Spectroscopic Studies of Ascorbate Oxidase. Electronic Structure of the Blue Copper Sites†

David M. Dooley,‡ John H. Dawson,§ Philip J. Stephens,‖ and Harry B. Gray*

ABSTRACT: Low-temperature electronic absorption and room temperature circular dichroism (CD) and magnetic circular dichroism (MCD) spectra are reported for ascorbate oxidase. Bands attributable to d–d electronic transitions in the type 1 (blue) coppers ($^2B_2$ ground state) have been observed at 5800, 10000, and 12000 cm^{-1} (1725, 1000, and 835 nm). The three bands are assigned to the transitions $^2B_2 \rightarrow {}^2E$, $^2B_2 \rightarrow {}^2B_1$, and $^2B_2 \rightarrow {}^2A_1$, respectively, in the slightly flattened tetrahedral blue copper centers. The ligand field theoretical treatment predicts a type 1 copper reduction potential of 417 mV. The MCD spectrum of ascorbate oxidase contains one major band at 14000 cm^{-1} (710 nm) and a very weak feature at 13200 cm^{-1} (550 nm). The intensities of the CD and MCD bands are consistent with two to three type 1 coppers in each enzyme molecule, and the near-infrared spectroscopic results suggest that the electronic structures of the blue coppers are closely similar.

Ascorbate oxidase (Dawson et al., 1975) (L-ascorbate:O_2 oxidoreductase, EC 1.10.3.3) is the most complex member of a small group of enzymes known as the blue copper oxidases (Malmström et al., 1975; Malkin & Malmström, 1970; Fee, 1975; Malkin, 1973; Mondovì et al., 1976; Scheinberg & Morell, 1973; Frieden & Hsieh, 1976) that catalyze the reduction of oxygen to two molecules of water with concomitant organic or inorganic substrate oxidation. In contrast to the four to seven intrinsic coppers of the laccases (Fee, 1975) and ceruloplasmin (Rydén & Björk, 1976), estimates for ascorbate oxidase run as high as 12 (Lee & Dawson, 1973a). Furthermore, it is the largest (M_r = 140000; Strothkamp & Dawson, 1974) of these enzymes, the only one with well-established subunit structure (Strothkamp & Dawson, 1974), and the most substrate specific.

The blue copper oxidases have three spectroscopically distinct copper sites whose properties have been extensively discussed: type 1 or blue, which exhibits an intense visible (~600-nm) electronic absorption band and small copper electron paramagnetic resonance (EPR)[1] hyperfine splittings; type 2 or nonblue, which has no detectable absorption bands and normal copper EPR hyperfine structure; and the EPR nondetectable binuclear type 3 unit, which exhibits an intense near-UV (~330-nm) absorption band. The number of coppers in each of these sites has been a matter of considerable debate. The laccases are thought to have the minimal number of each type (i.e., one each of types 1 and 2 and two type 3) (Fee, 1975). The complicated situation for ceruloplasmin has been discussed elsewhere (Rydén & Björk, 1976; Dawson et al., 1979). For ascorbate oxidase, EPR measurements suggest the presence of three type 1 and one type 2 coppers (Deinum et al., 1974; Van Leeuwen et al., 1975; Avigliano et al., 1979); the remaining coppers are EPR nondetectable. Based on the analogous values for laccase (Fee, 1975), the ranges of extinction coefficients reported for ascorbate oxidase (Strothkamp & Dawson, 1974; Deinum et al., 1974; Lee & Dawson, 1973b) are consistent with two to three type 1 coppers and two type 3 copper units. The presence of two apparently identical subunits (Strothkamp & Dawson, 1974) complicates the picture because it is inconsistent with an odd number of either type 1 or type 2 sites. Clearly, more work will be needed to clarify this matter.

Recent work in our laboratory has been concerned with the spectroscopic properties of blue copper proteins (Solomon et al., 1975, 1976a, 1980; Dooley et al., 1979; Hare et al., 1976; Dawson et al., 1978, 1979) and their cobalt(II) derivatives (McMillin et al., 1974a,b; Solomon et al., 1976b). In these studies were have employed absorption, circular and magnetic circular dichroism (CD and MCD), and EPR spectroscopic measurements to characterize the coordination environment and electronic structure of the copper atoms in these proteins. One of the principal findings of these studies has been the discovery of low energy d–d electronic transitions attributable to a distorted tetrahedral type 1 copper center (Solomon et al., 1976a, 1980; Dawson et al., 1979; Dooley et al., 1979). We have now extended our spectroscopic investigations to include the type 1 coppers of ascorbate oxidase, and the results are reported herein.

Experimental Procedures

Purification of Ascorbate Oxidase. Ascorbate oxidase was purified by slight modification of the method of Lee & Dawson (1973a) (steps 5 and 7 were omitted). A frozen ammonium sulfate precipitate (~900 g, kindly provided by Professor C. R. Dawson of Columbia University) was used as starting material; over 100 mg of purified protein was isolated, having a spectral purity ratio A_{280}/A_{610} of 25.6 [lit. value = 25.6 (Lee & Dawson, 1976b)]. On the basis of ϵ_{610} = 9600 M^{-1} cm^{-1} and M_r = 140000 (Strothkamp & Dawson, 1974), a copper stoichiometry of 8.2 ± 0.2 was determined by flame atomic absorption using Chelex-treated 0.05 M sodium acetate buffer (0.1 M NaCl, pH 5.5) for both protein and copper standards.

† Contribution No. 6193 from the Arthur A. Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125. *Received April 1, 1980; revised manuscript received November 5, 1980.* D.M.D. acknowledges a National Institutes of Health Predoctoral Traineeship (1974–1978), and J.H.D. acknowledges National Institutes of Health Postdoctoral Fellowship (1F32CA05748-01) (1976–1978). This research was supported by National Science Foundation Grant No. CHE77-11389 and by National Institutes of Health Grant No. AM19038.

‡ Present address: Department of Chemistry, Amherst College, Amherst, MA 01002.

§ Present address: Department of Chemistry, University of South Carolina, Columbia, SC 29208.

‖ Present address: Department of Chemistry, University of Southern California, Los Angeles, CA 90007.

[1] Abbreviations used: EPR, electron paramagnetic resonance; CD, circular dichroism; MCD, magnetic circular dichroism.

0006-2960/81/0420-2024$01.25/0

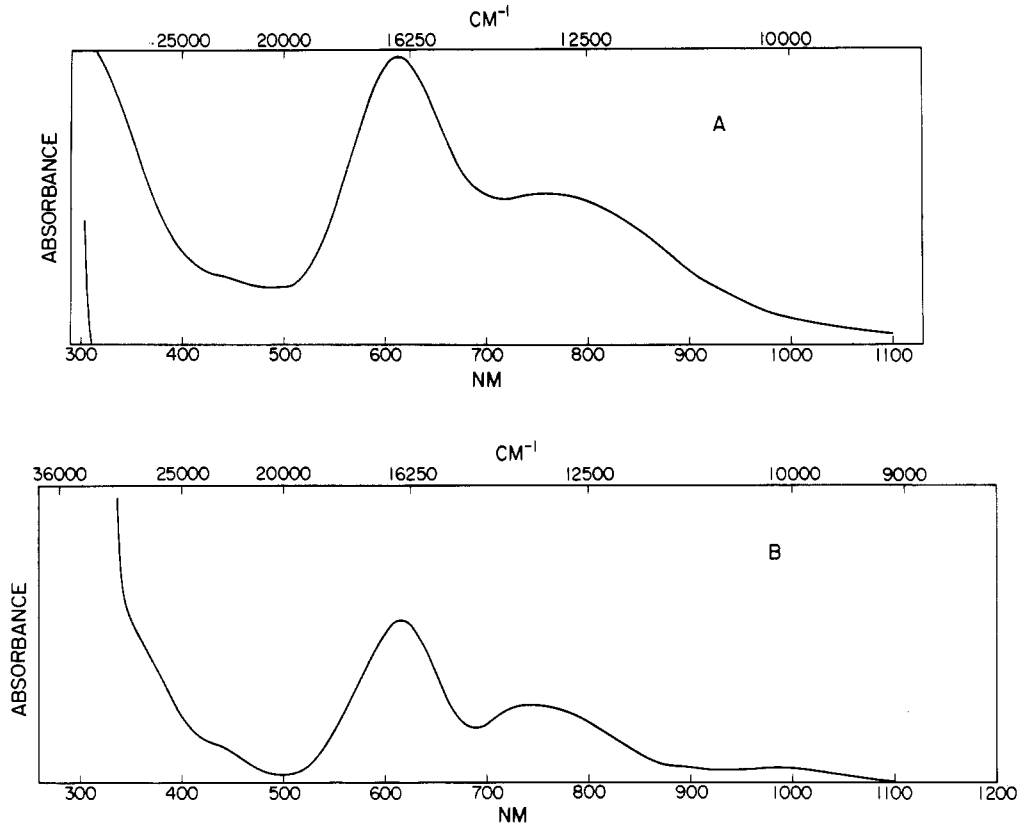

FIGURE 1: Absorption spectra of ascorbate oxidase: (A) solution at room temperature (ϵ_{610} = 9600 M^{-1} cm^{-1}); (B) thin film at 20 K.

This value is well within the range of 6–12 reported by others (Lee & Dawson, 1973a; Marchesini & Kroneck, 1977; Mondovì et al., 1975). Electrophoresis under nondenaturing conditions (Davis, 1964) revealed only minute impurities; no impurities were seen with electrophoresis under denaturing (sodium dodecyl sulfate) (Weber & Osborn, 1969) conditions.

General Procedures. Absorption spectra were obtained on a Cary 17 spectrophotometer. Jasco J-40 and Cary 61 instruments were used for CD measurements in the 300–800-nm region. Near-infrared (700–2000-nm) CD spectra were recorded on a laboratory-built instrument (Osborne et al., 1973; Nafie et al., 1976). Visible MCD measurements were made on the Cary 61 instrument with a field of 40 kG supplied by a Varian superconducting electromagnet. Instrument calibration and data manipulation were performed as described previously (Dawson et al, 1979). MCD spectra were corrected for natural optical activity (Dooley et al., 1979). CD and MCD spectra are reported in terms of differential molar extinction coefficient $\Delta\epsilon$. In the case of MCD, $\Delta\epsilon$ is normalized to a field of 10 kG.

A Brinkmann Model 101 pH meter equipped with a Metrohm combination glass electrode was used for pH measurements at room temperature. The relationship pD = pH meter reading + 0.4 was used to calculate pD (Covington et al., 1968; Glasoe & Long, 1960).

All spectroscopic measurements were made with protein in 0.02 M sodium phosphate solution in which the pD was adjusted to 7.4 with acetic acid-d_1. A thin film of ascorbate oxidase was prepared on a Plexiglass disk and its low-temperature absorption spectrum measured as previously described (Dooley et al., 1979) by using a Cryogenic Technology Model 20 cryocooler. Concentrated protein solutions were obtained by ultrafiltration (Amicon, PM-30 membrane or Millipore, immersible molecular separator). Protein concentrations were determined by using the published extinction coefficient (ϵ_{610} = 9600 M^{-1} cm^{-1}; Strothkamp & Dawson, 1974). Reagent grade chemicals were used without further purification. D_2O (Stohler Isotope Chemicals) was 99.8% D.

Results

The room temperature absorption spectrum of ascorbate oxidase (Figure 1A) is dominated by the intense blue band at 16 400 cm^{-1} (610 nm). Shoulders are seen at approximately 12 800, 22 200, and 30 300 cm^{-1} (780, 450, and 330 nm). The room temperature spectrum is nearly identical with those reported previously for ascorbate oxidase (Strothkamp & Dawson, 1974; Deinum et al., 1974; Van Leeuwen et al., 1975; Lee & Dawson, 1973b) except that the band at 880 nm reported by Lee & Dawson (1973b) is not resolved. Despite our use of their method of purification (Lee & Dawson, 1973a), only 8 coppers per enzyme were detected rather than 10–12 (Lee & Dawson, 1973a). This provides support for the suggestion by Van Leeuwen et al. (1975) that the band at 880 nm results from the additional coppers present in the Lee and Dawson preparation. The additional coppers do not significantly increase the specific activity of the enzyme (Marchesini & Kroneck, 1979).

The ascorbate oxidase spectrum is better resolved at 20 K (Figure 1B). Three new shoulders are observed at 20 K; their positions are approximately 10 000, 11 300, and 12 000 cm^{-1} (1000, 890, and 835 nm). The presence of the very weak feature at 11 300 cm^{-1} indicates that our enzyme preparation is not entirely free of adventitious copper. The 10 000- and 12 000-cm^{-1} bands have not been reported previously for ascorbate oxidase, although analogous absorption peaks have been observed in other blue copper proteins (Solomon et al., 1976a, 1980; Dawson et al., 1979; Dooley et al., 1979).

The near-infrared CD spectrum of ascorbate oxidase is shown in Figure 2. The lowest energy band at 5800 cm^{-1} (1725 nm) is between 2 and 3 times as intense as the analogous feature in the laccases (Dooley et al., 1979). A shoulder at ~9100 cm^{-1} (1100 nm) in the CD spectrum is most likely

Table I: Electronic Spectroscopic Properties of Ascorbate Oxidase[a]

low-temp absorption[b]		circular dichroism[c]			magnetic circular dichroism[d]			
cm^{-1}	nm	cm^{-1}	nm	$\Delta\epsilon$	cm^{-1}	nm	$\Delta\epsilon$	assignment (blue Cu)
		5 800	1720	1.6				$^2B_2 \rightarrow {}^2E$
10 000	1000	9 100	1100	−0.2				$^2B_2 \rightarrow {}^2B_1$
12 000	835							$^2B_2 \rightarrow {}^2A_1$
12 800	780	13 250	755	−17.0[e]	14 200	705	−1.2	$\pi S \rightarrow d_{x^2-y^2}$
16 400	610	16 530	605	7.3				$\sigma S \rightarrow d_{x^2-y^2}$
		18 200	550	4.6	18 200	550	0.05	$\sigma S^* \rightarrow d_{x^2-y^2}$
22 200	450	21 275	470	−4.9				$\pi N \rightarrow d_{x^2-y^2}$
		23 810	420	0.7				*f*
		30 300	330	−2.4				*g*

[a] Values listed correspond to apparent peak and shoulder positions and are not the result of Gaussian analyses. With overlapping bands present in some spectra, therefore, the apparent peak and shoulder positions will shift. [b] Figure 1; the weak feature at ~890 nm is attributed to adventitious copper. [c] Figures 2 and 3. [d] Figure 4. [e] Figure 3. Measurement on the near infrared CD spectrometer gave −25 (Figure 2). [f] Not assigned. [g] This band is associated with type 3 copper.

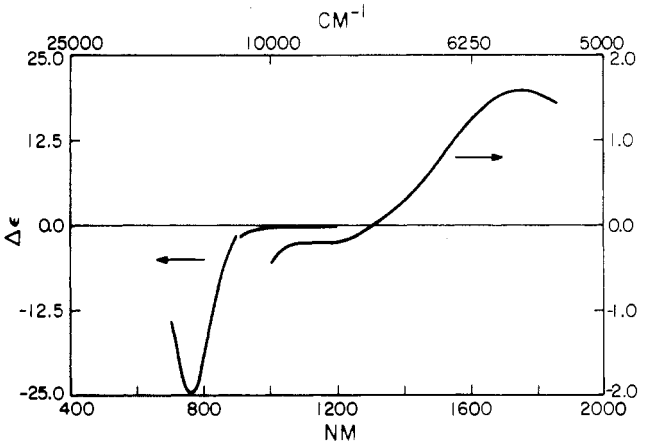

FIGURE 2: Near-infrared CD spectrum of ascorbate oxidase [0.8 mM in 0.02 M sodium phosphate/D_2O (pD = 7.4) solution] at room temperature.

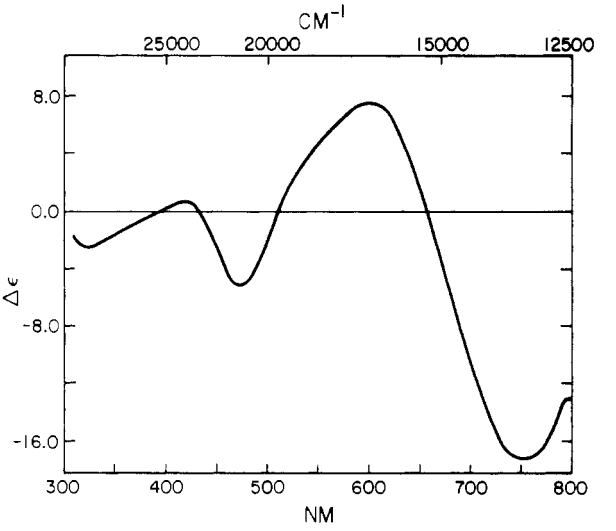

FIGURE 3: CD spectrum (300–800 nm) of ascorbate oxidase [0.8 mM in 0.02 M sodium phosphate/D_2O (pD = 7.4) solution] at room temperature.

attributable to the same transition as the lowest energy band observed in the low-temperature absorption spectrum (Figure 1). We assume that the CD band that corresponds to the 12 000-cm^{-1} shoulder in the low-temperature absorption spectrum is buried under the intense 13 250-cm^{-1} (755-nm) band.

The visible CD and MCD spectra of ascorbate oxidase are displayed in Figures 3 and 4. The CD spectrum features a five-band pattern of alternating sign similar to that reported by Lee & Dawson (1973b) but extended to 800 nm so as to observe the minimum at 755 nm (13 250 cm^{-1}) clearly. As with the other blue copper proteins (Dawson et al., 1979; Dooley et al., 1979), the CD spectrum in the 15 500–20 000-cm^{-1} region is asymmetric and likely consists of two overlapping transitions. The MCD spectrum of ascorbate oxidase (Figure 4) consists of one major negative band at 14 100 cm^{-1} (710 nm), having roughly 3 times the intensity of the corresponding feature in tree laccase (Dooley et al., 1979), and a very weak positive band at ~18 200 cm^{-1} (550 nm). All the spectral data we have collected for ascorbate oxidase are given in Table I.

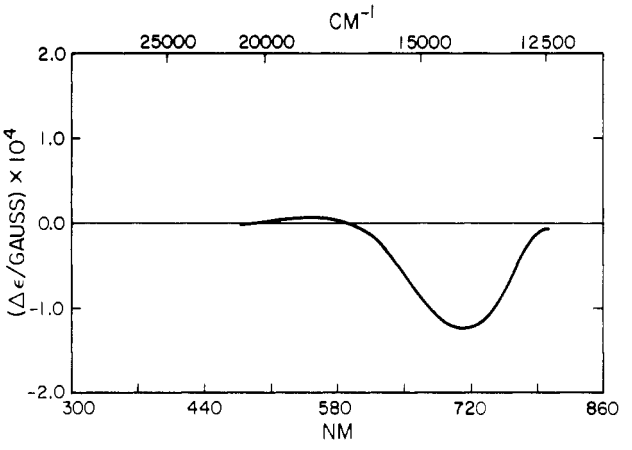

FIGURE 4: MCD spectrum of ascorbate oxidase [0.8 mM in 0.02 M sodium phosphate/D_2O (pD = 7.4) solution] at room temperature.

Discussion

Except for the shoulder at 330 nm attributable to type 3 copper (Fee, 1975), the multifeatured absorption spectrum of ascorbate oxidase (Figure 1) is quite similar to the spectra of proteins containing only the type 1 (blue) copper (Solomon et al., 1976a, 1980). On the basis of these spectral similarities, the bands other than that at 330 nm may clearly be ascribed to type 1 copper [$^2B_2(d_{x^2-y^2})$ ground state]. In previous papers we have shown that in the case of the single blue copper proteins the intense bands between 12 500 and 22 500 cm^{-1} are likely due to ligand to metal charge transfer transitions of the types $\pi S \rightarrow d_{x^2-y^2}$, $\sigma S \rightarrow d_{x^2-y^2}$, and $\pi N \rightarrow d_{x^2-y^2}$, in order of increasing energy (Solomon et al., 1976a, 1980). We have assigned the charge transfer bands in the spectrum of ascorbate oxidase to the same set of transitions (Table I).

The energies of the three d–d bands found in the near-infrared region (5800, 10 000, and 12 000 cm^{-1}) are in close agreement with those observed for other blue copper proteins we have examined (5000–6100, 8300–10 200, and 11 100–

12 000 cm^{-1}) (Solomon et al., 1976a, 1980; Dawson et al., 1979; Dooley et al., 1979), except for fungal laccase (Dooley et al., 1979), which has higher energy transitions throughout. Moreover, we have shown that the electronic transitions of copper types 2 and 3 do not fall in the near-infrared region (Dooley et al., 1979; Dawson et al., 1979). For these reasons we have assigned the three near-infrared bands observed in the spectrum of ascorbate oxidase as the transitions from $^2B_2$ to the ^2E, $^2B_1$, and $^2A_1$ states in a tetragonally distorted tetrahedral type 1 copper center ($^2E < {}^2B_1 < {}^2A_1$) (Solomon et al., 1980). Furthermore, the similarity in the d–d excitation energies to those of azurin and plastocyanin (Solomon et al., 1980), whose crystal structures have recently been reported (Colman et al., 1978; Adman et al., 1978), suggests that each of the type 1 sites of ascorbate oxidase has the same N_2SS^* (N = His, S = Cys, S* = Met) donor set.

In the previously elaborated flattened tetrahedral model for a blue copper site, the energies of the three d–d transitions are a function of the extent of distortion toward square-planar structure (expressed as a distortion angle β, where $\beta = 54.74°$ and 90° for the T_d and D_{4h} limits, respectively) and two radial integrals, *Ds* and *Dt* (Solomon et al., 1980). By methods described earlier (Solomon et al., 1980), we obtained $\beta = 61°$, $Ds = 670$ cm^{-1}, and $Dt = 490$ cm^{-1} for ascorbate oxidase blue copper. With these parameters, the $^2B_2 \rightarrow {}^2A_1$ excitation energy is calculated to be 12 220 cm^{-1}, (obsd, 12 000 cm^{-1}) and $^2B_{1g} \rightarrow {}^2A_{1g}$ in the square-planar (D_{4h}) limit ($\beta = 90°$) is predicted to be 21 000 cm^{-1}. The values for β, *Ds*, *Dt*, and the D_{4h} limit are close to the ranges observed for the other blue copper proteins we have examined: 60–61°, 700–800 cm^{-1}, 440–560 cm^{-1}, and 21 200–24 800 cm^{-1}. Again using standard procedures (Solomon et al., 1980), we have calculated that each of the blue copper sites of ascorbate oxidase is destabilized by 264 mV relative to $Cu(H_2O)_6^{2+}$ ($E° = 153$ mV); thus the predicted reduction potential is 417 mV. The blue copper reduction potential of ascorbate oxidase has not been experimentally determined.

The CD and MCD spectra of ascorbate oxidase in the visible region (Figures 3 and 4) show close correspondence to the analogous spectra of the other blue copper proteins (Solomon et al., 1980; Dooley et al., 1980; Dawson et al., 1979), except for stellacyanin (Solomon et al., 1980) and fungal laccase (Dooley et al., 1979). The CD spectrum also agrees well with that previously reported for ascorbate oxidase by Lee & Dawson (1973b) with the extension of their data to 800 nm to locate the lowest energy visible CD band at 13 250 cm^{-1} (755 nm). The intensities of the CD and MCD bands are ~3 times as great as the analogous features in tree laccase (Dooley et al., 1979). The asymmetric CD feature at ~18 000 cm^{-1} is attributed to overlapping $\sigma S \rightarrow d_{x^2-y^2}$ and $\sigma S^* \rightarrow d_{x^2-y^2}$ transitions (Table I), as is the case in several other blue proteins (Solomon et al., 1976a, 1980; Dawson et al., 1979; Dooley et al., 1979). Together, these data provide strong evidence that the coordination environment and electronic structure of the type 1 coppers of ascorbate oxidase are similar to those of plastocyanin and azurin.

In conclusion, a comment on the number and the degree of equivalence of the type 1 sites of ascorbate oxidase is in order. The intensities of the visible and near-infrared CD and visible MCD bands (Figures 2–4), as compared to the analogous intensities for tree laccase (Dooley et al., 1979), are consistent with two to three type 1 sites in ascorbate oxidase. Within the resolution of the techniques we have used, we find that these sites are equivalent. Particularly strong evidence for equivalent type 1 sites comes from the near-infrared CD measurements, since the d–d transition energies are very sensitive to slight perturbations in the structure of the copper center (Solomon et al., 1980). In this sense our findings for ascorbate oxidase parallel those reported previously (Dawson et al., 1979) for the type 1 coppers in ceruloplasmin.

Acknowledgments

We thank Professor C. R. Dawson and Dr. Ronald Strothkamp for numerous helpful discussions regarding details of the purification procedures and Dr. Maria Linder for the gel electrophoretic analysis.

References

Adman, E. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol. 123*, 35.
Avigliano, L., Desideri, A., Urbanelli, S., Mondovi, B., & Marchesini, A. (1979) *FEBS Lett. 100*, 318.
Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., & Venkatappa, M. P. (1978) *Nature (London) 272*, 319.
Covington, A. K., Paabo, M., Robinson, R. A., & Bates, R. G. (1968) *Anal. Chem. 40*, 700.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.
Dawson, C. R., Strothkamp, K. G., & Krul, K. G. (1975) *Ann. N.Y. Acad. Sci. 258*, 209.
Dawson, J. H., Dooley, D. M., & Gray, H. B. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4078.
Dawson, J. H., Dooley, D. M., Clark, R., Stephens, P. J., & Gray, H. B. (1979) *J. Am. Chem. Soc. 101*, 5046.
Deinum, J., Reinhammer, B., & Marchesini, A. (1974) *FEBS Lett. 42*, 241.
Dooley, D. M., Rawlings, J., Dawson, J. H., Stephens, P. J., Andrêasson, L.-E., Malmström, B. G., & Gray, H. B. (1979) *J. Am. Chem. Soc. 101*, 5038.
Fee, J. A. (1975) *Struct. Bonding (Berlin) 23*, 1.
Frieden, E., & Hsieh, H. S. (1976) *Adv. Exp. Med. Biol. 74*, 505.
Glasoe, P. K., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188.
Hare, J. W., Solomon, E. I., & Gray, H. B. (1976) *J. Am. Chem. Soc. 98*, 3205.
Lee, M. H., & Dawson, C. R. (1973a) *J. Biol. Chem. 248*, 6596.
Lee, M. H., & Dawson, C. R. (1973b) *J. Biol. Chem. 248*, 6603.
Malkin, R. (1973) in *Inorganic Biochemistry* (Eichhorn, G. L., Ed.) Vol. 2, pp 689–709, Elsevier, Amsterdam.
Malkin, R., & Malmström B. G. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol. 33*, 177.
Malmström, B. G., Andrêasson, L.-E., & Reinhammer, B. (1975) *Enzymes, 3rd Ed. 12*, 507–579.
Marchesini, A., & Kroneck, P. M. H. (1979) *Eur. J. Biochem. 101*, 65.
McMillin, D. R., Holwerda, R. A., & Gray, H. B. (1974a) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1339.
McMillin, D. R., Rosenberg, R. C., & Gray, H. B. (1974b) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4760.
Mondovì, B., Avigliano, L., Rotilio, G., Finazzi-Agrô, A., Gerosa, P., & Giovagnoli, C. (1975) *Mol. Cell. Biochem. 7*, 131.
Mondovì, B., Morpurgo, L., Rotilio, G., & Finazzi-Agrô, A. (1976) *Adv. Exp. Med. Biol. 74*, 424.
Nafie, L. A., Keiderling, T. A., & Stephens, P. J. (1976) *J. Am. Chem. Soc. 98*, 2715.
Osborne, G. A., Cheng, J. C., & Stephens, P. J. (1973) *Rev. Sci. Instrum. 44*, 10.
Rydên, L., & Björk, I. (1976) *Biochemistry 15*, 3411.

Scheinberg, I. H., & Morell, A. G. (1973) in *Inorganic Biochemistry* (Eichhorn, G. L., Ed.) Vol. 1, pp 306–319, Elsevier, Amsterdam.
Solomon, E. I., Clendening, P. J., Gray, H. B., & Grunthaner, F. J. (1975) *J. Am. Chem. Soc. 97*, 3878.
Solomon, E. I., Hare, J. W., & Gray, H. B. (1976a) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1389.
Solomon, E. I., Rawlings, J., McMillin, D. R., Stephens, P. J., & Gray, H. B. (1976b) *J. Am. Chem. Soc. 98*, 8046.
Solomon, E. I., Hare, J. W., Dooley, D. M., Dawson, J. H., Stephens, P. J., & Gray, H. B. (1980) *J. Am. Chem. Soc. 102*, 168.
Strothkamp, K. G., & Dawson, C. R. (1974) *Biochemistry 13*, 434.
Van Leeuwen, F. X. R., Wever, R., Van Gelder, B. F., Avigliano, L., & Mondovì, B. (1975) *Biochim. Biophys. Acta 403*, 285.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Ultraviolet Difference Spectroscopy of Myoglobin: Assignment of p*K* Values of Tyrosyl Phenolic Groups and the Stability of the Ferryl Derivatives†

M. Uyeda‡ and J. Peisach*

ABSTRACT: The ionization of tyrosyl phenolic groups of ferric myoglobins from red kangaroo, horse, and sperm whale has been studied by pH difference spectroscopy at 245 nm. As the number of tyrosyl residues in these proteins varies monotonically from one to three, respectively, we are able to make p*K* assignments for all of them. The apparent p*K* for tyrosine-146, an invariant residue in all myoglobins, is unusually high, 12.7–12.9, as this residue is in a hydrophobic region of the molecule and the tyrosyl phenolic group is hydrogen bonded to the peptide carbonyl of isoleucine-99. For the ferric cyanide and the oxy forms of the various myoglobins, this apparent p*K* is elevated by about 0.5 pH unit while in the deoxy proteins, it does not change significantly. A second tyrosine, at position 103, is found in the horse and sperm whale proteins, but not in the kangaroo protein. It has a significantly lower apparent p*K* than is observed for Tyr-146 in all the derivatives studied. A third tyrosyl residue, at position 151, is exclusive to the sperm whale protein and has an apparent p*K* of 10.3, almost equivalent to that of tyrosine in aqueous solution. The apparent p*K*s for the ferryl forms of the three myoglobins prepared with a 2-fold molar excess of H_2O_2 were also determined by pH difference spectroscopy. Although the sperm whale ferryl protein autoreduces 5 times faster than the horse or kangaroo ferryl proteins, the time resolution of the titrimetric procedure permitted the p*K* determination. As compared to the ferric forms of the proteins, it was found that the apparent p*K* for tyrosine-146 in the horse and kangaroo ferryl proteins was significantly elevated. Tyrosine-151, the residue exclusive to sperm whale myoglobin, could not be titrated at all. When the sperm whale ferryl protein was subsequently reduced to the ferric state, it was found that 85% of the optical contribution of this residue was no longer seen. Amino acid analysis showed that one of the three tyrosyl residues was lost. No analogous loss of tyrosine was observed for the ferric kangaroo and horse proteins treated with peroxide and subsequently reduced. It is suggested that the decreased stability of sperm whale ferryl myoglobin, as compared to horse and kangaroo ferryl myoglobins, is due, in part, to the interaction of the ferryl heme with Tyr-151, found in the sperm whale protein but not in the others.

Since 1943, pH difference spectroscopy in the ultraviolet has been used as a measurement of the extent of dissociation of tyrosyl phenolic hydroxyl groups which are components of protein structure (Crammer & Neuberger, 1943; Tanford & Roberts, 1952). Although these measurements are often made at the major absorption band for tyrosine near 295 nm, for heme proteins it is more convenient to employ shorter wavelengths, usually near 245 nm, in order to avoid complications arising from large spectral contributions of the heme Soret. Such a study has been carried out by Hermans (1962), who, from difference spectroscopy at 245 nm, was able to determine that in the CO derivative of sperm whale myoglobin two of the three tyrosyl groups could be titrated with p*K*s of 10.3 and 11.5, respectively. The third tyrosine did not titrate within the pH range employed in the study, and it was suggested that its p*K* must be greater than 12.8.

In 1964, Breslow also studied carbonmonoxy sperm whale myoglobin, as well as the cyanide derivative. For both forms of the protein, she reported that two of the three tyrosines ionized below pH 12. It remained, however, to decide which tyrosyl residues in the protein corresponded to which of the apparent p*K*s. In order to resolve this problem, we decided to study three different myoglobins in which the number of tyrosyl residues in each differed monotonically.

In the simplest case, myoglobin from *Macropus rufus*, the red kangaroo, only a single tyrosyl residue is found in the primary sequence (Air & Thompson, 1971). In myoblogin from horse, there are two while in the major chromatographic

† From the Departments of Molecular Pharmacology and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461. *Received September 16, 1980.* This investigation was supported in part by U.S. Public Health Service Research Grant HL-13399 and Program Project Grant HL-21016 from the Heart and Lung Institute and by National Science Foundation Grant GB-36422. This is Communication No. 335 from the Joan and Lester Avnet Institute of Molecular Biology. This work was reported at the Federation of American Biological Sciences Meeting in 1975.

‡ Permanent address: Department of Physiology, Gifu University School of Medicine, Gifu, Japan.

0006-2960/81/0420-2028$01.25/0