85]	369 (pH 7.0)
D. crassirhizoma plastocyanin (pH 4.5)	4 [N_2S(S)] trigonal pyramidal (2.94 Å)	1KDJ [86]	387 (pH 7.0)
Human ceruloplasmin (Cu41, Cu61)[b]	4 [N_2S(S)] trigonal pyramidal (~3.0 Å)	1KCW [87]	490, 580
C. cinereus laccase (type-2 Cu depleted)[b]	3 [N_2S] trigonal planar	1A65 [88]	550
Human ceruloplasmin (Cu21)[b]	3 [N_2S] trigonal planar	1KCW [87]	>1000 [Cu(I)] site[f]

[a]Average copper-ligand bond lengths are reported in cases where there are multiple molecules in the crystallographic asymmetric unit
[b]Resolution lower than 2 Å
[c]Molecules C and D only; molecules A and B have the same coordination as observed at pH 6.5 (see [65])
[d]Ref. [55]
[e]Kohzuma T, personal communication
[f]Ref. [56]

rates will be high (k_{ET} >10^9 s^{-1} for donor-acceptor distances <10 Å). Rates >10^9 s^{-1} are much higher than observed catalytic rates, so λ does not have to be rigorously controlled. However, in most of the reactions set out in Table 1, there is one step where the distance between donor and acceptor is >10 Å. In these long-range reactions, the coupling-limited k_{ET} values could be <10^9 s^{-1} and therefore the nuclear reorganization must be minimized (λ<1 eV) if reasonable rates are to be ensured at low driving forces (~0.1 eV). The way in which this can be brought about is by constraining the structure of the copper site. We therefore examine the problem of the function of blue copper sites starting from structures. On the basis of the structures and model reference states, we analyze two critical parameters, reduction potentials and reorganization energies. We then draw our conclusions.

Structure

The functional efficiency of copper in blue proteins can be related to the structures of ground and activated states involved in its biological reactions. Several ground states have been fully characterized by electronic structure calculations, spectroscopy, and X-ray crystallography (Tables 2 and 3) [10–16, 24–30]. As far as coordination structures are concerned, we

conclude that: (1) all ground states have three strong ligands (two imidazole-N; one thiolate-S); (2) there may be a weaker fourth or fifth ligand, but this is not required for a blue site; (3) trigonal planar coordination of the three strong ligands to the copper is not an essential feature; and (4) the structural features are to a large degree independent of oxidation state [i.e., Cu(I) and Cu(II) have virtually identical coordination].

In order to make comparisons with structures other than proteins, we asked C.K. Prout (Oxford) to search the Cambridge Structural Database System for all copper complexes having a CuSC fragment. This search was updated and then restricted to CuSC(NN) complexes by W.P. Schaefer (Caltech) in January, 1999. Schaefer examined 105 compounds containing monodentate, bidentate, and tridentate ligands (Table 4). Of the 77 Cu(II) structures, 61 are tetragonal (17 square planar; 27 square pyramidal; 17 square bipyramidal), 11 are trigonal bipyramidal, and 5 are tetrahedral. (The first example of a trigonal planar Cu(II)(NNS) structure was reported later in the year [31].) Of the 28 Cu(I) complexes, 18 are tetrahedral and 10 are trigonal planar. The message is clear: inorganic Cu(II) favors tetragonal coordination, whereas Cu(I) strongly prefers tetrahedral and trigonal planar geometries (Fig. 1).

Insight into these structures has been gained from high-level calculations of optimal geometries of blue copper coordination units in vacuum [24, 28]. We admit that we are surprised by the results – the derived structures are close to the observed coordina-

Table 3 Metal-ligand bond distances (Å)

***P. aeruginosa* azurin**	**M-N$^{\delta 1}$ (His46)**	**M-S$^{\gamma}$ (Cys112)**	**M-N$^{\delta 1}$ (His117)**	**M-S$^{\delta}$ (Met121)**	**M-O (Gly45)**	**PDB code**
Cu(II) (pH 5.5)[a]	2.08	2.24	2.01	3.15	2.97	4AZU [59]
Cu(I) (pH 5.5)[a]	2.14	2.25	2.04	2.97	3.15	[16][b]
Cu(II) (pH 9.0)[a]	2.06	2.26	2.03	3.13	2.93	5AZU [59]
Cu(I) (pH 9.0)[a]	2.14	2.27	2.15	3.10	3.17	[16][b]
Co(II)[a]	2.32	2.20	2.25	3.49	2.15	1VLX [89]
Ni(II)[a]	2.23	2.39	2.22	3.30	2.46	[90][b]
Zn(II)[a,c]	2.01	2.30	2.07	3.4	2.32	[91][b]
***A. denitrificans* azurin**	**M-N$^{\delta 1}$ (His46)**	**M-S$^{\gamma}$ (Cys112)**	**M-N$^{\delta 1}$ (His117)**	**M-S$^{\delta}$ (Met121)**	**M-O (Gly45)**	**PDB code**
Cu(II)	2.08	2.15	2.00	3.11	3.13	2AZA [61]
Cu(I)	2.13	2.26	2.05	3.23	3.22	[92][b]
Cd(II)	2.25	2.38	2.21	3.23	2.76	1AIZ [93]
***P. nigra* plastocyanin**	**M-N$^{\delta 1}$ (His37)**	**M-S$^{\gamma}$ (Cys84)**	**M-N$^{\delta 1}$ (His87)**	**M-S$^{\delta}$ (Met92)**		**PDB code**
Cu(II) (pH 6.0)	1.91	2.07	2.06	2.82	–	1PLC [79]
Cu(I) (pH 7.0)	2.13	2.17	2.39	2.87	–	5PCY [79]
Cu(I) (pH 7.8)[c]	2.12	2.11	2.25	2.90	–	4PCY [94]
Hg(II)	2.34	2.38	2.36	3.02	–	3PCY [95]
***S.* sp. PCC 7942 plastocyanin**	**M-N$^{\delta 1}$ (His37)**	**M-S$^{\gamma}$ (Cys84)**	**M-N$^{\delta 1}$ (His87)**	**M-S$^{\delta}$ (Met92)**		**PDB code**
Cu(II) (pH 5.0)	1.97	2.01	2.14	2.93	–	1BXU [96]
Cu(I) (pH 5.0)	2.09	2.37	2.17	2.80	–	1BXV [96]
***D. crassirhizoma* plastocyanin**	**M-N$^{\delta 1}$ (His37)**	**M-S$^{\gamma}$ (Cys87)**	**M-N$^{\delta 1}$ (His90)**	**M-S$^{\delta}$ (Met95)**		**PDB code**
Cu(II) (pH 4.5)	1.99	2.23	2.06	2.94	–	1KDJ [86]
Cu(I) (pH 4.5)	1.95	2.21	2.10	2.91	–	1KDI [86]
***A. faecalis* S-6 pseudoazurin**	**M-N$^{\delta 1}$ (His40)**	**M-S$^{\gamma}$ (Cys78)**	**M-N$^{\delta 1}$ (His81)**	**M-S$^{\delta}$ (Met86)**		**PDB code**
Cu(II) (pH 6.8)	2.16	2.16	2.12	2.76	–	1PAZ [78]
Cu(I) (pH 7.8)	2.16	2.17	2.29	2.91	–	1PZA [97]
Cu(II)	2.01	2.13	2.01	2.71	–	8PAZ [75]
Cu(I) (pH 7.0)	2.10	2.17	2.31	2.82	–	3PAZ [75]
***A. cycloclastes* pseudoazurin**	**M-N$^{\delta 1}$ (His40)**	**M-S$^{\gamma}$ (Cys78)**	**M-N$^{\delta 1}$ (His81)**	**M-S$^{\delta}$ (Met86)**		**PDB code**
Cu(II) (pH 6.0)	1.95	2.13	1.92	2.71	–	1BQK [98]
Cu(I) (pH 6.0)	2.04	2.19	2.11	2.85	–	1BQR [98]
***T. ferrooxidans* rusticyanin**	**M-N$^{\delta 1}$ (His85)**	**M-S$^{\gamma}$ (Cys138)**	**M-N$^{\delta 1}$ (His143)**	**M-S$^{\delta}$ (Met148)**		**PDB code**
Cu(II) (pH 4.6)	2.04	2.26	1.89	2.89	–	1RCY [81]
Cu(I)	2.22	2.25	1.95	2.75	–	1A3Z [81]
Cu(I)[c]	2.14	2.26	2.06	2.90	–	1A8Z [99]
Cu(I) (pH 3.4)[d]	2.09	2.16	1.90	2.60	–	1CUR [100]
Ascorbate oxidase	**M-N$^{\delta 1}$ (His445)**	**M-S$^{\gamma}$ (Cys507)**	**M-N$^{\delta 1}$ (His512)**	**M-S$^{\delta}$ (Met517)**		**PDB code**
Cu(II) (pH 5.5)[a]	2.11	2.08	2.08	2.86	–	1AOZ [80]
Cu(I)[a,c]	2.12	2.14	2.08	2.95	–	1ASO [101]

[a]Average metal-ligand bond distances are reported in cases where there are multiple molecules in the crystallographic asymmetric unit

[b]Atomic coordinates have not been deposited with the Protein Data Bank, Brookhaven National Laboratory

[c]Resolution lower than 2 Å

[d]NMR solution structure

Table 4 Copper coordination in inorganic compounds

Coordination number	Core (additional donor atoms)	Geometry	Cambridge structural database system codes[a] (additional donor atoms)
6	$Cu(II)N_2S(XYZ)$	Square bipyramidal	COGNAN01 (SSS); HAZKAU (OOO); JETMIE (SNN); JUXTIF (SNN); PANPID (SOO); PEWWET (SClCl); PEWWIX (SClCl); PITUCU (SOO); SEYNOZ (SNN) (3);[b] VUWGUP (SNN); VUWHAW (SNN) (2);[b] CIYNAZ (OOO); FICVIW (NNS); NAHDEF (SNN); PIATCU (SOO); RACMAJ (SOO) (2);[b] SOFXUG (OOO)
5	$Cu(II)N_2S(XY)$	Square pyramidal	BPYTCU (NN); BULTUX (NN); COHHIQ (NN); JECXEU (NN); ROQDEG (ON) (1);[b] TIVTAT (NO); TPAECU (OO); ZEDNIF (OO); FENMEQ (OO); FENMIU (OO); FIPFOZ (OO) (2);[b] GERPAU (OO); GLXZCU (ClCl); KEXVAC (ON); KEXVEO (NN); KUTSOH (SO) (4);[b] LESTIM (ClCl); LEYXUI (SCl); RIHMOK (NO); RONBOL (OO) (2);[b] SAHDOU (NN); SODZIU (NN); SOFXDA (OO); TIMQEL (SCl); TOQFAG (BrBr); VOBSAG (OO); ZEBLIB (ClCl)
		Trigonal bipyramidal	MAECUT (OO); PLTUCU (NN); RUQLOE (NO); TIVTEX (NO); KUCZEN (NO); LEYYAP (BrS); NEGWUR (OO); NIVDAX (NN); RUTBAJ (NN); RUTBEN (NN); ZAMCUL (NN)
4	$Cu(II)N_2S(X)$	Square planar	COGMUG (S); CXTPAC (S); LESBAM (S); MEQUCU10 (S); NAQPAW (N); QQQDSX02 (S); CONBUC (O); FIPFOZ (O) (2);[b] HEDSAK (Br); NEGXAY (O); NIJXOT (Br); VEPFAX10 (O); VEPFEB10 (O); WEWSAS (Br); YUNRII (Br); ZEBLEX (Cl); ZOWRAE (O)
		Tetrahedral	CIWVIN (S); FONXIP (S); SOFXOA (O); PANDAJ (N); TMCTCU (S)
	$Cu(I)N_2S(X)$	Tetrahedral	BUYBAY (S); CEWYOS10 (S); CUHBAI (N); CUHBOW (N) (2);[b] CUHBUC (N); DIYKOL (S); GIKDUZ (P); HEFJIL (S) (1);[b] JADDUN (S); NIRJED (P) (1);[b] PBTUCU (S); PYDSCU10 (N); RISWEV (S);[b] SISFEF (S); SISFIJ (S); TOYBOY (S) (1);[b] YINJIO (S); NBTPCU (N)
3	$Cu(I)N_2S$	Trigonal planar	CUGZUZ; HICVIY; JONZOB; NILBEP; NILBIT; PAFZUR; VETFEF; YOMJOZ (1);[b] YOMJUF (1);[b] BETYUU

[a]Codes as listed in January, 1999

[b]In structures with more than one copper atom in the asymmetric unit, the number of structures with the given geometry is listed in parentheses

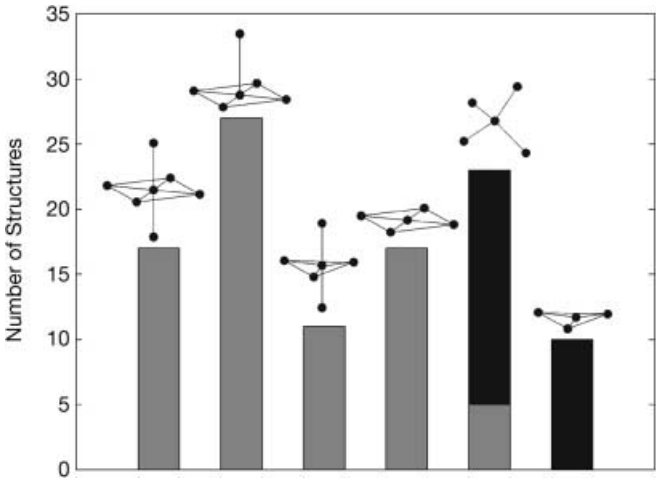

Fig. 1 Coordination geometries of the inorganic copper compounds examined by W.P. Schaefer: Cu(II), *gray*; Cu(I), *black*

tion geometries in blue proteins. It is obvious, however, that a realistic calculation of the structure must take into account the protein dielectric by inclusion of the environment around the site. Most importantly, the quantities that are relevant to function are redox free energies and reorganization energies at temperatures in the neighborhood of 300 K. Such quantities cannot be obtained from calculations of electronic potential surfaces of isolated complexes.

The effect of the folded protein structure on copper coordination is apparent upon examination of the bond distances set out in Table 3. Within the error of crystallographic measurement, there is very little change in the Cu(NNS) coordination core upon reduction of Cu(II) to Cu(I). What is exceedingly telling is that the structures of poplar apoplastocyanin [32], *P. aeruginosa* apoazurin (in one form in which there are probably $2H^+$ ions in the site) [33], *A. denitrificans* apoazurin [34], and *P. denitrificans* apoamicyanin [35] are closely similar to those of the corresponding holoproteins. Taken together, these data show convincingly that the protein fixes the geometry of the

site. We describe the constraints more fully below, but conformational changes of measurable significance are not apparent in the electron transfer step nor in the insertion of the metal ion.

Reduction potentials

We will start from known reference states in models as far as this is possible. James and Williams [36] showed that reduction potentials relative to the Cu(II)/Cu(I) aquated ions are dependent on: (1) the type of ligand (nitrogen π-acceptors and sulfur donors generally raise the potential; anionic ligands lower the potential); and (2) steric hindrance (distortion of the coordination sphere tends to increase the potential, especially in cases where the geometry is forced toward tetrahedral).

Subsequently, many authors have pointed out that all metal redox couples have high potentials in hydrophobic sites if the redox centers are positively charged [37, 38]. Thus, the trigonal core of three donors (NNS) in a blue copper complex of unit positive charge will be unfavorable for Cu(II) and therefore will generate a high potential. When placed in the low dielectric medium of the protein the potential can only be raised further. Accordingly, we shall assume that the high potentials of ceruloplasmin and fungal laccase (Table 1) are typical of the Cu(NNS) core in the absence of any further ligation. The significance of these high potentials in reactions will be discussed below. Here we concentrate on the fact that some of the potentials are much lower and one (*R. vernicifera* stellacyanin: 184 mV) [39] is almost as low as that of the aquated ion.

Based on extensive spectroscopic work, Solomon and co-workers [26, 29] have suggested that constrained coordination regulates the axial interactions in blue proteins. The role of axial ligation in tuning reduction potentials has been established by a combination of mutagenesis and X-ray structural results [39–41]. Hydrophobic residues in the axial positions raise the potential (fungal laccase), whereas strong O ligands, for example, in the azurin Met121Glu mutant, lower it. Relatively strong axial ligation is found in stellacyanin, which has one of the lowest potentials. Axial ligation is somewhat weaker in native azurin, which has long methionine S-Cu(II) and carbonyl O-Cu(II) distances. The carbonyl O-Cu(II) interaction is much weaker in plastocyanin, and the potential is somewhat higher. Also, in rusticyanin, the protein fold forces the carbonyl oxygen to point away from the Cu(II) ion; in this very hydrophobic site, the potential is even higher (680 mV). In summary, the protein fold in blue copper proteins encapsulates the metal in sites of different degrees of hydrophobicity and with varying availability of axial ligands, and these effects of the native fold regulate the reduction potentials most powerfully against a background set by the positions of the major ligands. We recognize, of course, that other factors [42–46], especially site exposure to solvent [43], also can modulate the potentials of blue proteins.

The large range of reduction potentials in blue copper proteins is a result of evolutionary pressure, since it matches differential biological functions of the individual proteins (Table 1). In the photosynthetic transport chain cytochrome *f*-plastocyanin-P700$^+$, for example, the potential of the blue protein (370 mV) falls between that of the cytochrome (340 mV) and P700$^+$ (490 mV). Thus the thermodynamic properties of copper are constrained to match function.

Reorganization energies

Since, as shown above, the reduction potentials of blue copper proteins fit closely between those of their respective donors and acceptors, the driving forces for one or other of the biologically relevant reactions are often relatively small, say as low as 0.1 eV; at such driving forces, and given the distances to one or other of the reactants is >10 Å, rapid long-range electron transfer is possible only if the nuclear reorganization energy is below 1 eV. Nature had to deal with this problem in order to use copper for electron transport because, unlike hemes, copper redox couples in aqueous solution have $\lambda > 1$ eV, owing to the large structural changes that accompany Cu(II)/Cu(I) redox reactions. (One system that has been analyzed in detail is $[Cu(phen)_2]^{2+/+}$, where λ is 2.4 eV [44].) By placing a copper complex in a constrained protein environment, however, the overall nuclear reorganization energy is dramatically lowered [44], as depicted in Fig. 2. Indeed, extensive experimental work on Ru-modified *P. aeruginosa* azurin has fixed λ between 0.6 and 0.8 eV [47, 48], with the latter value established as a rigorous upper limit by the observation of rapid Cu(I) to Ru(III) electron transfer at cryogenic temperatures [48]. Since λ(Ru)~0.7 eV, λ(azurin) also must be ~0.7 eV [47]. Thus the azurin [Cu(II)/Cu(I)] reorganization energy is more than 1 eV lower than typical λ values for self-exchange reactions of inorganic copper complexes in aqueous solution.

It is our view that protein-enforced constraints are an important factor in the overall lowering of the reorganization energy of blue copper relative to that of an unconstrained complex with the same ligand set. The lowering of λ in a blue protein can be attributed to two main constraining factors: one is the exclusion of water from the copper site in the folded polypeptide; and the second is the inner-sphere coordination inside the rigid hydrophobic cavity [44, 49]. We might be able to assess the relative importance of those two factors if we knew the λ(inner) and λ(outer) contributions to λ(total). Calculations by Ryde and co-workers [28, 50] on the optimal vacuum geometries of blue copper model sites give 0.6 eV for λ(inner). What is

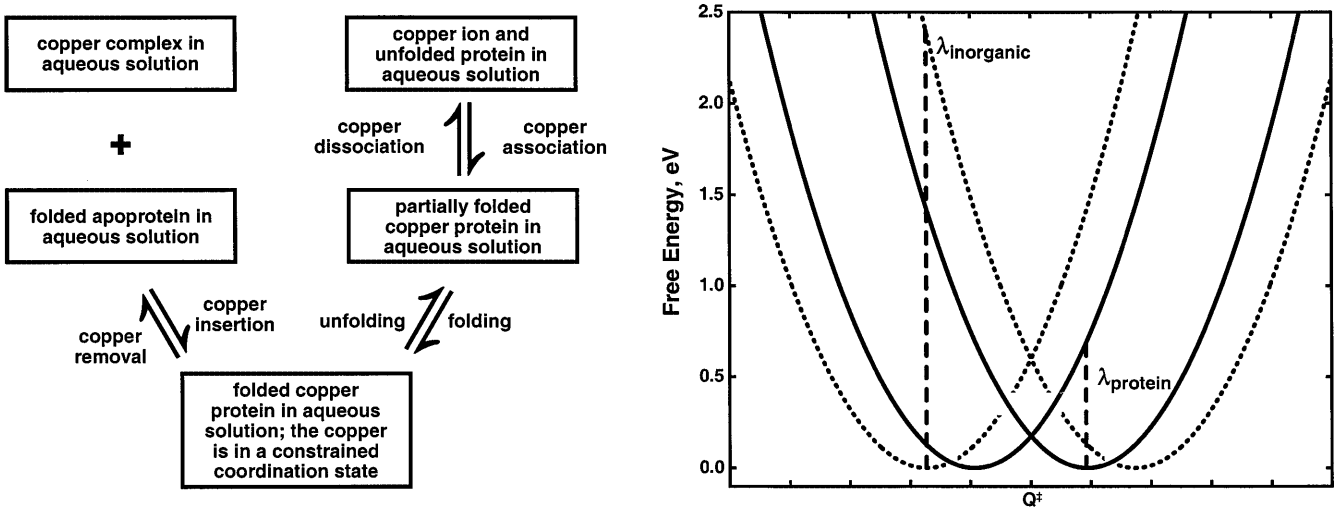

Fig. 2 Constrained coordination is enforced in the folded polypeptide structure; it enhances the activity of the copper ion in reactions that define or generate the biological function of the protein. The free energy profiles (free energy versus dimensionless reaction coordinate Q) for $[Cu(phen)_2]^{2+/+}$ and azurin Cu(II)/Cu(I) self-exchange reactions in aqueous solution show that the nuclear reorganization energy for electron transfer between Cu(II) and Cu(I) is much smaller for the protein

needed, however, is a first principles calculation of a blue copper reorganization energy that includes all inner-sphere and outer-sphere (protein and solvent) contributions.

Other copper proteins

It is obvious that if, as we state, the blue copper proteins have evolved with constrained sites to match a simple electron transfer function, then a similar examination to the one given above of copper sites that serve different functions in other proteins should reveal quite different constraints. Reference to reviews of biological copper chemistry [11–16, 51] confirms this prediction. The variety of copper sites now known in proteins can be shown in fact to match the functional requirement such as dioxygen-carrying (hemocyanin), dioxygen activation (oxidases taking O_2 to H_2O), hydroxylation (taking O_2 to bound OH), and control of copper concentrations (copper exchange as in metallothionein or ATPase pumps) [51].

Concluding remarks

Three properties of blue copper proteins distinguish them from inorganic copper complexes: an intense blue color; a high reduction potential; and an unusual ground-state EPR spectrum. These properties were explicable once crystal structures became available. We have shown here that the site structures in the proteins are unusual by reference to known structures of inorganic complexes, and that some of the variations in the reduction potentials of blue proteins can be explained by structural perturbations of the coordination sphere and its surroundings based on a common trigonal NNS donor core. Of special interest is recent work that implicates certain hydrogen bonds as the structural elements that constrain copper coordination in plastocyanin [52]. Another recent paper also discusses protein control of blue copper properties [53].

In summary, our analysis of the experimental evidence indicates that blue copper sites are constrained to functional advantage. Indeed, we have shown that the reduction potentials of copper centers are modulated to fit the overall ΔG° of the catalyzed enzymic reaction; and that the reorganization energy for electron transfer to and from a blue copper protein is very low. We have noted further that copper proteins that have evolved for different functions have different sites.

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