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Chemistry

Preparation and characterization of pentaammineruthenium-(histidine-83)azurin: Thermodynamics of intramolecular electron transfer from ruthenium to copper

(ruthenium–histidine binding/protein modification/blue copper/oxido-reduction/reorganization energy)

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ABSTRACT **The reaction between $a_5RuH_2O^{2+}$ (a is NH_3) and *Pseudomonas aeruginosa* azurin at pH 7, followed by oxidation, yields $a_5Ru(His\text{-}83)^{3+}$-azurin($Cu^{2+}$) as the major product. Spectroscopic measurements (UV-visible, CD, EPR, and resonance Raman) indicate that the native structure is maintained in the modified protein. The site of ruthenium binding (His-83) was identified by peptide mapping. The a_5RuHis/Cu ratio in the modified protein, determined from the EPR spectrum, is 1:1, and the reduction potentials (vs. normal hydrogen electrode, pH 7.0, 25°C) are blue copper ($Cu^{2+/1+}$), 320 ± 2 mV; $a_5Ru(His\text{-}83)^{3+/2+}$, 50 ± 10 mV. From measurements of the reduction potentials at several temperatures in the 5–40°C range, ΔH° for intramolecular $Ru^{2+} \rightarrow Cu^{2+}$ electron transfer was estimated to be −12.4 kcal mol^{-1} (1 cal = 4.184 J). Analysis of kinetic data in light of the electron transfer exothermicity indicates that the reorganizational enthalpy of the blue copper site can be no larger than 7.1 kcal mol^{-1}.**

Electron transfer reactions of metalloproteins have been studied extensively in several laboratories in recent years (1–5). An important conclusion from these studies is that the rate of electron transfer depends, among other factors, on the distance between the redox sites involved (ref. 6 and references therein). In an attempt to shed additional light on this subject, we have begun to study the electron transfer kinetics of metalloproteins in which redox-active metal complexes are bonded to histidines on the polypeptide chains. The redox centers in these modified proteins are fixed and their distances may be estimated fairly accurately from the crystal structures of the corresponding native proteins.

In previous work we have employed methods similar to that of Matthews *et al.* (7, 8) to prepare pentaammineruthenium derivatives of horse heart cytochrome *c* (9, 10) and sperm whale myoglobin (11). Although the ruthenium redox site in the modified cytochrome *c* is well over 10 Å from the heme site, kinetic studies have established that the intramolecular $Ru^{2+} \rightarrow Fe^{3+}$ electron transfer rate is at least 30 s^{-1} (10, 12, 13).

We report here the preparation and characterization of a derivative in which an a_5Ru^{3+} (a is NH_3) group is bonded to the His-83 residue in *Pseudomonas aeruginosa* azurin (Az), a well-characterized blue copper protein that acts as an electron transfer agent in the bacterial respiratory chain (refs. 14 and 15 and references therein). Interpretation of the kinetics of intramolecular electron transfer (16) in this modified protein has been aided by our studies of the temperature dependences of the reduction potentials of both the $Cu^{2+/1+}$ and the $Ru^{3+/2+}$ sites.

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MATERIALS AND METHODS

P. aeruginosa Az, obtained from the Public Health Laboratory Service in Great Britain, has an A_{625}/A_{280} ratio of 0.58. Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was obtained from Worthington. Baker reagent-grade acetonitrile was used for HPLC. Ampholine plates for isoelectric focusing in the pH range 3.5–9.5 were obtained from LKB. Column chromatography of the modified Az was carried out with Whatman CM52 cellulose. $[a_5RuH_2O](PF_6)_2$ was prepared according to a published procedure (17) and Na[Co(EDTA)] was prepared by a slight modification (Na_2CO_3 instead of $BaCO_3$) of the published method (18). Distilled and deionized water was used throughout. Ultrafiltration was performed with Amicon YM-5 membranes, under argon with gentle stirring at 5°C.

Az Modification and Chromatography. Az was allowed to react with a 50-fold excess of freshly prepared $[a_5RuH_2O](PF_6)_2$ under argon at room temperature in 50 mM Tris buffer (pH 7.2) for 3 hr. In a typical experiment, a deaerated solution of 27 mg of the ruthenium reagent in Tris buffer was added anaerobically to a deaerated solution of 14 mg of Az in the same buffer, and the reaction mixture was deaerated briefly by evacuation and argon and left under a constant pressure of argon without bubbling through the liquid. The total volume was 10 ml, so that the concentration of the protein was 0.1 mM and of the ruthenium reagent, 5 mM. Small variations in the reagent ratio, concentrations, pH, or reaction time did not affect the properties of the modified protein and affected the yields only slightly. To stop the reaction, the ruthenium reagent was removed by repeated ultrafiltration of the reaction mixture into water or 50 mM NH_4OAc buffer (pH 4.0). Solution of *ca.* 15 mg of Na[Co(EDTA)] in *ca.* 1 ml of water or this buffer, about 15-fold excess with respect to both Cu and Ru, was added to oxidize the concentrated protein. The oxidation was allowed to proceed for several hours in the unbuffered solution or overnight in the NH_4OAc buffer. The oxidant was then removed by ultrafiltration into the acetate buffer (pH 4.0). Recovery of the protein from the reaction was 98–100%.

All three components of the reaction mixture were separated efficiently by cation-exchange chromatography on a 2 × 5 cm column of CM52 cellulose, previously equilibrated with 50 mM NH_4OAc buffer (pH 4.0). The separation was carried out at room temperature and the elution rate was *ca.* 100 ml/hr. The first fraction (A) was eluted quickly with 50 mM buffer and elution with this buffer continued until the

Abbreviations: a, NH_3; NHE, normal hydrogen electrode; Az, azurin.

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second band reached the bottom of the column. This second fraction (B) was then eluted with 75 mM NH_4OAc buffer (pH 4.0). The third fraction (C), which had remained near the top of the column, was then eluted with 0.15 M NaCl solution in 50 mM NH_4OAc buffer (pH 4.0). The average yields are as follows: fraction A, 4.0 mg or 27%; fraction B, 4.9 mg or 34%; fraction C, 2.7 mg or 18%. The isoelectric points (pI values in pH units) are the following: fraction A, 5.4; fraction B, 7.2; fraction C, 8.1. The pI values did not change after exhaustive dialysis by ultrafiltration.

Peptide Mapping. Procedures described elsewhere (9) for tryptic hydrolysis, separation of the resulting peptides by reversed-phase HPLC, and amino acid analysis were used with minor modifications. A sample containing 5–6 mg of fraction B in 2 ml of water was denatured for 4 min in boiling water prior to tryptic hydrolysis. The linear gradient for the HPLC separation of the tryptic peptides consisted of 45 ml each of phosphate buffer (49 mM KH_2PO_4/5.4 mM H_3PO_4, pH 2.85) and acetonitrile, the concentration of the latter increasing from 0% to 45%; the elution rate was 1 ml/min. The absorbances were recorded at 220 nm (peptide bonds) and at 300 nm, which is near the absorption maximum (303 nm) of a_5RuHis^{3+} (19).

Spectra. The following instruments were used for physical measurements and characterization: Cary 219 and Cary 17 for the UV-visible absorption; Cary 60 for CD; and Varian E-line Century-series X-Band EPR spectrometer for the EPR spectra at 9 K. Resonance Raman spectra were obtained with a Spex Industries model 14018 double monochromator equipped with 2400-line/mm holographic gratings and a Hamamatsu R955 photomultiplier tube; the excitation (622 nm) sources were model 170 argon-ion and model 375-A dye (rhodamine 6G) lasers, produced by Spectra-Physics (Mountain View, CA); spectral bandpass was 3 cm^{-1}. The spectra were taken at the ambient temperatures of 0°C and 24°C, with the scan times of 4000 and 4800 s, respectively. Cyclic voltammograms were obtained with a PAR 173 potentiostat and PAR 175 Universal instrument, coupled with a Houston Instruments X-Y recorder model 2000. The concentrated solution of fraction B was allowed to evaporate on the surface of the working graphite electrode; the reference electrode was a saturated NaCl calomel electrode and the solvent was 0.100 M phosphate buffer (pH 7.0). The experiments were carried out at room temperature and the scan rate was 100 mV/s. The spectroelectrochemical measurements from 5°C to 40°C were carried out with an optically transparent thin-layer electrochemical (OTTLE) cell in a nonisothermal configuration. The apparatus, sample preparation, and mediator, $[a_5Rupy](ClO_4)_3$, were the same as in the experiment with native Az (20).

RESULTS AND DISCUSSION

The Reaction and pI Values. The pI of fraction A (5.4) is the same as that of native Az under identical conditions, which indicates that this fraction consists of unreacted protein. The higher pI of fraction B (7.2) is compatible with the presence of additional positively charged group(s) in the protein. [The pI of sperm whale myoglobin (11) increases similarly when it is modified with a_5Ru^{3+}.] Since exhaustive dialysis (by ultrafiltration) of fraction B did not affect its pI and since isoelectric focusing did not show a separate band attributable to the species with high positive charge (the ruthenium complex), we conclude that the ruthenium reagent is strongly bound to, and not merely adsorbed on, the protein. The relatively good yield (>30%) of the modified protein in fraction B may be due in part to electrostatic attraction at pH 7.2 between the $a_5RuH_2O^{2+}$ complex and Az.

The yield of fraction C increased when the reaction time was prolonged. The pI of 8.1 is compatible with the presence of >1 equivalent of the ruthenium complex in this modified Az.

Spectroscopic Characterization. The EPR and resonance Raman spectroscopic properties of the native (21) and modified (fraction B) Az are listed in Table 1. Evidently the electronic and geometric structures of the blue copper site are unaffected by the protein modification. Since the resonance Raman frequencies are the same at 0°C and 24°C, the copper site also must be insensitive to small changes in temperature. The CD spectra from 280 to 700 nm of the fully oxidized native and modified proteins and from 200 to 350 nm of the fully reduced proteins are similar to each other, which indicates that labeling with the ruthenium complex does not perturb the protein conformation appreciably.

The UV-visible spectra of the native and modified Az are shown in Fig. 1 *A* and *B*, respectively. The sole qualitative difference between them is a shoulder at *ca.* 300 nm in the modified protein; it can be attributed to the a_5RuHis^{3+} chromophore, whose band maximum is at 303 nm (19). The A_{625}/A_{280} ratio for fraction B from every preparation is 0.50 or greater, which is an indication of purity. Since the a_5RuHis^{3+} chromophore absorbs appreciably in the region about 280 nm (19), this ratio is smaller than the corresponding value for native Az (0.58).

To elucidate the effect of Az labeling upon its absorption spectrum, we normalized spectra in Fig. 1 *A* and *B* and subtracted the former from the latter. The resulting difference spectrum (Fig. 1*C*) can be compared with the spectrum of the $[a_5RuHis]Cl_3$ solution (Fig. 1*D*), which is equimolar with the native protein solution. The band maxima at 299 and 302 nm in Fig. 1 *C* and *D*, respectively, indicate unambiguously that a histidine residue is labeled with pentaammineruthenium in the modified protein. Moreover, their nearly identical absorbances indicate that the a_5RuHis/protein molar ratio is 1:1. However, the difference spectrum contains an additional band at *ca.* 258 nm, which is absent from the spectrum of a_5RuHis^{3+}. If not an artifact of the normalization and subtraction procedures, this band might be due to pentaammineruthenium-labeled methionine residue(s) or to some compound in solution, not attached to the protein.

Modified Site. *P. aeruginosa* Az contains four histidines and six methionines per molecule. Because His-46, His-117, and Met-121 are coordinated to the copper atom (22), two histidines and five methionines are potential sites of covalent binding to the a_5Ru^{2+} complex (10). The method for identification of the binding site by peptide mapping has been described in detail elsewhere (9), so only the salient features of this analysis, as applied to fraction B, will be presented here. Since only the peptide that eluted at *ca.* 12 ml in the high-performance liquid chromatogram of the tryptic hydrolyzate absorbs significantly at 300 nm, we concluded that only this peptide contains an $_5RuHis^{3+}$ group. The similarity of the absorption spectra of this labeled peptide (Fig. 2*A*) and of a_5RuHis^{3+} (Fig. 2*B*) proves that labeling takes place at a histi-

Table 1. Spectroscopic properties of the blue copper sites in native and modified Azs

Spectroscopic property	Native Az	Fraction B
EPR		
$g_{\|\|}$	2.271	2.271
$a_{\|\|}$(G)	57	57
g_x	2.065	2.065
g_y	2.043	2.044
Resonance Raman, $\nu(cm^{-1})$	369*	370
	404*	404
	424*	425

*Ref. 21.

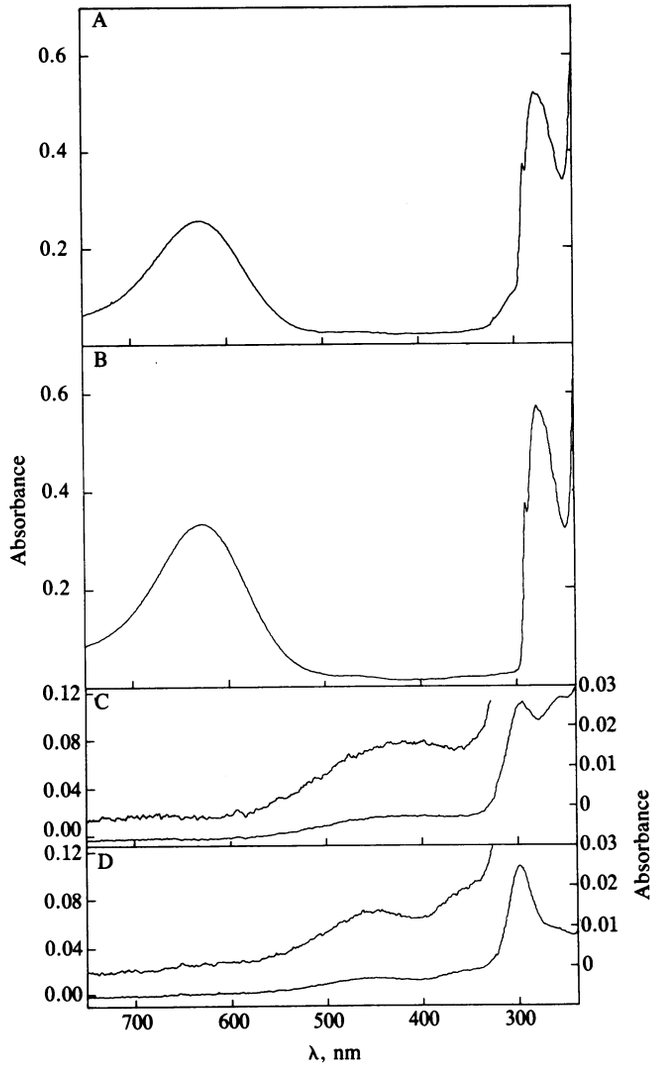

FIG. 1. (*A*) Absorption spectrum of a 40 μM solution of a_5Ru-(His-83)$^{3+}$-Az(Cu^{2+}). (*B*) Absorption spectrum of a 60 μM solution of native Az[Az(Cu^{2+})]. (*C*) Difference between the normalized spectra of modified and native Az. (*D*) Absorption spectrum of a 60 μM solution of [a_5RuHis]Cl$_3$. The absorbance scale for the upper traces in *C* and *D* is expanded 5-fold and shown on the right.

dine residue. The maximum at *ca.* 260 nm in the peptide spectrum declines and eventually disappears upon bubbling oxygen through the solution. This observation is compatible with an assignment of this maximum to a_5RuHis^{2+}, a chromophore that indeed has a band at 260 nm (19). The amino acid analysis showed this peptide to contain valine, isoleucine, alanine, histidine, threonine and lysine, a composition that fits exactly the segment 80–85 of *P. aeruginosa* Az (23). We conclude that a pentaammineruthenium group is attached to the imidazole ring of His-83. This Ru—N bond in a_5Ru(His-83)-Az evidently is not affected by denaturation of the sample at 100°C and by conditions under which tryptic hydrolysis is carried out (9).

The structure of Az (22) reveals that the imidazole of His-83 is located on the surface of the protein and protrudes into the surrounding medium (Fig. 3) (24). This exposure makes the imidazole easily accessible to the labeling reagent in solution. Several aspartic acid and glutamic acid residues are found in the vicinity of His-83; their negative charges presumably facilitate the attack of $a_5RuH_2O^{2+}$ at His-83. The N1 atom in the imidazole ring of His-83 and the S atom of Cys-112 (a ligand to Cu) are 11.8 Å apart, so the Ru^{3+} and Cu^{2+} sites are well isolated from each other in the modified protein.

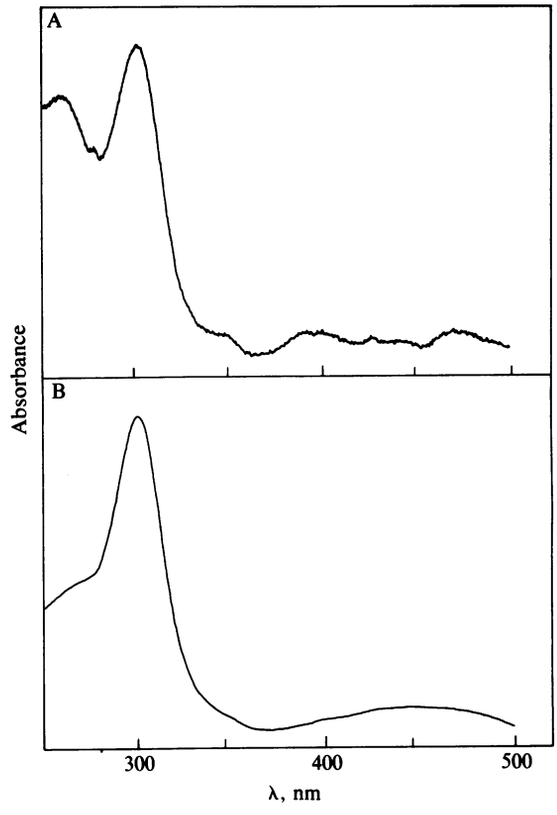

FIG. 2. (*A*) Absorption spectrum of the hexapeptide containing His-83 (Val-Ile-Ala-His-Thr-Lys), obtained by tryptic hydrolysis of the modified Az and HPLC separation of the resulting peptides. (*B*) Absorption spectrum of a 0.3 mM solution of [a_5RuHis]Cl$_3$.

The Cu/Ru Ratio. To determine the number of a_5RuHis units per molecule of the modified Az (fraction B), we compared its EPR spectrum (Fig. 4*A*) with the EPR spectrum of an equimolar mixture of Az and a_5RuHis^{3+} (Fig. 4*B*). The samples were fully oxidized, so that both Ru and Cu atoms were "EPR-visible." The positions of the Ru peaks in the two spectra are virtually identical (*g* values are 2.956 and 2.951 for the modified protein and the mixture, respectively), thereby confirming that pentaammineruthenium is bound to histidine. The ratio of the Ru^{3+} and Cu^{2+} signal heights is 0.52 in the modified protein and 0.53 in the equimolar mixture. On the basis of these ratios, we conclude that a molecule of the modified Az (fraction B) contains 1 equivalent of a_5RuHis^{3+}—i.e., that His-83 is the only histidine residue labeled.

Reduction Potentials. The reduction potential of blue copper in a_5Ru(His-83)$^{3+}$-Az(Cu$^{2+/1+}$) was determined spectroelectrochemically at pH 7.0 (0.100 M phosphate buffer). The value of 320 ± 2 mV vs. normal hydrogen electrode (NHE) at 25°C is very similar to the reduction potential of native Az, which is 308 ± 2 mV under identical conditions (20). The temperature dependence of the blue copper reduction potential permitted calculation of the thermodynamic parameters. The reduction potential of the ruthenium site in a_5Ru(His-83)$^{3+/2+}$-Az(Cu^{1+}), measured by cyclic voltammetry, was found to be 50 ± 10 mV vs. NHE at 25°C and pH 7.0. This value is somewhat lower than that for free a_5Ru-His$^{3+/2+}$, 80 ± 5 mV (25). Since the difference between these two values is not large, we believe that the thermodynamic redox properties of the ruthenium site in the protein will not differ much from the corresponding properties of a_5Ru-His$^{3+/2+}$, determined by variable-temperature cyclic voltammetry (25). The thermodynamic parameters are listed in Table 2.

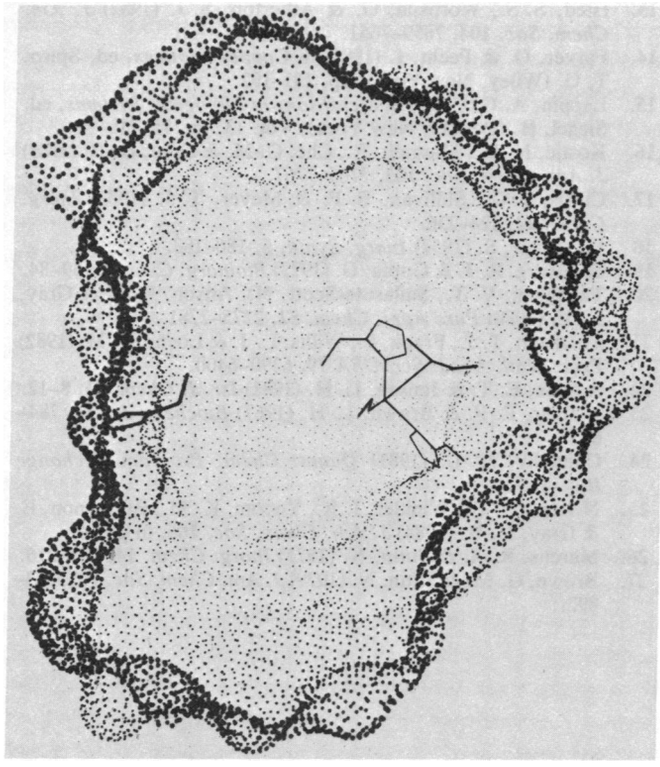

FIG. 3. Surface of Az, with the front section removed to show the imidazole ring of His-83 and the copper atom with its four ligands. The surface consists of points of contact between a sphere of 3-Å radius (chosen to simulate $a_5RuH_2O^{2+}$) and the atomic van der Waals spheres of the protein. The surface was calculated by program MS (ref. 24).

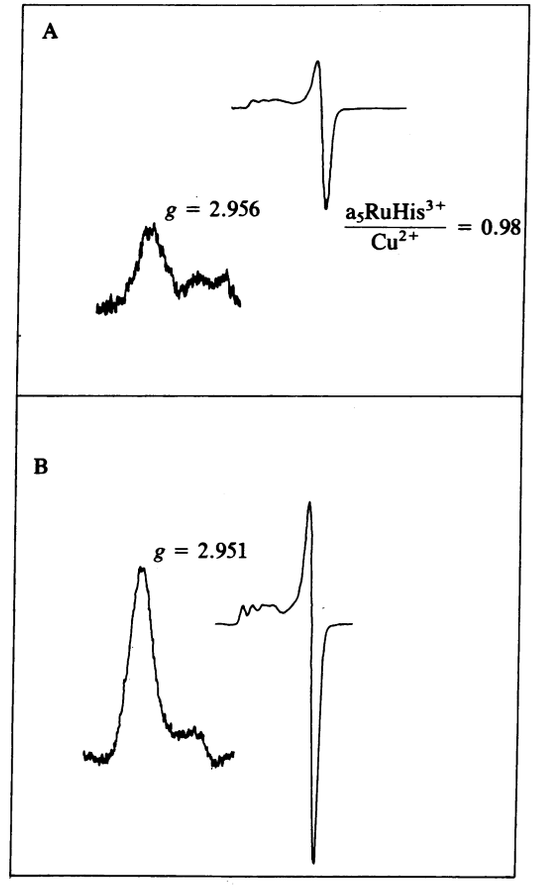

FIG. 4. (*A*) EPR spectrum of $a_5Ru(His\text{-}83)^{3+}$-Az($Cu^{2+}$). (*B*) EPR spectrum of an equimolar mixture of $[a_5RuHis]Cl_3$ and Az(Cu^{2+}).

In a previous paper (16) we reported the kinetics of intramolecular electron transfer from $a_5Ru(His\text{-}83)^{2+}$ to the Az blue copper (Cu^{2+}). The rate constant is $1.9 \pm 0.4\ s^{-1}$ at pH 7.0 and varies only within the experimental error limits from −8°C to 53°C. From these experiments we were able to estimate that the activation enthalpy for intramolecular electron transfer, $\Delta H^{\ddagger}_{obs}$, is <0.80 kcal mol^{-1}. The thermodynamic result is of special interest because for intramolecular electron transfer the observed activation enthalpy is equal to the reorganizational enthalpy (ΔH^{*}_{Cu-Ru}) (25):

$$\Delta H^{\ddagger}_{obs} = \Delta H^{*}_{Cu-Ru}. \quad [1]$$

After Marcus and Sutin (26), we write Eq. 2:

$$\Delta H^{*}_{Cu-Ru} = \frac{1}{2}\left(\Delta H^{*}_{Cu} + \Delta H^{*}_{Ru} + \Delta H^{\circ}_{Cu-Ru}\right), \quad [2]$$

where ΔH^{*}_{Cu} and ΔH^{*}_{Ru} are the reorganizational enthalpies accompanying electron exchange reactions of the blue copper ($Cu^{2+/1+}$) and $a_5RuHis^{3+/2+}$ sites, respectively, and ΔH°_{Cu-Ru} is the standard enthalpy change for the electron transfer reaction. For ΔH^{*}_{Ru} we take 6.9 kcal mol^{-1}, which is the calculated value of the reorganizational energy of electron exchange for $a_5Rupy^{3+/2+}$ (27) and, on the basis of the data in Table 2, ΔH°_{Cu-Ru} is calculated to be −12.4 kcal mol^{-1}. Thus, with $\Delta H^{*}_{Cu-Ru} \leq 0.80$ kcal mol^{-1}, an upper limit of 7.1 kcal mol^{-1} is obtained for the total (inner- and outer-sphere) reorganizational enthalpy of the blue copper site.

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Table 2. Thermodynamic parameters for the reduction of a_5RuHis^{3+} and the blue copper (Cu^{2+}) in native and modified Azs in 0.100 M phosphate buffer (pH 7.0)

Thermodynamic parameter	Native Az($Cu^{2+/1+}$)*	$a_5Ru(His\text{-}83)^{3+}$-Az($Cu^{2+/1+}$)	$a_5RuHis^{3+/2+}$†
E°, mV vs. NHE, 25°C	308 ± 2	320 ± 2	80 ± 5
ΔS°, entropy units	−31.7 ± 1.2	−26.8 ± 0.8	−3.4 ± 0.2
$S^{\circ}_{red} - S^{\circ}_{ox}$, entropy units	−16.1 ± 1.2	−11.2 ± 0.8	12.2 ± 2
ΔG°, kcal mol^{-1}	−7.10 ± 0.05	−7.39 ± 0.05	−1.96 ± 0.12
ΔH°, kcal mol^{-1}	−16.6 ± 0.4	−15.4 ± 0.3	−3.0 ± 0.8

One calorie = 4.184 joules.
*Ref. 20.
†Ref. 25.

1. Wherland, S. R. & Gray, H. B. (1977) in *Biological Aspects of Inorganic Chemistry*, eds. Addison, A. W., Cullen, W. R., Dolphin, D. R. & James, B. R. (Wiley, New York), pp. 289–367.
2. Freeman, H. C. (1981) in *Coordination Chemistry-21*, ed. Laurent, J. P. (Pergamon, Oxford), pp. 29–51.
3. Brunori, M., Colosimo, A. R. & Silvestrini, M. (1983) *Pure Appl. Chem.* **55,** 1049–1058.
4. Moore, G. R. & Williams, R. J. P. (1976) *Coord. Chem. Rev.* **18,** 125–197.
5. Bennett, L. E. (1973) *Prog. Inorg. Chem.* **18,** 1–176.
6. Mauk, A. G., Scott, R. A. & Gray, H. B. (1980) *J. Am. Chem. Soc.* **102,** 4360–4363.
7. Matthews, C. R., Erickson, P. M., van Vliet, D. L. & Petersheim, M. (1978) *J. Am. Chem. Soc.* **100,** 2260–2262.
8. Matthews, C. R., Erickson, P. M. & Froebe, C. L. (1980) *Biochim. Biophys. Acta.* **624,** 495–510.
9. Yocom, K. M., Shelton, J. B., Shelton, J. R., Schroeder, W. A., Worosila, G., Isied, S. S., Bordignon, E. & Gray, H. B. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7052–7055.
10. Yocom, K. M., Winkler, J. R., Nocera, D. G., Bordignon, E. & Gray, H. B. (1983) *Chem. Scripta* **21,** 29–33.
11. Margalit, R., Pecht, I. & Gray, H. B. (1983) *J. Am. Chem. Soc.* **105,** 301–302.
12. Winkler, J. R., Nocera, D. G., Yocom, K. M., Bordignon, E. & Gray, H. B. (1982) *J. Am. Chem. Soc.* **104,** 5798–5800.
13. Isied, S. S., Worosila, G. & Atherton, S. J. (1982) *J. Am. Chem. Soc.* **104,** 7659–7661.
14. Farver, O. & Pecht, I. (1981) in *Copper Proteins*, ed. Spiro, T. G. (Wiley, New York), pp. 151–192.
15. Lappin, A. G. (1981) in *Metal Ions in Biological Systems*, ed. Siegel, H. (Dekker, New York), Vol. 13, pp. 15–71.
16. Kostić, N. M., Margalit, R., Che, C.-M. & Gray, H. B. (1983) *J. Am. Chem. Soc.* **105,** 7765–7767.
17. Curtis, J. C., Sullivan, B. P. & Meyer, T. J. (1983) *Inorg. Chem.* **22,** 224–236.
18. Kirschner, S. (1957) *Inorg. Synth.* **5,** 186–188.
19. Sundberg, R. J. & Gupta, G. (1973) *Bioinorg. Chem.* **3,** 39–84.
20. Taniguchi, V. T., Sailasuta-Scott, N., Anson, F. C. & Gray, H. B. (1980) *Pure Appl. Chem.* **52,** 2275–2281.
21. Thamann, T. J., Frank, P., Willis, L. J. & Loehr, T. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6390–6400.
22. Adman, E. T. & Jensen, L. H. (1981) *Isr. J. Chem.* **21,** 8–12.
23. Ambler, R. P. & Brown, L. H. (1967) *Biochem. J.* **104,** 784–825.
24. Connolly, M. L. (1981) *Quant. Chem. Program Exchange Bull.* **1.** 75,
25. Nocera, D. G., Winkler, J. R., Yocom, K. M., Bordignon, E. & Gray, H. B. (1984) *J. Am. Chem. Soc.* **106,** in press.
26. Marcus, R. A. & Sutin, N. (1975) *Inorg. Chem.* **14,** 213–216.
27. Brown, G. M. & Sutin, N. (1979) *J. Am. Chem. Soc.* **101,** 883–892.