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Spectroscopic Studies on Spinach Ferredoxin and Adrenodoxin*

Graham Palmer, Hans Brintzinger, and Ronald W. Estabrook

ABSTRACT: Two non-heme iron proteins, adrenodoxin and spinach ferredoxin, which are similar to each other in many respects but differ in that their electron paramagnetic resonance (epr) signals are axially symmetric and rhombic, respectively, have been investigated for their optical activity, in order to characterize further the symmetry of the ligand field of iron in these proteins. Circular dichroism spectra of the oxidized and reduced proteins were obtained from 700 to 300 mμ and analyzed in terms of individual Gaussian components. Unexpectedly, it was found that the optical activity of the two proteins is very similar, differing only by minor shifts in wavelength and intensity of the individual components, and by the occurrence of weak additional bands at the fringes of the spectra of ferredoxin.

Low-temperature optical spectra of the proteins are given.

In recent years there has been increasing interest in a new family of iron proteins in which iron is not a component of heme, but rather appears to be bonded directly to the protein. Although there are numerous proteins which would nominally belong in this class, *e.g.*, ferritin, conalbumin, rubredoxin, the designation non-heme iron protein (NHIP),[1] as this new group of proteins has unfortunately been labeled, is usually only applied to those iron proteins which liberate H_2S on acid denaturation, *i.e.*, those which contain acid-labile sulfur. Furthermore, the NHIP all exhibit characteristic optical absorption, although the visible spectra are rather plain by comparison to those obtained with the heme proteins.

Many of these NHIP are further characterized by unique magnetic resonance properties exhibiting a novel electron paramagnetic resonance (epr) signal at $g = 1.94$ after reduction. Until recently, it appeared

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[1] Abbreviations used: NHIP, non-heme iron proteins; TPNH, reduced triphosphopyridine nucleotide; CD, circular dichroism; epr, electron paramagnetic resonance.

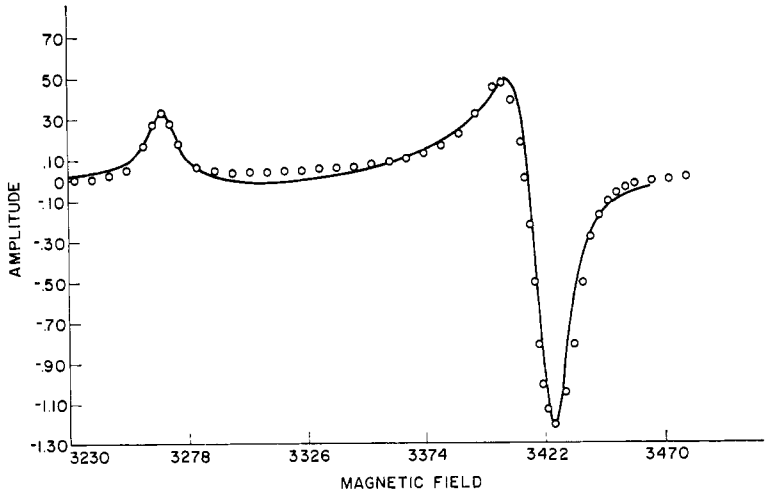

FIGURE 1: Comparison between computed (———) and experimental (O) epr spectrum of adrenodoxin. The experimental conditions were: microwave power, 2 mw; microwave frequency, 9.252 Gc; field modulation, 6 gauss; temperature, 95°K. The parameters used to compute the spectrum were $g_x = 1.930$, $g_y = 1.935$, $g_z = 2.0214$ and the linewidth parameters $A_x = 7.5$ gauss, $A_y = 9.0$ gauss, $A_z = 7.0$ gauss; a Lorentzian line shape was assumed.

that an equally large proportion of the NHIP (the so-called ferredoxins) did not possess such an epr signal. Recent developments in technique have allowed us (Palmer and Sands, 1966; Palmer *et al.*, 1966) to observe $g = 1.94$ resonances in the ferredoxins from spinach and *Clostridium pasteurianum*. It would thus appear that there is no fundamental difference between the ferredoxins and the mitochondrial NHIP at least insofar as their gross epr behavior is concerned. This work reports on the visible optical activity and other spectral properties of two typical NHIP, the ferredoxin obtained from spinach and adrenodoxin isolated from beef adrenal mitochondria.

Materials and Methods

Spinach ferredoxin was prepared by the method of Tagawa and Arnon (1962); the product had an absorbancy ratio $A_{420}:A_{276}$ of 0.44. Adrenodoxin was prepared from beef adrenal cortex by the method described by Omura *et al.* (1967). The product had a ratio $A_{415}:A_{280}$ of 0.87 compared to a value of 0.76 obtained with ultracentrifugally pure protein (Kimura and Suzuki, 1967). Reduction of adrenodoxin was achieved by the addition of 1 mg of dithionite to 3 ml of a solution containing 1–2 $\times$ 10^{-4} M adrenodoxin, TPNH (10^{-4} M), and TPNH adrenodoxin reductase (10^{-7} M) (Omura *et al.*, 1967).

Typically, ferredoxin was reduced by addition of *ca.* 1 mg of dithionite to 3 ml of 10^{-4} M ferredoxin solution. In addition to reducing the protein, dithionite produces a slow irreversible denaturation ($k \approx 0.1$ hr^{-1}) which can be detected as a decrease in the residual visible absorption and concomitant attenuation of the optical activity. Consequently, in experiments with the reduced enzyme we have found it necessary to run alternately optical and circular dichroism (CD) spectra monitoring the denaturation as a function of time. In the spectra reported here the extent of denaturation was either zero or negligibly small as judged by the change in absorbance at 420 mμ before and after the measurement of optical activity.

CD spectra were obtained with a Durrum-Jasco UV-5 recording spectropolarimeter with the CD accessory, and optical spectra with a Cary 15 recording spectrophotometer. All spectra were obtained in 10-mm light path cylindrical quartz cells (Opticell Manufacturing Co.) so that CD and optical spectra could be readily obtained on the same sample. These spectra were obtained at ambient temperatures (23–24°). CD spectra were run under a variety of conditions, both instrumental sensitivity and protein concentration being varied, thus enabling the various regions of the spectrum to be observed under conditions of adequate resolution between spectrum and base line. Both spectra and base lines were run repeatedly as a check on instrument performance.

Because of instrumental limitations, the data are least reliable at the extremes of the spectral range studied; however, the region from 650 to 700 mμ has been thoroughly studied and is believed to be correct within 0.1 unit of ($\epsilon_L - \epsilon_R$). The region below 320 mμ is not so reliable because of high noise problems associated with the increasing absorbancy in this region.

Epr spectra were obtained at X band with a Varian V-4500-10A spectrometer using a variable temperature ensemble similar to the Varian accessory. Epr quantitation was performed by manual double integration and comparison to a standard of copper in excess EDTA. Low-temperature (liquid nitrogen) optical spectra were obtained on a specially constructed spectrophotometer, described in detail by Chance (1957).

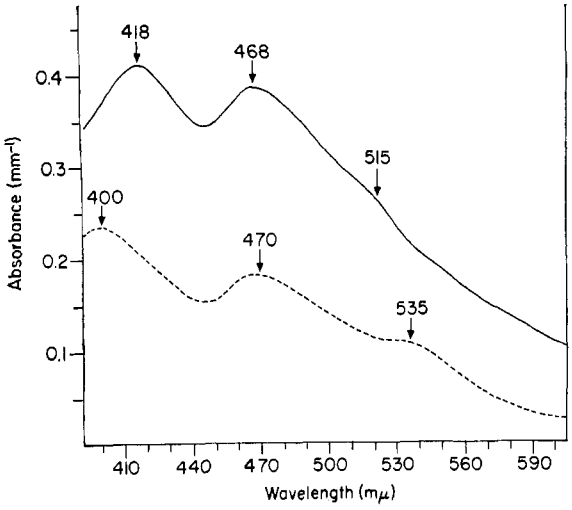

FIGURE 2: Low-temperature optical spectrum of oxidized and reduced spinach ferredoxin. (———) Oxidized (0.76 mM Fe). (– – – –) Reduced with $Na_2S_2O_4$.

The CD spectra were resolved empirically by means of a program written for the IBM 7090, calculating individual component Gaussians as a function of frequency. The epr spectrum of adrenodoxin was computed by a program (written in collaboration with Dr. R. H. Sands) which follows the analytical derivation of Kneubuhl (1960).

Epr Measurements on Adrenodoxin. The epr spectrum of reduced spinach ferredoxin has been reported previously. On reduction it exhibits an intense temperature-sensitive resonance of the $g = 1.94$ type with $g_x = 1.89$, $g_y = 1.95$, $g_z = 2.04$ (Palmer and Sands, 1966).

As previously noted (Kimura, 1967) adrenodoxin exhibits an axially symmetric epr spectrum in the reduced form (Figure 1). Full development of the signal

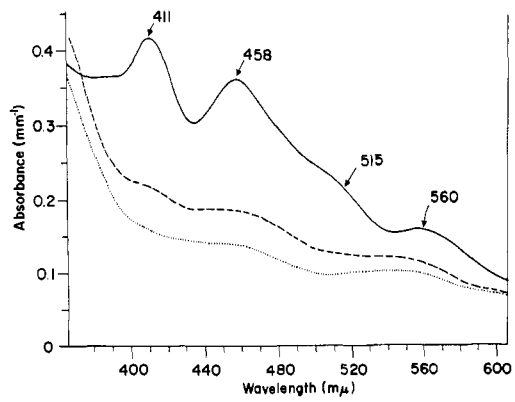

FIGURE 3: Low-temperature optical spectra of oxidized and reduced adrenodoxin. (———) Oxidized (1 mM Fe). (– – – –) Reduced + fp + TPNH. (· · · ·) Reduced + $Na_2S_2O_4$.

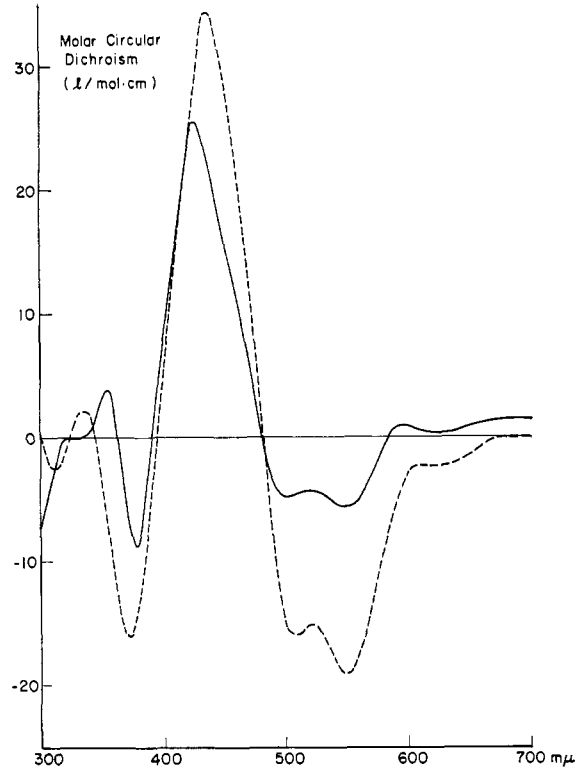

FIGURE 4: CD spectra of oxidized ferredoxin and adrenodoxin (for details see Experimental Section). (———) Ferredoxin. (– – – –) Adrenodoxin.

requires TPNH plus the flavoprotein TPNH adrenodoxin reductase, reduction with dithionite being extremely slow. Double integration of the epr spectrum and comparison with a standard of copper-EDTA indicate that the epr spectrum accounts for 0.75 atom of iron/mole of protein. This compares well with a value of 0.95 atom/mole recently reported by Kimura (1967).

The measured g values are $g_\perp = 2.02$ and $g_{||} = 1.93$. Computer simulation indicates that the best fit is obtained with $g_x = 1.93$, $g_y = 1.935$, and $g_z = 2.0214$ (*cf.* Figure 1). The epr spectrum is relatively insensitive to temperature, good spectra having been obtained over the range 20–150 °K. A plot of log signal amplitude *vs.* temperature is linear until excessive broadening sets in at about 190 °K.

Low-Temperature Optical Spectra of Oxidized and Reduced Ferredoxin and Adrenodoxin. To facilitate the interpretation of the optical activity of these two proteins it is convenient to have also their optical spectra. The solution spectra of NHIP exhibit one or two poorly defined maxima superimposed on a long-absorption tail. Some improvement in resolution is obtained at low temperature (Figures 2 and 3). The comparison of the low-temperature absorption spectra with solution CD spectra appears valid, since the sharpness of the CD peaks (*vide infra*) indicates that the temperature-dependent vibrational broadening

TABLE I: Parameters Necessary to Fit the CD Spectra in Figures 4 and 5 by Superposition of the Gaussians $I\exp((\nu_{max} - \nu)/\Delta\nu)^2$.[a]

Ferredoxin oxidized	ν_{max} (cm^{-1})	14,500	17,000	18,150	20,000	22,400	23,500	24,500	26,500	28,200	31,500
	λ_{max} (mμ)	712	588	551	500	446	426	408	372	355	317
	$\Delta\nu$ (cm^{-1})	1,150	600	1,100	850	1,000	1,000	1,100	850	800	1,500
	I	1.4	2.5	−5.6	−4.6	12.1	20.3	6.5	−9.1	4.2	2.6
	R	0.051	0.040	−0.149	−0.086	0.239	0.379	0.128	−0.128	0.052	0.054
Ferredoxin reduced	ν_{max} (cm^{-1})	15,800	17,000	18,600	19,400	21,150	22,600	24,950	26,900	30,300	
	λ_{max} (mμ)	632	588	538	515	473	442	401	372	330	
	$\Delta\nu$ (cm^{-1})	1,000	1,000	600	1,300	1,200	1,200	1,250	1,450	1,750	
	I	2.0	3.4	2.5	−4.8	−13.3	−8.8	10.1	−16.0	−14.0	
	R	0.057	0.088	0.035	−0.141	−0.331	−0.206	0.222	−0.379	−0.355	
Adrenodoxin oxidized	ν_{max} (cm^{-1})	15,800	18,200	20,000	21,600	23,000	24,400	26,800	29,800	32,100	
	λ_{max} (mμ)	632	549	500	463	435	410	373	335	312	
	$\Delta\nu$ (cm^{-1})	550	1,120	900	1,100	1,100	1,100	1,300	800	900	
	I	−2.2	−18.9	−15.1	13.5	29.9	7.7	−16.1	2.2	−2.2	
	R	−0.034	−0.511	−0.299	0.302	0.628	0.152	−0.342	0.027	−0.031	
Adrenodoxin reduced	ν_{max} (cm^{-1})	15,000	16,900	18,600	19,750	21,400	22,700	25,000	27,350	31,250	
	λ_{max} (mμ)	667	592	538	506	467	441	400	366	320	
	$\Delta\nu$ (cm^{-1})	600	1200	500	1,100	1,000	900	1,100	1,150	1,400	
	I	1.0	7.0	−1.0	−8.5	−14.2	−7.1	5.3	−22.7	−15.5	
	R	0.018	0.218	−0.012	−0.208	−0.291	−0.123	0.103	−0.420	−0.305	

[a] Intensities I in liters per mole per centimeter, center frequencies (ν_{max}) and band widths (Δ_ν) in cm^{-1}. From these parameters the rotatory strength R of the respective transition was obtained according to $R = 0.439I\Delta\nu/\nu_{max}$ (see, *e.g.*, Tinoco, 1965); R is in Debye units × Bohr magnetons.

of the optical absorption is irrelevant for rotational strengths. Most striking are the maxima at 411, 458, and 560 (adrenodoxin) and 418 and 468 (ferredoxin) in the oxidized form. Minor inflections are discernible upon reduction, as indicated in Figures 2 and 3. Because of limitations in the low-temperature recording spectrophotometer it was not possible to obtain spectra below 380 mμ; thus the peak at 330 mμ (shoulder in adrenodoxin) which does not change markedly on reduction has not been investigated at low temperature.

CD of the Oxidized Proteins. The CD spectra of the oxidized proteins are shown in Figure 4. Both samples show intense and detailed spectra indicating the presence of multiple Cotton effects between 300 and 700 mμ. Qualitatively the spectra of both proteins are very similar with two negative Cotton effects at 510 and 550 mμ, and intense positive Cotton effects at *ca.* 430 mμ, a negative Cotton effect at 370 mμ, and a small positive Cotton effect at *ca.* 330–350 mμ. The only region where the spectra are qualitatively different lies between 590 and 700 mμ where ferredoxin shows two positive Cotton effects at 590 and 700 mμ and adrenodoxin shows a single negative Cotton effect at 630 mμ.

As an aid in the understanding of these spectra we have resolved each of the recorded spectra into a family of Gaussian components by means of a computer. The parameters necessary to fit the spectra are recorded in Table I. The analysis shows clearly that the spectra are more complicated than is obvious from cursory examination. This is particularly apparent for the intense Cotton effect observed at *ca.* 430 mμ in each protein, which has been resolved into three positive components.

Considering the spectra in more detail we find that the CD spectrum of ferredoxin supports the previously published optical rotatory dispersion (ORD) spectrum of this protein, which we have also confirmed except that we have been unable to resolve the trough at 350 mμ observed with both the parsley (Gillard *et al.*, 1965) and spinach proteins (Ulmer and Vallee, 1963). However, the Cotton effect at 355 mμ in the CD spectrum confirms the existence of this trough. The small positive Cotton effect at 317 mμ corresponds to the 330-mμ peak in the absorption spectrum. Similarly the positive rotation centered at 712 mμ agrees with a weak optical transition ($\epsilon \sim 800$) observed at 710 mμ in concentrated solutions of the protein. The two minima at 551 and 500 mμ have no obvious counterpart in the visible spectrum which does, however, have a small inflection at 522 mμ most convincingly seen in the original records. The three positive transitions occur at 408, 426, and 446 mμ; the two longer wavelength components of this trio presumably correspond to the absorption

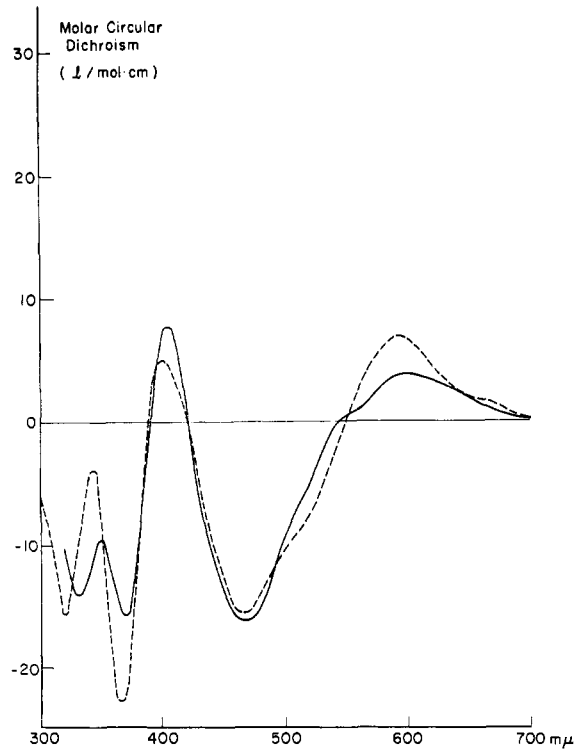

FIGURE 5: CD spectra of reduced ferredoxin and adrenodoxin (for details see Experimental Section). (———) Ferredoxin. (– – – –) Adrenodoxin.

maxima at 418 and 468 mμ seen in the low-temperature optical spectra. The optically active transitions at 372 and 355 mμ cannot be resolved in the visible spectrum although there is considerable absorption in this region.

The CD spectrum of adrenodoxin is consistent with the published ORD (Kimura, 1967). Although the 632-mμ trough has no obviously defined counterpart in the optical spectrum there is considerable end absorption in this region which presumably reflects the presence of several weak overlapping bands. The two negative Cotton effects at 549 and 500 mμ correspond to the peak at 560 and inflection at 515 mμ (Figure 3) while the two positive rotations at 463 and 410 mμ correspond to the peaks at 458 and 411 mμ in the visible spectrum. Again the near-ultraviolet negative rotation at 373 mμ has no obvious counterpart in the visible spectrum while the two transitions at 335 and 312 mμ in the CD spectrum may explain why the optical absorption shows only a shoulder in this region (Kimura, 1967) whereas a peak is clearly observable with the spinach protein.

CD Spectra of the Reduced Proteins. On reduction both proteins show gross changes in their CD spectra (Figure 5). The spectra are qualitatively very similar, exhibiting a large number of optically active transitions with positive Cotton effects at *ca.* 600 and 400 mμ and negative Cotton effects at 473, 372, and 330 mμ. Computer analysis again emphasizes the complicated nature of these spectra, indicating the presence of unresolved transitions particularly in the region of the large negative Cotton effect at 470 mμ.

Certain of the optically active transitions are again visible in the optical spectrum. Thus the peaks at 400 and 470 mμ in the visible spectrum of ferredoxin correspond to the positive and negative extrema at 401 and 473, respectively, while the shoulder at 535 mμ in the optical spectrum has its counterpart in the positive Cotton effect at 538 mμ. Furthermore, both optical and CD spectra show a peak at 330 mμ. The inflections at 416, 455, and 555 which can be observed in the optical spectrum of reduced adrenodoxin are presumably to be correlated with the optically active transitions at 400, 467, and 538 mμ.

Discussion

Although the nature of the chromophoric group in NHIP has not been positively identified, it is generally supposed that the iron atoms present in the protein are responsible for the color. Thus whereas the native protein is red-brown, the metal-free protein is colorless, while reduction of the iron either chemically or enzymically leads to a substantial decrease in the visible color. It is further generally believed that the unusual features of the color of these metalloproteins is due to a special coordination environment involving sulfur as one or more of the ligands. Certainly the characteristic color is rapidly and completely bleached by stoichiometric quantities of mercurial, and these proteins invariably contain the curious form of sulfur called acid-labile sulfur.

By contrast to the spectra of the heme proteins the visible absorption of the NHIP exhibits little detail consisting of a pronounced end absorption on which is superimposed a small number of poorly defined maxima. Thus, examination of the optical spectra is not a very stringent test for comparison of the properties of the NHIP. The large intensity and wealth of detail to be found in the CD of these proteins make the latter technique a much more sensitive method for a comparative study of these proteins.

The striking similarity of the CD spectra of spinach ferredoxin and adrenodoxin in both the oxidized and reduced forms is direct evidence for believing that the environment of the iron chromophores is essentially identical in both proteins. The spectra of these proteins differ markedly from those reported for the ferredoxin from *C. acidi urici* and rubredoxin from *P. elsdenii* (Atherton *et al.*, 1966) both qualitatively and quantitatively. The reduced form of rubredoxin does not exhibit optical activity at wavelengths greater than 400 mμ (this is in agreement with the absence of any light absorption in this region) and the reduced bacterial ferredoxin has little optical activity at wavelengths greater than 500 mμ despite considerable optical absorption in this region. In contrast, both plant ferredoxin and adrenodoxin exhibit prominent positive maxima at about 600 mμ with evidence for additional transitions in the 500-

600-mμ region. A comparison of the CD spectra of these four proteins (this paper and Atherton *et al.*, 1966) suggests that there probably are three classes of proteins: (I) spinach ferredoxin and adrenodoxin which show intense and detailed optical activity throughout the whole visible region[2] in both valence states; (II) bacterial ferredoxin showing elaborate CD in the oxidized form but having little optical activity at wavelengths greater than 500 mμ on reduction even though the visible absorption is still quite intense in this region; and (III) rubredoxin which has neither visible absorption nor visible optical activity in the reduced form, and which lacks acid-labile sulfur.

One might be tempted to ascribe the differences between the plant and bacterial ferredoxins to some consequence of their differing iron contents, *i.e.*, two and six atoms of iron per mole of protein, respectively. However, milk xanthine oxidase which has eight atoms of iron per mole (although probably only four atoms per active center) exhibits a CD spectrum qualitatively similar to adrenodoxin in its oxidized form (G. Palmer and V. Massey, unpublished work) although the spectrum of the reduced protein is unique with characteristic Cotton effects out to 700 mμ.

The simple classifications detailed above are supported by epr data. Reduced proteins which on the basis of their CD spectra belong to class I give rise to epr spectra of the $g = 1.94$ type. No resonances are observed in the oxidized form. Bacterial ferredoxin also gives a $g = 1.94$ epr signal on reduction (Palmer *et al.*, 1966) but the resonance is poorly resolved and contains absorptions in the wings of the spectrum which are not understood. Furthermore, the oxidized protein shows variable amounts of an isotropic resonance at $g = 2.0$. Rubredoxin stands alone in that it shows no resonance in the reduced form but shows the $g = 4.28$ resonance in the oxidized form. The origin of this latter signal is well understood (Blumberg, 1967, and references therein), and is characteristic of high-spin ferric iron in a crystal field of low symmetry. Under these conditions second-order effects of spin-orbit coupling create a zero-field splitting with large rhombic components which gives rise to an isotropic g factor of $^{30}/_{7} = 4.28$.

The origin of the $g = 1.94$ epr resonance is a matter of debate, neither the valence nor spin state of the iron atom responsible for the signal being unambiguously defined. We have recently proposed a model for spinach ferredoxin which points out that low-spin ferric iron in a tetrahedral ligand field would yield the principal resonance below 2.00. To obtain the spatially defined, nondegenerate ground state with the unpaired electron in the d_{xy} orbital it is necessary to elongate the tetrahedron; a complex of this geometry would belong to point group D_{2d}. In this situation $d_{yz} \equiv d_{xz}$; therefore, $g_x = g_y$ and we obtain the simple spectrum exhibited by adrenodoxin (Figure 1) for which deviations from axial symmetry must at most be marginal. The minimum requirement necessary to introduce optical activity in such a chromophore is to reduce the symmetry to D_2 corresponding to an angular displacement of the two ligand pairs. In the lower D_2 symmetry, however, the x and y coordinates are no longer equivalent and different ligand fields would be experienced by the d_{xz} and d_{yz} orbitals with the consequence that $g_x \neq g_y$. Rhombic epr spectra such as found with spinach ferredoxin (Palmer and Sands, 1966) would then be observed. Inasmuch as optical activity is only exhibited by those systems which lack both a center and plane of symmetry it follows that in this simple crystal field approximation, a tetrahedral structure of the kind just described cannot simultaneously be the origin of optical activity and exhibit an axially symmetric epr spectrum.

A similar problem exists with certain coordination compounds of copper. Thus the serum protein ceruloplasmin (Blumberg, 1966) and many copper–amino acid complexes (Bryce, 1966; Zand and Palmer, 1967) also exhibit optical activity associated with the copper absorption bands despite an axially symmetric copper epr spectrum. Indeed, with bis(alinato)copper(II) the epr spectrum is still axially symmetric when observed in a 35-Gc epr spectrometer (Zand and Palmer, 1967); at this higher frequency even small deviations from axial symmetry would be detected.

The crystal structure of many copper–amino complexes have been elucidated (*cf.* Freeman, 1966) and it appears generally true that the chelate rings are buckled with both the copper and amino nitrogen atoms lying out of the plane of the adjacent $CCOO^-$ group. Thus, in these complexes, the absence of a plane of symmetry explains the observed optical activity in the d–d transition but complicates the interpretation of the axially symmetric epr spectrum. The existence of this parallel between the copper–amino acid complexes and the NHIP suggests that there is no compelling reason to question the model of Brintzinger *et al.* (1966) on the grounds of these symmetry arguments.

Attempts to compute the optical spectra from the parameters used to fit the CD spectra (Table I) have not been successful. The line widths of the CD transitions are so narrow that the computed absorption spectra exhibit considerable detail that is not observed experimentally. Improvement in the fit is found if the line width of the absorption bands are arbitrarily broadened, and indeed it is true that CD bands are generally narrower than the corresponding absorption bands. However, there is no means of knowing whether our line-shape analysis is unique. Rather than arbitrarily deciding on a Gaussian line shape it may be more meaningful to use both the absorption and CD spectra to locate the positions of the bands and to use an arbitrary line-shape function. Absorption bands are seldom actually Gaussian, but their shape varies from case to case depending on the relative positions of ground- and excited-state potential surfaces. This

[2] Recent measurements reveal that the optical activity of the oxidized spinach protein extend out to 1100 mμ (R. G. Denning and G. Palmer, unpublished data) with an additional positive Cotton effect at 840 mμ and a negative Cotton effect at 940 mμ.

approach would obviously improve some of the poorer correlations observed between maxima in the optical and CD spectra but would not eliminate the strong positive CD band at 440 mμ seen in both oxidized proteins for which no evidence is available in the optical spectrum. Further complications arise if the sign of the CD changes within a single electronic absorption, as has been found with certain n–π* transitions (Weigang, 1966).

Some insight into the nature of the electronic transitions involved is given by the Kuhn dissymmetry factor *g* defined as the ratio of the CD to the absorption (Kuhn, 1958). For electric dipole transitions the Kuhn *g* is less than 10^{-2}, whereas for magnetic dipole allowed transitions the Kuhn *g* will be between 10^{-2} and 2 (Mason, 1963). In these proteins the molar absorbancy increases from about 800 at 700 mμ to about 13,500 at 320 mμ in the oxidized form and from 600 at 700 mμ to about 13,000 at 300 mμ in the reduced form. From the intensities of the various CD transitions noted in Table I it is found that the Kuhn *g* factor for the various bands is of the order 10^{-4}–10^{-3}, indicating that the observed transitions are electric dipole allowed. This could be taken as evidence for a tetrahedral ligand field where d–d transitions are indeed formally electric dipole allowed. However, any metal–ligand complex which has appreciable covalent bonding will exhibit charge-transfer bands of moderate-to-large extinctions and these could acquire enough magnetic dipole character from the metal to become optically active. This is particularly true when the metal and ligand has oxidation–reduction capabilities, as would be the case for iron and sulfur.

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