ELSEVIER

Biophysical Chemistry 80 (1999) 129–138

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Elastic control of electron transfer enthalpy and intensity of light absorption by cupric blue proteins

Arthur S. Brill*

Department of Physics and Biophysics Program, University of Virginia, Charlottesville, VA 22901, USA

Received 18 January 1999; received in revised form 13 May 1999; accepted 13 May 1999

Abstract

The experimental data available shows that the change in enthalpy accompanying electron transfer to cupric blue proteins decreases as the ratio of the strengths of two visible light absorption bands increases. A compact mathematical expression for this inverse relation is formulated, the derivation of which demonstrates that the unusual geometry imposed by the protein upon the redox site is responsible both for the optical band intensity ratio and for a significant fraction of the enthalpy change. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Copper proteins; Elastic energy; Electron transfer; Enthalpy; Light absorption; Redox site

1. Introduction

Respiring systems are energized by 'burning' food fragments, a set of processes in which electrons fall through a series of drops in free energy terminating in the reduction of molecular oxygen. In photosynthetic systems, electrochemical potential converted from photon energy drives electrons through processes leading to the synthesis of foodstuffs. In both systems, copper-containing proteins are involved in a few of the electron transfer steps, including the class of small ($\sim 10^4$ Da) proteins, with single redox sites, which are intensely blue when cupric [1]. The latter copper proteins, ubiquitous in living systems, are the subjects of this paper. Within the constraints of their defining optical (and magnetic resonance) features, they exhibit significant ranges of thermodynamic and spectroscopic features. Analysis of the data from many kinds of measurement is leading to correlation of the functional and structural properties over these ranges. In the following exposition, it is shown how the results of EPR, visible light absorption, and amino acid composition measurements taken together enable one to quantify mechanical and electrical contributions to the change in enthalpy accompany-

* Tel.: +1-8049243781; fax: +1-8049247909
E-mail address: asbox@virginia.edu (A.S. Brill)

0301-4622/99/$ - see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0301-4622(99)00068-X

ing electron transfer to the blue cupric proteins. The formulation developed below expresses succinctly and quantitatively the thesis, initially proposed by Williams [2], (see also Vallee and Williams [3]), and now subject to considerably more experiment and theory, that the unusual magnetic, optical, and thermodynamic parameters arise from stereochemical constraint imposed by the protein upon the redox site.

2. The change in torsional elastic energy accompanying reduction of the cupric site in blue proteins

The influence of site geometry upon the change in elastic energy of harmonic mode j accompanying transfer of an electron to a metal complex in a protein can be expressed $\Delta H_{\text{elastic},j} = [k_{\text{P},j}k_j/(k_{\text{P},j}+k_j)]q_{\text{D},j}(0.5q_{\text{D},j}-q_{\text{P},j})$, Eq. 13 of Brill [4]. Here $k_{\text{P},j}$ is the stiffness of mode j in the protein site without the metal, and k_j (taken the same for the acceptor, A, and donor, D, states) is that of mode j in the metal complex when not in the protein; $q_{\text{D},j}$ and $q_{\text{P},j}$ are the coordinates of the minima of the mode j harmonic potentials of the donor (reduced) complex and the apoprotein site, respectively. We will consider below changes in the angular geometry of a particular harmonic mode of the redox site in the blue proteins, so that, dropping the mode-identifying index j, k_{P} is a torsional force constant (expressed in terms of linear displacement at a copper–ligand bond length of 2 Å), and q_{D} and q_{P} are angles giving the tetrahedrally disposed directions from planar. q_{D} corresponds to the true tetrahedral angle, 35.26°. For the acceptor site, the stiffness k_{PA} and the coordinate q_{PA} (the average out-of-planarity angle of the cupric–ligand bonding orbitals, to be called η below) can be determined from spectroscopic measurements [5,6]. From electron paramagnetic resonance measurements at 77 and 110 K on cupric azurin from *Pseudomonas aeruginosa*, the values $k_{\text{PA}} = 17\times10^4$ mN m^{-1} and $\eta = 18.6°$ were obtained [7]. [The force constant k_{PA} was determined by applying equipartition to the angular (i.e. η) distribution frozen in at 200 K.] Because thermal excitation of vibrational states at 298 K is somewhat greater than at 200 K, the stiffness is expected to be slightly smaller, and η slightly greater, at the warmer temperature. A method, originally described in Brill [6], for determining η from visible light absorption spectra is outlined in Section 3 below. k_{P} and k_{PA} are related by Eq. 6 of Brill [4]; with $k_{\text{A}} = k_{\text{D}} = k$

$$k_{\text{PA}} = k_{\text{P}} + k. \tag{1}$$

k is small compared with k_{P} and k_{PA}. q_{P} and q_{PA} are related by Eq. 3 of Brill [4]; with $k_{\text{A}} = k_{\text{D}} = k$

$$\eta \equiv q_{\text{PA}} = (k_{\text{P}}q_{\text{P}} + kq_{\text{A}})/(k_{\text{P}}+k) = k_{\text{P}}q_{\text{P}}/k_{\text{PA}}. \tag{2}$$

where q_{A} has been set equal to 0 (which can be done without loss of generality [4]). η is seen to be proportional to q_{P}, and these angles are within a few degrees of each other [7,8]. Both angles are tetrahedrally displaced from planar, q_{P} more than η. It follows that, for this mode,

$$\Delta H_{\text{elastic}} = kq_{\text{D}}[0.5\,q_{\text{D}}(1-k/k_{\text{PA}}) - \eta] \tag{3}$$

where k is the only parameter which cannot be determined experimentally. An estimate for k will be obtained in Section 5 below from a least squares fit to ΔH_{total} from a formula in which Eq. (3) appears.

3. Relation of optical band intensities to angular geometry

The intensity or strength of an optical absorption band is quantified by its oscillator strength f_k for band k, given by its area according to

$$f_k = 4.32\times10^{-9}\int_{\text{band k}} a_k(\bar{\nu})\mathrm{d}\bar{\nu} \tag{4}$$

where $\bar{\nu} = 1/\lambda$ is the frequency in cm^{-1} (i.e. in wave numbers), λ is the wavelength in cm, and $a_k(\bar{\nu})$ is the molar absorptivity in M^{-1} cm^{-1} at $\bar{\nu}$.

Absorption spectra have to be resolved into their constituent bands before the integral (area)

for each band can be determined. The bands to be considered in this paper are those centered at approximately 16×10^3 cm^{-1} (620 nm) and at approximately 22×10^3 cm^{-1} (450 nm). The ratios of the oscillator strengths of these bands for four cupric blue proteins are shown in Table 1 wherein the redox potentials for these proteins also appear. Clearly more rigorous information is obtained from comparison of spectroscopic and thermodynamic properties when the measurements are taken under the same conditions. The traditional standard condition for thermodynamic data is for the protein to be in aqueous buffer at pH 7 and 295–298 K. All of the available band analysis data which satisfies the latter condition is in Table 1. For plastocyanin under this condition, only the ratio of the aborptivity maxima in the recorded spectrum (bands unresolved) is published. [For resolved bands of the same shape and width, the oscillator strengths are proportional to the peak absorptivities. The ratios of the widths of the 620 nm to the 450 nm bands for azurin (0.96), stellacyanin (0.85) and umecyanin (0.86) are in the range -4.5 to $+7.9\%$ of their average (0.89), and the ratio is likely to be within this range for plastocyanin. Rather, the uncertainty in employing the ratio of absorptivity maxima in unresolved spectra is the extent of contributions from adjacent bands.] With regard to buffer, ionic strength has some effect upon W, the relatively small electrostatic term to be discussed in Section 4, below; 0.1 M PO_4 is often used.

Quantum mechanical modeling of the redox site in cupric blue proteins is discussed in much more detail in Brill [6] than in the brief exposition that follows.

For strong bands, which arise from electric dipole transitions, oscillator strengths are related to electronic structure by the formula

$$f_k^{x(\text{or } y \text{ or } z)} = 1.085 \times 10^{-5} \bar{\nu}_{k,\text{center}} |\langle \text{ground}| \, x(\text{or } y \text{ or } z)|k\rangle|^2 \quad (5)$$

where x (or y or z) is in Å and $\langle \text{ground} \,|\, x \,|\, k \rangle$ is (apart from the charge of the electron included in the numerical coefficient) the x electric dipole matrix element between the ground state and excited state k. In order to apply Eq. (5), one needs the ground and excited state orbitals. For the cupric site, which is electron deficient, it is convenient (and correct) to use hole states for these (and other) calculations. The radial functions of the states are necessary for calculating the absolute intensities of the transitions, but we

Table 1
Ratios of optical band strengths and standard redox potentials for those four cupric blue proteins on which both have been measured

Protein	$f_{620\text{nm}}/f_{450\text{nm}}$	Redox potential
Stellacyanin (*Rhus vern.*)	4.0[a]	191 mV (-18.4 kJ mol^{-1})[b] in 0.1 M PO_4
Umecyanin (horseradish)	7.9[c]	283 (-27.3)[d] in 0.02 M Na cacodylate
Azurin (*Ps. aerug.*)	12.9[e]	308 (-29.7)[b] in 0.1 M PO_4
Plastocyanin (french bean)	$a_{597\text{nm}}/a_{445\text{nm}} = 9.7$[f]	360 (-34.7)[b] in 0.1 M PO_4

[a] Brill [6].
[b] Taniguchi et al. [9].
[c] Stigbrand and Sjöholm [10].
[d] Stigbrand [11].
[e] Aqualino [12].
[f] Sykes [13].

are considering here the relative intensities of transitions for which the angular distributions of the hole molecular orbitals determine the ratio of the strengths of pairs of bands. The hybrid-atomic-orbital model (HAOM) for hole molecular orbitals has been demonstrated to be effective in quantitatively expressing the role of angular geometry in the optical, magneto-optical, and magnetic resonance properties of low-symmetry cupric sites [6,7,14–16], and will be used now.

The simplest manifold of states that produces the relevant optical properties is shown in Table 2 where the five orbitals are labeled by the irreducible representations of the point group (D_2) to which they belong [6]. While this single parameter set of orbitals is not sufficient to deal with all the optical, magnetic, and magneto-optical properties of the cupric blue proteins, the states which are left out are not required for the transitions being considered here, and the small admixtures of other states removed from the ground state primarily affect magnetic parameters. The directions in which the four lobes of the ground state orbital have their maximum concentration are above and below the *xy* plane at the angle η required for the elastic energy calculation of Eq. (3). This bond angle and the hybridization parameter β are related by [17]

$$\sin\eta = \{(1-\beta^2)/5\beta^2\}^{1/2}. \tag{6}$$

β, and hence η, can be obtained directly from band analysis of the experimental visible absorption spectrum, as follows. With the orbitals of Table 2, one finds

$$f_{16000}/f_{22000} = (\bar{\nu}_{16,\mathrm{center}}/\bar{\nu}_{22,\mathrm{center}})4\beta^2(1-\beta^2) /\{1-4\beta^2(1-\beta^2)\} \tag{7}$$

from which the experimentally-determined parameter r is defined:

$$r \equiv (\bar{\nu}_{22,\mathrm{center}}/\bar{\nu}_{16,\mathrm{center}})(f_{16000}/f_{22000}). \tag{8}$$

Measurement is then related to the orbital model by

$$r = 4\beta^2(1-\beta^2)/\{1-4\beta^2(1-\beta^2)\}. \tag{9}$$

Combining Eqs. (6) and (9), one obtains the required out-of planarity angle η as a function of r from

$$\sin^2\eta = (\sqrt{1+r}-1)/5(\sqrt{1+r}+1). \tag{10}$$

For cupric azurin, $r = 17.4$ with the protein in aqueous buffer at 298 K and Eq. (10) gives $\eta = 20.65°$. From electron paramagnetic resonance measurements at 77 K, $\eta = 18.6°$. The difference in angle of 2° arises in part from the simplicity of the five-orbital model wherein small admixtures of states are omitted, but also because of effects caused by the room vs. cryogenic temperature difference.

Shown in Fig. 1 is $\Delta H_{\mathrm{elastic}}$ as a function of r (with η and β marked on the same axis) for several values of k in the range expected for small cupric complexes.

4. Thermodynamic parameters and an electrostatic contribution

The thermodynamic parameters relevant to this paper, shown in Table 3, are from the laboratory of H.B. Gray [9,18]. While band analyses of the optical spectra from *Alcaligenes faecalus* and *Alcaligenes denitrificans* azurins are not available, the $\Delta G°$ and $\Delta H°$ values for these two proteins are given to help with the following estimation of

Table 2
Five orbital model for optical band strength ratio

	Energy
$\left.\begin{aligned}\lvert\beta'_3\rangle &= \sqrt{1-\beta^2}\lvert 3d_{yz}\rangle - \beta\lvert 4p_x\rangle \\ \lvert\beta'_2\rangle &= \sqrt{1-\beta^2}\lvert 3d_{xz}\rangle - \beta\lvert 4p_y\rangle\end{aligned}\right\}$	22×10^3 cm^{-1}
$\left.\begin{aligned}\lvert\beta_3\rangle &= \beta\lvert 3d_{yz}\rangle + \sqrt{1-\beta^2}\lvert 4p_x\rangle \\ \lvert\beta_2\rangle &= \beta\lvert 3d_{xz}\rangle + \sqrt{1-\beta^2}\lvert 4p_y\rangle\end{aligned}\right\}$	16×10^3 cm^{-1}
$\lvert\beta_1\rangle = \beta\lvert 3d_{xy}\rangle + \sqrt{1-\beta^2}\lvert 4p_z\rangle$	0

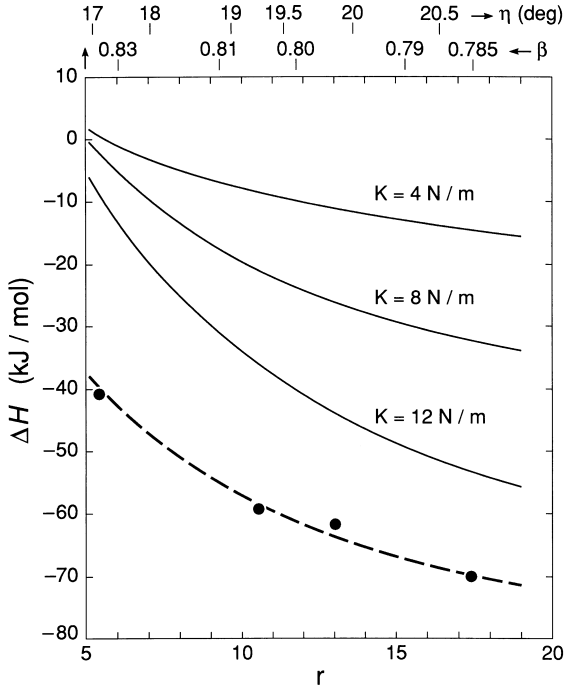

Fig. 1. Enthalpy changes vs. r (ratio of oscillator strengths of 625-nm band to 450-nm band). The solid lines are $\Delta H_{\text{elastic},\eta}$ vs. r for three values of k (torsional force constant of the metal complex when not in the protein). The solid circles (●) are $\Delta H°$ (measured) $- W$ (calculated electrostatic contribution) from Table 5. The dashed line is a least squares fit to the solid circles.

$\Delta H°$ for umecyanin (not in the literature). For the five proteins in Table 3 for which both $\Delta G°$ and $\Delta H°$ have been measured, the average $T\Delta S°$ is -30.6 kJ mol^{-1} and the range ± 9 kJ mol^{-1}. It is reasonable then to expect that, for umecyanin, $\Delta H°$ $(= \Delta G° + T\Delta S°) = -58 \pm 9$ kJ mol^{-1}.

There are electrostatic and elastic components of $\Delta H°$.

$$\Delta H° = \Delta H_{\text{elastic},\eta} + \Delta H_{\text{elastic,other}} + W + \Delta H_{\text{electrostatic,other}} \quad (11)$$

where W is the electrostatic work (energy) required to bring the electron from infinity to the acceptor site through the electric field due to the net charge on the protein. This energy is the same as the electrostatic energy required to remove a proton when a protein is titrated (with base), except that the final location of the electron and initial location of the proton determine interactions that depend upon details of the protein structure [19]. $\Delta H_{\text{electrostatic,other}}$ includes changes in bond energy which are not of elastic origin. In the formulation of Tanford [20]

$$W = -2RTwZ \quad (12)$$

where Z is the net charge on the protein molecule and w is a parameter which, because it depends upon dielectric constant, the size of the protein, and the Debye–Hückel parameter, is not exactly inversely proportional to the temperature. At 298 K, $w \approx 0.1$ and $W \approx 0.5\ Z$ kJ mol^{-1}. At low pH, Z is the positive charge corresponding to the number of sites on side chains, and to the terminal amino group, with which protons are associated. With increasing pH, Z decreases as protons dissociate from ionizable groups in accordance with [20]

$$\log\{x_l/(1-x_l)\} = \text{pH} - \text{p}K_{\text{intr},l} + 0.868\,wZ \quad (13)$$

where x_l is the fractional degree of dissociation of group l.

Hydrogen ion titration curves are not yet available for the blue cupric proteins. However, the p$K_{\text{intr},l}$ of ribonuclease from Table 30-1 of Tanford [20] can be shown to be consistent with the known pIs (pI ≡ isoelectric pH) [1,22–25] and amino acid compositions [26] of the blue cupric proteins. This check was performed by dropping $0.868\,wZ$ from Eq. (13) and summing over all the ionizable groups, increasing the pH until $Z = 0$ and comparing that pH with the published measured value of pI. In the pH range in which the cupric complex is fully formed, it has a net charge of $+1$; i.e. the two imidazoles and one sulfhydryl involved in the complex are deprotonated (and are not being titrated as the pH is raised further). Charge from bound salt ions is taken to be negligible within the pH bounds of the calculations. The codes used for this calculation and, below, for obtaining Z at pH 7 derive from the program of Brill and Venable [27] for computing proton dissociation functions of insulin. Two of the columns of Table 4 give the measured values of pI and those calculated with $0.868\,wZ = 0$ for the four proteins treated in this paper. For stella-

Table 3
Thermodynamic parameters for electron transfer at pH 7.0, 100 mM PO_4

Protein	At 298 K		ΔH° (kJ mol^{-1})	At 298 K $T\Delta S^\circ$ (kJ mol^{-1})	ΔS° (J/K mol^{-1})
	E_0 (mV)	ΔG° (kJ mol^{-1})			
Stellacyanin					
Rhus vern.[a]	191	−18.4	−43.1	−24.7	−82.9
Umecyanin					
horseradish[b]	283 (in 20 mM Na cacodylate)	−27.3	n.a.[d]	n.a.	n.a.
Azurin					
Alc. faec.[c]	266	−25.7	−62.7	−37.0	−124.2
Alc. denitr.[c]	276	−26.6	−55.6	−29.0	−97.3
Ps. aerug[a]	308	−29.7	−69.4	−39.7	−133.2
Plastocyanin					
french bean[a]	360	−34.7	−57.3	−22.6	−75.8

[a] Taniguchi et al. [9].
[b] Stigbrand [11].
[c] Ainscough et al. [13].
[d] From $\langle T\Delta S^\circ \rangle = -30.6$ kJ mol^{-1}, range $= \pm 9$ kJ mol^{-1}, expect that ΔH° for umecyanin is in the range -58 ± 9 kJ mol^{-1}.

cyanin it is known that there are a total of 16 aspartate plus asparagine residues, but the distribution is somewhat uncertain; it can be seen by comparing $pI_{measured}$ with $pI_{calculated}$ for the three allowed possibilities in Table 4 that the first stellacyanin entry (nine aspartates) is favored over the other two. Note that the heterogeneity in the covalent structure of umecyanin involves only residues with non-ionizing sidechains [28]. Agreement between $pI_{calculated}$ and $pI_{measured}$ is good for

Table 4
Measured and calculated values of the isoelectric pH of proteins of known amino acid composition

Protein	Numbers of ionizable groups[a]							$pI_{measured}$	pI calculated with $0.868\ wZ = 0$	pI calculated with $0.868\ wZ = 0.2\ (pI_{measured} - pH)$
	D	E	Y	C	H	K	P			
Stellacyanin								9.86[b]		
Case 1	9	0	7	3	4	10	4		9.66	9.57
Case 2	10	0	7	3	4	10	4		9.51	9.41
Case 3	11	0	7	3	4	10	4		9.36	9.22
Umecyanin	10	5	4	3	3	8	3	5.85[c]	5.50	5.41
Azurin	11	4	2	3	4	11	1	5.4[d]	6.10	6.27
Plastocyanin	5	9	3	1	2	5	0	4.2[e]	4.60	4.69
$pK^f_{int\ r}$	4.70	4.70	9.95	10.00	6.50	10.20	12.20			

[a] Protein Data Bank [26].
[b] Reinhammar [22].
[c] Stigbrand [23].
[d] Horio et al. [24].
[e] Sykes [25].
[f] Tanford [20].

all four proteins, indicating that, at least in the pH region around the pI values, the $pK_{intr,l}$ can be used for approximate calculations.

Values for W can now be obtained. For ribonuclease (13 700 Da) and for the insulin dimer (12 400 Da), proteins of the same size as the cupric blue proteins, the average rate of decrease in net charge in the pH range 4–10 is 2.3 per unit increase in pH [[20], Fig. 30-5; [21], Table I]. With .868 $w \approx .0868$, Eq. (13) becomes

$$\begin{aligned}\log\{x_l/(1-x_l)\} &= \mathrm{pH} - pK_{intr,l} \\ &\quad + 0.2(\mathrm{pI} - \mathrm{pH}) \\ &= 0.8\ \mathrm{pH} - pK_{intr,l} \\ &\quad + 0.2\ \mathrm{pI}. \end{aligned} \tag{14}$$

In applying Eq. (14), essentially the same result is obtained whether one uses the published (measured) values for pI or the values computed in the preceding paragraph. This can be seen in two ways: (1) the results of calculations of the pH at which $Z = 0$, with $\mathrm{pI} = \mathrm{pI}_{measured}$ in Eq. (14) (and the same $pK_{intr,l}$ used above), are given in the final column of Table 4 and differ very little from those of the preceding column; (2) the results of calculations of W at pH 7.0 from Eq. (14) with $\mathrm{pI} = \mathrm{pI}_{measured}$ and with $\mathrm{pI} = \mathrm{pI}_{calculated}$ are given in the second and third columns respectively of Table 5, and the values obtained are essentially the same. Table 5 also presents in the final two columns, for each of the four proteins (rows): $\Delta H°$ (experimentally-determined change in enthalpy, Table 3) reduced by W (average of the values in the preceding two columns), and r [experimentally-determined band intensity ratio parameter of Eq. (8)].

5. Conclusion

The entries for $\Delta H° - W$ in Table 5 are plotted as the solid circles in Fig. 1 vs. the corresponding r. According to Eq. (11), $\Delta H° - W = \Delta H_{elastic,\eta} + \Delta H_{elastic,other} + \Delta H_{electrostatic,other}$ in which $\Delta H_{elastic,\eta}$ is the function of the unknown k given by Eq. (3), and $\Delta H_{elastic,other} + \Delta H_{electrostatic,other}$ is expected to be essentially independent of the out-of-planarity angle η. This leads one to define

$$\Delta H_{other} \equiv \Delta H_{elastic,other} + \Delta H_{electrostatic,other}. \tag{15}$$

With ΔH_{other} taken to be a constant and η given in terms of r by Eq. (10), a non-linear least squares fit of

$$\Delta H_{fit} \equiv \Delta H_{other} + kq_D[0.5q_D(1 - k/k_{PA}) - \eta] \tag{16}$$

to the solid circles of Fig. 1 produced the dashed curve in excellent agreement, and the parameters $\Delta H_{other} = -37.6(5)$ kJ mol^{-1}, $k = 0.79(6) \times 10^4$ mN m^{-1}. The resulting $\chi^2 \equiv \Sigma_i[(\Delta H° - \mathrm{W}) - \Delta H_{fit}]^2_{protein\,i}/(N_i - N_{terms})$, with $N_i = 4$ and $N_{terms} = 2$, is 2.44 kJ^2 mol^{-2}.

The goodness of the fit indicates that $\Delta H_{elastic,other}$ and $\Delta H_{electrostatic,other}$ depend very little upon η, as expected. The value of k is realistic; for example, it is hardly different than the force constant of 0.82×10^4 mN m^{-1} for the carbon–metal–carbon bending mode of tetrahedral nickel carbonyl, $Ni(CO)_4$, complex in solu-

Table 5
Enthalpic and optical parameters

Protein	W calculated with $0.868\ wZ = 0.2\ (\mathrm{pI}_{measured} - \mathrm{pH})$[a] (kJ mol^{-1})	W calculated with $0.868\ wZ = 0.2\ (\mathrm{pI}_{calculated} - \mathrm{pH})$[a] (kJ mol^{-1})	$\Delta H° - W$ (kJ mol^{-1})	$r = \frac{(\lambda * f)_{620\ nm\ band}}{(\lambda * f)_{450\ nm\ band}}$
Stellacyanin	−2.87	−2.89	−40.2	5.4
Umecyanin	1.31	1.27	−59.(3)	10.6
Azurin	0.56	0.67	−70.0	17.4
Plastocyanin	3.90	3.92	−61.2	13.0

[a] W is electrostatic work done at pH 7.0 in bringing a distant electron to a uniformly charged protein surface.

tion [29]. In the case of stellacyanin, $\Delta H_{\text{elastic},\eta}$ is a very small fraction of the total change in enthalpy, $\Delta H°$. For umecyanin, azurin and plastocyanin, the elastic contributions from the out-of-plane torsional mode are substantial fractions of $\Delta H°$. The out-of-planarity angles η for the four proteins are within the range 17–21° which, at a bond length of 2 Å, corresponds to an atomic displacement range of only .14 Å. While the need for more data is apparent, the measurements and analysis at hand enable one to conclude, tentatively, that: (1) by means of the structural feature η, which determines the size of the torsional elastic energy contribution with great sensitivity, the protein exerts considerable control over the change in enthalpy accompanying electron transfer; and (2) the same structural feature determines the relative strengths of two characteristic absorption bands in the visible spectra of the blue cupric proteins.

5.1. Responses to comments of the reviewers

All but one of the comments are suggestions for additional references.

Response to first referee:

(1) '... rack mechanism introduced … by Lumry and Eyring [J. Phys. Chem. 58 (1954) 110–120]'.

The rack mechanism concept which Lumry and Eyring pioneered is cited as Lumry and Eyring [3] in Brill [4]. The earlier Brill paper [4], also mentions Lumry et al., J. Am. Chem. Soc. 84 (1962) 157–231. The present manuscript relates *light absorption* and an enthalpy change to a sterochemical constraint, a relation considered by Williams and his coauthors but not by Lumry and his (who also do not deal with the class of proteins which is the subject of the paper at hand).

(2) '... application of the rack concept is presented in Winkler et al. [Proc. Natl. Acad. Sci. USA 94 (1997) 4246–4249]...; ... Wittung-Stafshede et al. [J. Biol. Inorg. Chem. 3 (1998) 367–370]....'.

These two papers report the results of measurements of redox potential on unfolded (by guanidine hydrochloride) and folded cytochrome *c*, azurin (blue when cupric), and the copper protein from *Thermus thermophilus* (purple when cupric). Enthalpy changes are not mentioned, nor are optical or magnetic measurements on the unfolded proteins. Large electrostatic changes in going from the folded to the unfolded state, and 'increasing axial interaction' in going from Cu(I) ('favoring a linear or trigonal structure') to Cu(II) ('preferring a tetragonal geometry') are discussed; these effects are not modeled quantitatively.

(3) '... recent theoretical work indicating that there is no strain in oxidized blue sites [Ryde et al., J. Mol. Biol. 261 (1996) 586–596, and several later papers by the same authors]'.

These theoretical papers present the results of quantum chemical computations on site models consisting of 40 or less atoms. When considered, the effect of the surrounding protein and solvent is estimated by point charges. Employing a difference in calculated energy of 'less than 5 kJ mol^{-1}, i.e within the error limits of the method' (uncertainty ± 5 kJ mol^{-1} ?), as the criterion for absence of strain between the geometrically distinct oxidized and reduced (computed) structures, the authors conclude that mechanical control by the protein is not significant. The latter viewpoint is one with which those dealing with function may not agree. Analogous (apart from sign) energy differences can be obtained from the manuscript at hand by subtracting $\Delta H_{\text{other}} = -37.7$ kJ mol^{-1} [just below Eq. (16)] from the $\Delta H° - W$ entries of Table 5; these range from −2.5 for stellacyanin to -32.3 kJ mol^{-1} for azurin. Two of the papers in this series by Ryde and colleagues deal with spectroscopic properties of plastocyanin and stellacyanin. The abstract of the stellacyanin paper [De Kerpel et al., J. Phys. Chem. B (1998) 4638] states '... The ground state singly occupied orbital is... predominantly π antibonding involving Cu3d and $S_{cys}3p_{\pi}$. However it also contains a significant amount (18%) of Cu–S_{cys} σ antibonding character. This σ interaction is responsible for the appearance in the absorption spectrum of a band at 460 nm, with a significantly higher intensity than observed for other, axial, type 1 copper proteins (i.e. plastocyanin or azurin)...' This description is reminiscent of that of LaCroix et al. [LaCroix et al., J. Am. Chem. Soc. 118 (1996) 7755] who deal with the Type 1 site of nitrite reductase, which also has strong absorption at 460 nm, and state '... HOMO rotation increases the

psuedo-σ interaction and decreases the π interaction of the cysteine (Cys) sulfur with Cu $d_{x^2-y^2}$. Furthermore, significant methionine (Met) sulfur character is mixed into the HOMO due to increased overlap with Cu $d_{x^2-y^2}$.... Additionally, the new S(Met)–Cu interaction accounts for the unexpectedly high sulfur covalency in the HOMO. ...' However, Veselov et al. [Biochemistry 37 (1998) 6095], reporting the results of ENDOR measurements on the Type 1 site of nitrite reductase and a mutant form which does not have the unusually strong absorption at 460 nm, say in their abstract '... Surprisingly, in (the mutant form) there was *no change* from the native Type 1 copper either in the histidine or cysteine hyperfine couplings... '. The discrepancies in covalency between salient (placed in the abstracts) predictions of the quantum chemical computations and the experimental results discourage one from dwelling upon results from the electronic structure calculations/models which, apparently, leave out some important feature(s) of the sites as they are in the proteins and/or have some technical limitation(s).

6. Response to second referee:

(1) '... it may be of interest to include nitrite reductase,... (see L.B. LaCroix et al., J.A.C.S. 118 (1996) 7755... '

Reference is made in the preceding paragraph to LaCroix et al. (1996), and Veselov et al. (1998), papers that deal with nitrite reductase.

(2) '... intensity of the optical absorption bands in the visible... it may be useful to provide... more information on their assignment. This will also introduce a problem that is not addressed in the manuscript, i.e. that the intensity and energy of the absorption bands may not depend only on the out of plane distortion.... '

Within the context of the model employed, the assignment of the optical transitions is apparent in Table 2. Possibly the referee would like to see chemically-defined molecular/bond orbitals associated with the symmetry-labeled states of Table 2, but a description of this kind which satisfies all the experimental information is not available at present. As the referee implies, the intensity (and energy) of the absorption bands do not depend only upon out-of-plane distortion; i.e. the radial parts of the orbitals involved in Eq. (5) do not depend directly upon out-of-planarity. Useful information relevant to atoms and radial factors involved in the transitions has been obtained from a line of experiments originating in the laboratory of H.B. Gray and often discussed in terms of charge-transfer bands (e.g. Tennent and McMillin, J. Am. Chem. Soc. 101 (1979) 2307).

(3) '... Gewirth et al., J.A.C.S. 110 (1988) 3811... suggested that both the length of the Cu–S(cys) bond and the S(met)–Cu–S(cys)–C(cys) dihedral angle can play a role in defining the spectroscopic properties of blue copper sites'.

The reservations expressed above about the quantum chemical computations that have been carried out for models of the cupric site in blue proteins apply here as well. Furthermore, this electronic structure calculation of Gewirth and Solomon proceeds from the statement '... Single-crystal EPR spectroscopy on plastocyanin in conjunction with a crystal field calculation indicated that the blue copper site was best described as having elongated C_{3v} effective symmetry... '. In contrast, from a later, more complete, single-crystal study on azurin [Coremans et al., J. Am. Chem. Soc. 116 (1994) 3097], it is concluded that '... The g-tensor does not corroborate descriptions of the copper site in terms of effective C_{3v}, C_{2v}, or C_s symmetry....'.

References

[1] A.G. Sykes, Adv. Inorg. Chem. 36 (1991) 377–408.
[2] A.S. Brill, R.B. Martin, R.J.P. Williams, in: B. Pullman (Ed.), Electronic Aspects of Biochemistry, Academic Press, New York, 1964, p. 519.
[3] B.L. Vallee, R.J.P. Williams, Proc. Natl. Acad. Sci. USA 59 (1968) 498–505.
[4] A.S. Brill, Mol. Phys. 85 (1995) 727–734.
[5] A.S. Brill, in: B. Chance et al. (Eds), Tunneling in Biological Systems, Academic Press, New York, 1979, p. 561.
[6] A.S. Brill, Transition Metals in Biochemistry, Ch. 3, Sections 3 and 4, Springer-Verlag, Berlin, Heidelberg, New York, 1977.
[7] A. Aqualino, A.S. Brill, G.F. Bryce, B.S. Gerstman, Phys. Rev. A 44 (1991) 5257–5271.

[8] A.S. Brill, Biophys. J. 22 (1978) 139–142.
[9] V.T. Taniguchi, N. Sailasuta-Scott, F.C. Anson, H.B. Gray, Pure Appl. Chem. 52 (1980) 2275–2281.
[10] T. Stigbrand, I. Sjöholm, Biochim. Biophys. Acta 263 (1972) 244–257.
[11] T. Stigbrand, FEBS Lett. 23 (1972) 41–43.
[12] A. Aqualino, Structural Distributions in Azurin, Ph.D. Thesis, University of Virginia (1982), Table II.
[13] A.G. Sykes, Chem. Soc. Rev. 14 (1985) 283–315.
[14] B.S. Gerstman, A.S. Brill, J. Chem. Phys. 82 (1985) 1212–1230.
[15] B.S. Gerstman, A.S. Brill, Phys. Rev. A 37 (1988) 2151–2164.
[16] B.S. Gerstman, A.S. Brill, Phys. Rev. A 39 (1989) 5903–5913.
[17] G.F. Bryce, A.S. Brill, J. Chem. Phys. 48 (1968) 4398–4404.
[18] E.W. Ainscough, A.G. Bingham, A.M. Brodie et al., Biochemistry 26 (1987) 71–82.
[19] A.-S. Yang, M.R. Gunner, R. Sampogna, K. Sharp, B. Honig, Proteins 15 (1993) 252–265.
[20] C. Tanford, Physical Chemistry of Macromolecules, Ch. 8, Section 30d, Wiley, New York, London, 1961.
[21] C. Tanford, J. Epstein, J. Am. Chem. Soc. 76 (1954).
[22] B. Reinhammar, Biophys. Biochim. Acta 205 (1970) 35–47.
[23] T. Stigbrand, Biophys. Biochim. Acta 236 (1971) 246–252, 2163–2169.
[24] T. Horio, I. Sekuzu, T. Higashi, K. Okunuki, Haematin enzymes, Int. Union Biochem. Symp. 19 (1959) 302–311.
[25] A.G. Sykes, Struct. Bonding 75 (1990) 175–233.
[26] Protein Data Bank, Brookhaven National Laboratory (1998).
[27] A.S. Brill, J.H. Venable, Jr., J. Am. Chem. Soc. 89 (1967) 3622–3626.
[28] G. Van Driessche, C. Dennison, A.G. Sykes, J. Van Beeumen, Protein Sci. 4 (1995) 209–227.
[29] L.H. Jones, R.S. McDowell, M. Goldblatt, J. Chem. Phys. 48 (1968) 2663–2670.