Studies of the Thermodynamics of Electron Transfer Reactions of Blue Copper Proteins

Napapon Sailasuta, F. C. Anson, and Harry B. Gray*

Contribution No. 5744 from the Arthur A. Noyes Laboratory, California Institute of Technology, Pasadena, California 91125.
Received May 15, 1978

Abstract: The temperature dependences of the reduction potentials of *Pseudomonas aeruginosa* azurin, bean plastocyanin, and *Rhus vernicifera* stellacyanin have been studied, giving $\Delta H°$ values of −26, −23, and −17 kcal mol^{-1} and $\Delta S°$ values of −63, −52, and −44 eu, respectively. The results have been interpreted in terms of solvation changes that accompany reduction of the blue copper sites of the three proteins. The deduced order of increasing hydrophobicity of the redox sites is stellacyanin < plastocyanin < azurin, which accords with the decreasing kinetic accessibility found for these proteins in reactions with inorganic complexes.

Introduction

In the vast literature relating to the physicochemical properties of proteins,[1-26] surprisingly little can be found on the subject of the thermodynamics of metalloprotein electron transfer reactions. For this reason, we have measured the temperature dependences of the reduction potentials of several metalloproteins, in order to determine the standard enthalpies and entropies of the redox processes involved. For these measurements we have employed an optically transparent thin layer electrochemical cell[27] and a redox mediator to accelerate the rate of electron transfer between the metalloprotein and the electrode. A detailed review of this spectroelectrochemical application of thin layer cells is available.[28]

Our initial efforts have been concentrated on three blue copper proteins, namely, azurin from *Pseudomonas aeruginosa*, bean (*Phaseolus vulgaris*) plastocyanin, and *Rhus vernicifera* stellacyanin. This choice would appear felicitous because X-ray structural studies are in progress on the former two proteins.[29,30] Furthermore, we have previously elucidated the kinetics of the redox reactions of the three blue proteins with a variety of inorganic reagents.[31-33] It is therefore of interest to see if any systematic relationships exist among the structural, kinetic, and thermodynamic properties of this closely related series of proteins.

Experimental Section

Apparatus. The optically transparent thin layer electrode used was similar to that described by Heineman.[27] A precalibrated thermocouple was inserted at the middle of one edge of the thin layer nonisothermal electrochemical cell in contact with the solution. Electrical contact between the thermocouple and the gold-minigrid electrode was avoided by appropriate trimming of the latter. The solution temperature was measured to ±0.1 °C. The electrochemical cell consisted of the transparent gold-minigrid electrode confined between two microscope slides, a Pt auxiliary electrode, and a specially constructed small-diameter saturated calomel reference electrode (SCE) that was separated from the thermostated cell by a long KCl salt bridge and was maintained at room temperature. The minigrid and auxiliary electrodes were immersed in a small cup containing ca. 3 mL of the test solution. For anaerobic measurements, the cup was replaced by a specially constructed Lucite block that contained ca. 1 mL of electrolyte along with the three electrodes.

Dissolved oxygen was removed from the electrolyte in the thin layer region by maintaining the electrode potential at −0.6 V for 10 min. The potential of the miniature SCE reference electrode was compared with that of several commercial reference electrodes and found to match their potentials within ±3 mV.

A Wenking Model 6357 TR potentiostat was used to control the potential of the minigrid electrode. The precise values of the potentials were measured with a Tektronix Model DM 501 digital voltmeter.

The electrochemical cell was placed in a thermostated cell holder which fit in the cell compartment of a Cary 17 or 15 spectrophotometer where the optical measurements were performed.

Proteins and Mediators. *Pseudomonas aeruginosa* azurin was purified according to the method of Ambler and Wynn[34] to an A_{625}/A_{280} ratio of 0.58. Stellacyanin from the Japanese lacquer tree (*Rhus vernicifera;* obtained as an acetone powder from Saito and Co., Ltd., Tokyo) was prepared by the method of Reinhammar[35] and purified to an A_{604}/A_{280} ratio of 0.18. Plastocyanin from bean leaves (*Phaseolus vulgaris*) was purified by the method of Milne and Wells[36] to an A_{597}/A_{280} ratio of 0.90. All protein solutions were dialyzed against phosphate buffer, pH 7.0, ionic strength (μ) = 0.1 M.

Pentamminepyridineruthenium(III) ($Ru(NH_3)_5py^{3+}$) and tris(1,10-phenanthroline)cobalt(III) ($Co(phen)_3^{3+}$) were prepared as described previously.[32,33]

Solutions were prepared immediately before use (the appropriate amount of mediator was dissolved in the protein solutions). The mediator used and the wavelength monitored for each protein are given in Table I.

Spectral Measurements. The concentrations of reduced and oxidized protein corresponding to each potential applied to the transparent electrode were determined spectrophotometrically. The spectra were recorded after equilibrium among the electrode, mediator, and protein had been attained as indicated by the cessation of current flow and the stability of the spectrum. Typical absorbance differences between solutions of the fully oxidized and fully reduced protein were ca. 0.1 absorbance unit. For temperature-dependence studies, thermal equilibrium was established by allowing the cell to equilibrate in the thermostated cell holder for approximately 45 min.

Data Analysis. The reduction potential of each protein was obtained from a plot of the applied potential ($E_{applied}$) vs. the logarithm of the ratio of concentrations of oxidized to reduced forms of the protein:

$$E_{applied} = E^f + \frac{RT}{nF} \ln \frac{[O]}{[R]}$$

The E^f values reported are averages of at least three independent measurements on each protein.

The enthalpy and entropy changes for the redox reactions of each of the proteins were obtained from the intercept and slope of plots of the free energy ($\Delta G° = -nFE^f$) vs. temperature. Standard deviations were obtained from a linear least-squares analysis of the data. Since the temperature of the reference electrode was held constant, the entropy difference obtained approximates the difference in entropy between the oxidized and reduced forms of each redox couple, $S°_{red} - S°_{ox}$.[37-39]

Results and Discussion

Figure 1 shows spectra obtained with the thin layer cell for two of the mediators employed. Figure 2 shows the spectra obtained for each of the proteins studied. Nernst plots (applied potentials vs. the logarithm of the ratio of the concentration of the oxidized and the reduced forms of the protein) at various temperatures are displayed in Figure 3. The corresponding formal potentials and the calculated thermodynamic parameters for the proteins are given in Table II. Values of $\Delta S°$ were

0002-7863/79/1501-0455$01.00/0

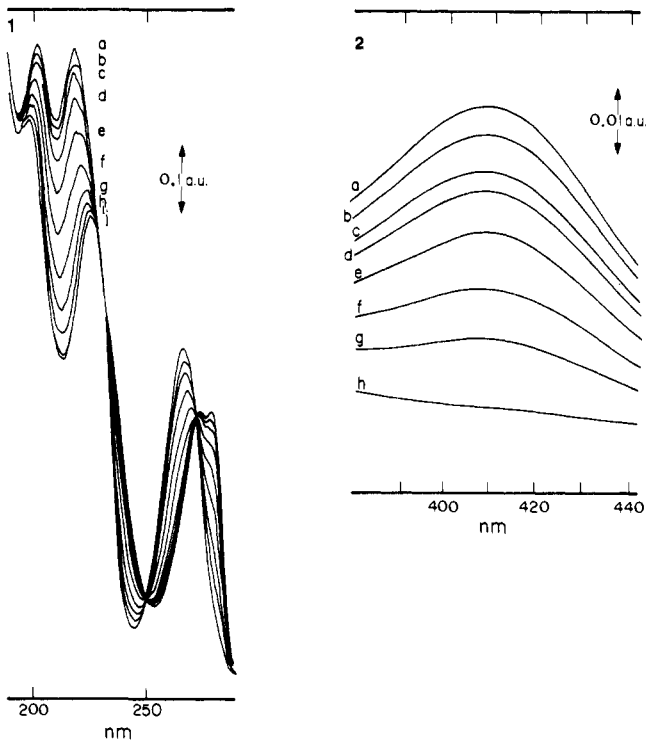

Figure 1. Thin layer spectra of mediators for different values of applied potential, E_{app} (mV vs. NHE). (1) $Co(phen)_3(ClO_4)_3$, 1.84×10^{-3} M, cell thickness 0.051 cm: (a) 490, (b) 440, (c) 420, (d) 400, (e) 380, (f) 360, (g) 340, (h) 320, (i) 300, (j) 240. (2) $Ru(NH_3)_5py(ClO_4)_3$, 1.80×10^{-3} M, cell thickness 0.051 cm: (a) 90, (b) 190, (c) 215, (d) 225, (e) 240, (f) 260, (g) 280, (h) 390.

Table I. Mediators Employed with Each Protein and the Wavelengths at Which the Protein Concentrations Were Monitored

protein[a]	mediator[a]	E^f [b] of mediator, mV vs. NHE	species monitored (λ, nm)
stellacyanin	$Ru(NH_3)_5py^{3+}$	253	$Cu^{2+/1+}$ (604)
plastocyanin	$Co(phen)_3^{3+}$	360	$Cu^{2+/1+}$ (597)
azurin	$Co(phen)_3^{3+}$	360	$Cu^{2+/1+}$ (625)

[a] See Figure 2 for concentrations used. [b] E^f values were obtained from cyclic voltammograms and/or from spectral measurements with the optically transparent thin layer cell.

Table II. Formal Potentials (25 °C), $\Delta H°$, and $\Delta S°$ Values[a]

protein	E^f, mV vs. NHE	$\Delta H°$, kcal mol^{-1}	$(S°_{red} - S°_{ox})$,[d] eu	$\Delta S°$,[e] eu
stellacyanin[b]	184 (±1)	−17 (±1)	−24 (±3)	−44 (±3)
plastocyanin[c]	347 (±1)	−23 (±2)	−32 (±5)	−52 (±5)
azurin[c]	330 (±1)	−26 (±1)	−43 (±3)	−63 (±3)

[a] These values refer to the process protein(ox) + $\frac{1}{2}H_2$ → protein(red) + H^+(aq). [b] pH 6.5, 8.3×10^{-3} M NaH_2PO_4, 5.9×10^{-3} M Na_2HPO_4, 8.3×10^{-3} M Na_2SO_4; total ionic strength 0.1 M. [c] pH 7, 1.9×10^{-3} M KH_2PO_4, 2.7×10^{-3} M Na_2HPO_4; total ionic strength 0.1 M. [d] Evaluated from dE^f/dT; see text. [e] $\Delta S° = (S°_{red} - S°_{ox}) - 20$; see text.

calculated from the $(S°_{red} - S°_{ox})$ values by taking $\Delta S°$ for H^+ (aq) + e^- → $\frac{1}{2}H_2$ as 20 eu.[40]

The formal reduction potential of bean plastocyanin is

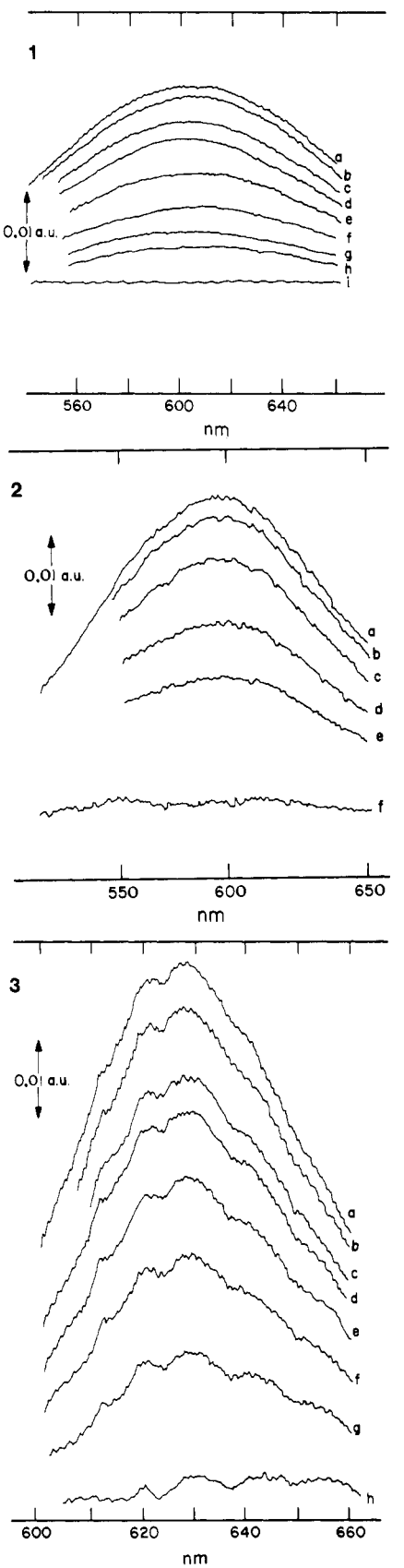

Figure 2. Thin layer spectra of proteins with different mediators for different values of applied potentials, E_{app} (mV vs. NHE). (1) Stellacyanin, 2.7×10^{-3} M with $Ru(NH_3)_5py^{3+}$ 2.8×10^{-3} M, cell thickness 0.021 cm: (a) 240, (b) 230, (c) 220, (d) 210, (e) 190, (f) 175, (g) 160, (h) 145, (i) 90. (2) Plastocyanin, 1.8×10^{-3} M with $Co(phen)_3^{3+}$ 2×10^{-3} M, cell thickness 0.051 cm: (a) 540, (b) 430, (c) 390, (d) 360, (e) 340, (f) 240. (3) Azurin, 1.0×10^{-3} M with $Co(phen)_3^{3+}$ 1.0×10^{-3} M, cell thickness 0.051 cm: (a) 540, (b) 385, (c) 360, (d) 350, (e) 340, (f) 325, (g) 305, (h) 240.

slightly lower than the value reported for spinach plastocyanin.[41,42] The potentials measured by us for stellacyanin and azurin are in good agreement with the values frequently cited.[43]

The fact that plastocyanin and azurin have similar reduction potentials very likely reflects close structural similarities. Indeed, preliminary crystallographic results[29] in combination with spectroscopic data[44] indicate that the copper binding site in azurin is very much like that of plastocyanin. The crystal structure of oxidized poplar plastocyanin at 2.7-Å resolution shows that the copper(II) atom has a highly distorted tetrahedral coordination geometry.[30] The four copper coordination positions are occupied by the sulfur atoms of cysteine 84 and methionine 92 and the δ-nitrogen atoms of the imidazole groups of histidines 37 and 87. Furthermore, the copper site, which carries unit positive charge in the oxidized form, is located beneath a hydrophobic patch formed by a number of residues.

The above suggestion that there may be some relationship between copper binding structure and the redox potential is all the more intriguing in view of the fact that stellacyanin, with a potential much less positive than any other blue protein, is known *not* to possess any methionine.[45] Thus the ligand environment of the copper in stellacyanin must be at least slightly different from that of the plastocyanin structure. It is likely that one contributing factor to the unusually low potential of stellacyanin is in fact its different copper coordination environment.

The values of $\Delta S°$ for the electron transfer reactions of the three blue proteins are large and negative. Interpretation of these results is facilitated if we first consider the corresponding thermodynamic parameter for the reduction of $Fe(H_2O)_6^{3+}$, which is $\Delta S° = 27$ eu.[46] The major contribution to the change in the entropy upon reduction of $Fe(H_2O)_6^{3+}$ is generally viewed as a decrease in the solvent ordering around the ion owing to partial charge neutralization. Specifically, reduction of $Fe(H_2O)_6^{3+}$ decreases the charge within the inner sphere and expands its radius, thereby releasing a few water molecules from the outer sphere to the bulk water. The entropy change associated with this process is positive. In contrast to $Fe(H_2O)_6^{3+}$, the value of $\Delta S°$ for reduction of $Fe(phen)_3^{3+}$, which possesses hydrophobic ligands, is approximately -20 eu.[47] In the latter case, the propeller-like configuration of the planar phenanthroline ligands provides open channels that allow water molecules to come into relatively close contact with the Fe(III) in the oxidized complex. The partial charge neutralization (+3 to +2) upon reduction causes a net rearrangement of these water molecules, resulting in formation of cages of structured water of abnormally low entropy[48,49] in the hydrophobic phenanthroline channels. We believe that the latter effect is important in the blue proteins, as the copper environment is hydrophobic and full charge neutralization of the redox center (+1 to 0) occurs upon reduction. Thus, any partially disordered water molecules near the +1 site will be converted to a highly ordered state (an internally hydrogen-bonded network of very low entropy) in the reduced protein.

We attribute the differences in $\Delta S°$ among the three blue proteins to variations in the hydrophobic character of the copper redox centers. The results suggest, therefore, that the hydrophobicity of the metal site environment increases in the order stellacyanin < plastocyanin < azurin.[50] It should be noted that the proposed order is inversely related to the accessibility of the redox sites to hydrophilic inorganic agents that has been derived[31] from kinetic experiments, as is reasonable. Further evidence that the blue copper center in stellacyanin is the least hydrophobic, and thus most kinetically accessible, is the fact that we were able to measure the reduction potential of this protein in the absence of mediator and the resulting value was the same as that obtained in the presence of a mediator. The only difference was that the time required to reach equilibrium after each new potential applied was a few minutes longer. The other two blue proteins required mediator in order to obtain stable potential readings.

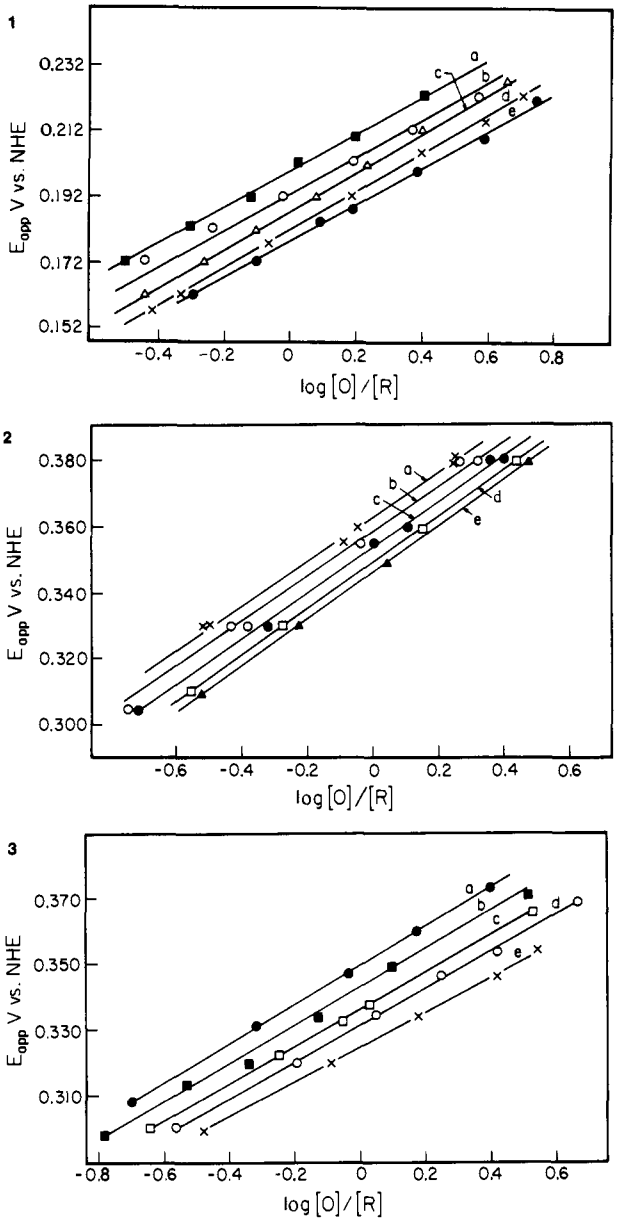

Figure 3. Nernst plots (E_{app} vs. log [O]/[R]) for the proteins at different temperatures (°C). Experimental conditions are the same as those given in Figure 2. (1) Stellacyanin: (a) 6.5, (b) 11.2, (c) 16.2, (d) 21.7, (e) 25.0. (2) Plastocyanin: (a) 6.5, (b) 15.0, (c) 18.0, (d) 23.0, (e) 25.0. (3) Azurin: (a) 12.2, (b) 15.0, (c) 19.0, (d) 21.0, (e) 24.2.

Acknowledgments. We thank Bob Scott for many helpful comments. This work was supported by the National Science Foundation. N.S. thanks Chulalongkorn University for financial support.

References and Notes

(1) C. Tanford, *Adv. Protein Chem.*, **24**, 1 (1969).
(2) W. M. Jackson and J. F. Brandts, *Biochemistry*, **9**, 2295 (1970).
(3) P. Y. Chou and H. A. Scheraga, *Biopolymers*, **10**, 657 (1971).
(4) H. Klump and T. Ackermann, *Biopolymers*, **10**, 513 (1971).
(5) J. Levy and R. Biltonen, *Biochemistry*, **11**, 4145, 4138 (1972).
(6) S. Lapanje and J. A. Rupley, *Biochemistry*, **12**, 2370 (1973).
(7) D. B. Millar, *Biochim. Biophys. Acta*, **359**, 152 (1974).
(8) R. Lecanidon and E. G.-Richards, *Eur. J. Biochem.*, **57**, 127 (1975).
(9) J. J. Christensen, *Biochemistry*, **9**, 4907 (1970).
(10) C. A. Spilburg, *J. Biol. Chem.*, **247**, 4219 (1972).

(11) A. C. I. Anusiem and R. Lumry, *J. Am. Chem. Soc.*, **95**, 904 (1973).
(12) N. M. Allewell, J. Friedland, and K. Niekamp, *Biochemistry*, **14**, 224 (1975).
(13) J. F. Halsey and R. L. Biltonen, *Biochemistry*, **14**, 800 (1975).
(14) S. M. Lam and P. R. Schimmel, *Biochemistry*, **14**, 2775 (1975).
(15) S. C. Cooper, J. Lambek, and M. Erecinska, *FEBS Lett.*, **59**, 241 (1975).
(16) R. Cereijo-Santalo, *Can. J. Biochem.*, **49**, 721 (1971).
(17) L. Rafflenbeul et al., *Z. Naturforsch. C*, **28**, 533 (1973).
(18) W. L. Stone, *J. Biol. Chem.*, **250**, 4368 (1975).
(19) R. Margalit and A. Schejter, *FEBS Lett.*, **6**, 278 (1970).
(20) L. S. Kaminsky, V. J. Miller, and A. J. Davison, *Biochemistry*, **12**, 2215 (1973).
(21) G. L. Dohm, R. Kisner, E. J. Kisner, and R. K. Burkhard, *J. Biol. Chem.*, **245**, 2773 (1970).
(22) N. V. Beaudette and N. Langerman, *Arch. Biochem. Biophys.*, **161**, 125 (1974).
(23) A. Mangold and N. Langerman, *Arch. Biochem. Biophys.*, **169**, 126 (1975).
(24) M. Erecinska and J. M. Vanderkoii, *Arch. Biochem. Biophys.*, **166**, 495 (1975).
(25) R. C. Prince and J. M. Olson, *Biochim. Biophys. Acta*, **423**, 357 (1976).
(26) M. Goldberg and I. Pecht, *Biochemistry*, **15**, 4197 (1976).
(27) W. R. Heineman, B. J. Norris, and J. G. Goelz, *Anal. Chem.*, **47**, 79 (1975).
(28) T. Kuwana and N. Winograd, "Electroanalytical Chemistry", Vol. 7, A. J. Bard, Ed., Marcel Dekker, New York, N.Y., 1974, pp 1–74.
(29) E. T. Adman, R. E. Stenkamp, L. C. Sieker, and L. H. Jensen, *J. Mol. Biol.*, **123**, 35 (1978).
(30) P. M. Colman, H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw, and M. P. Venkatappa, *Nature (London)*, **272**, 319 (1978).
(31) R. A. Holwerda, S. Wherland, and H. B. Gray, *Annu. Rev. Biophys. Bioeng.*, **5**, 363 (1976).
(32) J. V. McArdle, C. L. Coyle, H. B. Gray, G. S. Yoneda, and R. A. Holwerda, *J. Am. Chem. Soc.*, **99**, 2483 (1977).
(33) D. Cummins and H. B. Gray, *J. Am. Chem. Soc.*, **99**, 5158 (1977).
(34) R. P. Ambler and M. Wynn, *Biochem. J.*, **131**, 485 (1973).
(35) B. Reinhammar, *Biochim. Biophys. Acta*, **205**, 35 (1970).
(36) P. R. Milne and J. R. E. Wells, *J. Biol. Chem.*, **245**, 1566 (1970).
(37) J. N. Agar, *Adv. Electrochem. Electrochem. Eng.*, **3** (1963).
(38) E. L. Lee, R. J. Cave, K. L. Guyer, P. D. Tyma, and M. J. Weaver, *J. Am. Chem. Soc.*, submitted for publication.
(39) V. T. Taniguchi, N. Sailasuta, F. C. Anson, and H. B. Gray, to be submitted for publication.
(40) C. M. Criss and M. Salomon in "Physical Chemistry of Organic Solvent Systems", A. K. Covington and T. Dickinson, Ed., Plenum Press, New York, N.Y., 1973, Chapter 2.
(41) R. Malkin, D. B. Knaff, and A. J. Bearden, *Biochim. Biophys. Acta*, **305**, 675 (1973).
(42) S. Kotoh, I. Shivatori, and A. Takamiya, *J. Biochem.*, **51**, 32 (1962).
(43) J. A. Fee, *Struct. Bonding (Berlin)*, **23**, 1 (1975).
(44) E. I. Solomon, J. W. Hare, and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1389 (1976).
(45) C. Bergman, E. Grandolk, P. Nyman, and L. Strid, *Biochem. Biophys. Res. Commun.*, **77**, 1052 (1977).
(46) G. I. H. Hanania, D. H. Irvine, W. A. Eaton, and P. George, *J. Phys. Chem.*, **71**, 2022 (1967).
(47) Results from three independent measurements are as follows: −18 (±2),[38] −25 (±4),[39] and −20 (±2) eu (P. George, G. I. H. Hanania, and D. H. Irvine, *J. Chem. Soc.*, 3548 (1959)). The first two values were calculated assuming that ΔS° (H^+ (aq) + e^- → $\frac{1}{2}H_2$) = 20 eu.
(48) R. Lumry in "Probes of Structure and Function of Macromolecules and Membranes", Vol. 2, B. Chance et al., Ed., Academic Press, New York, N.Y., 1971, p 353.
(49) C. Tanford, "The Hydrophobic Effect", Wiley, New York, N.Y., 1973.
(50) The extent to which conformational changes contribute to the ΔS° values is unknown. However, our interpretation in terms of rearrangement of water structure does not require accompanying conformational changes in the blue proteins. Detailed discussion of this point will be part of the presentation of our study of the redox thermodynamics of various *c*-type cytochromes, as in these cases much more direct structural information is available (N. Sailasuta, S. Schichman, F. C. Anson, and H. B. Gray, to be submitted for publication).

Mechanism of Alkaline Hydrolysis of *S*-Adenosyl-L-methionine and Related Sulfonium Nucleosides

Ronald T. Borchardt

Contribution from the Department of Biochemistry, McCollum Laboratories, The University of Kansas, Lawrence, Kansas 66044. *Received February 7, 1978*

Abstract: Sulfonium nucleosides such as *S*-adenosylmethionine (SAM) and 5′-deoxy-5′-dimethylthioadenosine (DMTA) are very labile to mild alkaline conditions resulting in the cleavage of the glycosidic bond. This glycoside cleavage results from an elimination reaction where proton abstraction occurs at the carbon atom (5′ position) adjacent to the sulfonium center with subsequent elimination to form a 4′,5′ double bond. Cleavage of the glycosidic bond can be envisioned as a concerted part of the elimination reaction or via the formation of a "hemiacetal" intermediate which rapidly breaks down. The rate of glycosidic cleavage of SAM (DMTA) was found not to be linearly dependent on hydroxide ion concentration. This nonlinearity resulted because of the existence of two reacting species. SAM^- (or $DMTA^-$), which has the 2′- (or 3′-) hydroxyl group ionized (pK_a = 12.1), undergoes hydrolysis at a substantially slower rate (k_1 = 0.0363 M^{-1} s^{-1}) than the nonionized species (k_1 = 0.790 M^{-1} s^{-1}). The hydrolysis of 3′-deoxy-SAM, which does not have an acidic functionality in the pH range studied, exhibits a linear dependence on hydroxide ion concentration (k_1 = 0.488 M^{-1} s^{-1}). NMR experiments using DMTA in $NaOD/D_2O$ revealed that during the hydrolysis only a single hydrogen atom was exchanged with deuterium at the 5′ position. The small primary deuterium isotope effects (k_H/k_D = ~1.4) observed for the hydrolysis of both DMTA and $DMTA^-$ suggest asymmetric transition states for these proton abstractions. For the hydrolysis of the nonionized DMTA a significant solvent isotope effect was observed ($k_1(H_2O)/k_1(D_2O)$ = 0.524) suggesting a transition state with substantial bond making to the hydrogen acceptor. In contrast, for the hydrolysis of $DMTA^-$ the lack of a solvent isotope effect ($k_2(H_2O)/k_2(D_2O)$ = 1.02) suggested a transition state with little bond breaking in the reactant.

The role of *S*-adenosyl-L-methionine (SAM) in enzymatically catalyzed group transfer reactions is now well recognized.[1] In addition to SAM's unique properties as a biological methyl or aminopropyl donor, the molecule also exhibits some interesting chemical properties, many of which are inherent in its sulfonium nucleoside structure.[2] For example, SAM has been shown to be very labile to mild alkaline conditions resulting in the cleavage of the glycoside bond.[3] In sharp contrast, *S*-adenosylhomocysteine is stable under similar alkaline conditions, suggesting that the labilization of the glycosidic bond of SAM results from the presence of the sulfonium center. This sensitivity to alkaline hydrolysis appears to be a general characteristic of sulfonium nucleosides, since various purine and pyrimidine 5′-deoxy-5′-dimethylsulfonium nucleosides have been shown to undergo similar hydrolytic reactions.[3b,d] Using 5′-deoxy-5′-dimethylthioadenosine and methyl (5-deoxy-5-dimethylthio)-β-D-ribofuranoside as model compounds, Baddiley et al.[3d] and Frank et al.[3b] showed that

0002-7863/79/1501-0458$01.00/0