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Low Temperature Electronic Absorption Spectra of Oxidized and Reduced Spinach Ferredoxins. Evidence for Nonequivalent Iron(III) Sites

(ligand field spectra/iron-sulfur proteins/iron(III) coordination)

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ABSTRACT **The electronic absorption spectra of oxidized and reduced spinach ferredoxins have been measured between 1200 and 600 nm at low temperature in D_2O/ethylene glycol glasses. Relatively weak absorption bands are observed at 720, 820, and 920 nm in oxidized ferredoxin, and at 652, 820, and 920 nm in reduced ferredoxin. The spectral results show that the two Fe(III) centers in oxidized ferredoxin are not equivalent, and that the 820- and 920-nm bands are associated with the nonreducible site. Assignment of the reducible site as tetrahedral Fe(III) is indicated. The 720-nm (13.9 kcm^{-1}) band in oxidized ferredoxin is attributed to an intensity-enhanced $^6A_1 \rightarrow {}^4T_1$ *d–d* transition, whereas the 652-nm (15.3 kcm^{-1}) feature of reduced ferredoxin could be due either to $^5E \rightarrow {}^3T_1$ in tetrahedral Fe(II)S_4 or an Fe(II) → Fe(III) intervalence excitation.**

There has been a considerable amount of detailed spectroscopic and magnetic work on several of the redox-active iron sulfur proteins. Rubredoxin, the one-iron protein from *Clostridium pasteurianum*, is known from x-ray structural studies to have a distorted tetrahedral [FeS_4] coordination (1). Magnetic susceptibility studies have established high-spin Fe^{2+} and Fe^{3+} configurations for the reduced and oxidized proteins, respectively (2). Eaton and coworkers have recently measured the near infrared spectrum of Fe^{2+}-rubredoxin and have located absorption bands near 4 and 6 kcm^{-1} attributable to the components of the $^5E \rightarrow {}^5T_2$ transition of a distorted tetrahedral [Fe(II)S_4] core structure (3, 4).

Extension of this electronic spectroscopic work to the two-iron, two-labile-sulfur ferredoxins showed that one of the sites in the reduced spinach protein (Fd_{red}) is probably very similar to the distorted tetrahedral [Fe(II)S_4] site found in reduced rubredoxin (4). No conclusions were reached concerning the assignment of the electronic spectrum of the oxidized spinach ferredoxin (Fd_{ox}), however. Several magnetic susceptibility studies have been reported for oxidized and reduced two-iron ferredoxins. The most recent work, which covered the range 77–300°K, established antiferromagnetic coupling between two $^6A_1$ Fe^{3+} centers in Fd_{ox} (5). The interpretation of the magnetic data for Fd_{red} was not as definitive, but the evidence favored an antiferromagnetically coupled $Fe^{2+}(^5E)$–$Fe^{3+}(^6A_1)$ model (5). Mössbauer (6, 7) and ENDOR (8) experiments are also consistent with a spin-coupled $Fe^{2+}(^5E)$–$Fe^{3+}(^6A_1)$ site in Fd_{red}. A recent paper summarizes the rather extensive characterization of the binuclear site in the spinach protein (9). The core structure is generally represented as two $(cys\text{-}S)_2Fe$ units connected by two sulfide bridges.

Several important details concerning the coordination structures of Fd_{ox} and Fd_{red} remain to be elucidated. The Mössbauer spectrum of Fd_{ox} exhibits a single quadrupole-split doublet (6, 7) but the possibility of nonequivalent Fe^{3+} sites has not been eliminated. Indeed, the relatively broad Mössbauer line widths observed have been interpreted (7) in terms of a slight nonequivalence, although other factors could be responsible (6). Additional experimental information relating to the structures of the Fe^{3+} sites in both Fd_{ox} and Fd_{red} is clearly needed.

We have found previously that the spin-forbidden sextet-quartet *d–d* bands of spin-coupled $^6A_1$ Fe^{3+} binuclear complexes are often much more intense than in monomeric reference systems (10). Intensity enhancements as great as 10^3 would not be unreasonable for the sextet → quartet (Fe^{3+}) and quintet → triplet (Fe^{2+}) bands associated with the strongly spin-coupled binuclear sites of the proteins. Identification of the lowest bands could add significantly to the site characterization, as the *d–d* transition positions are often diagnostic of geometrical structure. For this reason, we have carefully examined the electronic absorption spectra of Fd_{ox} and Fd_{red} at low temperature in the 1200 to 600-nm region. Our analysis of the results is presented in this paper.

MATERIALS AND METHODS

The compounds $KFeS_2$ (11) and $KFeSe_2$ (12) were synthesized by published procedures. Tris(tetraphenylthioxoimidodiphosphinato)iron(III) was prepared by mixing $Na(OPPh_2NPPh_2S)$ (ref. 13) and $FeCl_3$ in a 3:1 molar ratio in water. The dark red precipitate was filtered, dried under vacuum, and recrystallized from acetone-petroleum ether solutions. Analytical calculations $C_{72}H_{60}N_3O_3FeP_6S_3$: C, 63.91; H, 4.47; N, 3.11; O, 3.55; Fe, 4.13; P, 13.73; S, 7.11. Found: C, 64.21; H, 4.59; N, 3.19; O, 3.29; Fe, 4.09; P, 13.74; S, 7.42.

Fd_{ox} was prepared by a standard method (14). A sample of purified protein gave an absorbance ratio (420/275 nm) of 0.47. The protein sample used for the low temperature glass was lyophilized and then dissolved in D_2O. Enough ethylene glycol was added to make the solution a 1:1 mixture. Addition of ethylene glycol did not significantly affect either the band positions or molar extinction coefficients in the visible spectrum of the protein. The protein concentration was measured by the absorbance at 420 nm (ϵ 9400 M^{-1} cm^{-1}) of a portion of the sample. Thin films of the oxidized protein were ob-

Abbreviations: Fd_{ox}, oxidized spinach ferredoxin; Fd_{red}, reduced spinach ferredoxin; Ph, phenyl (in chemical formula).

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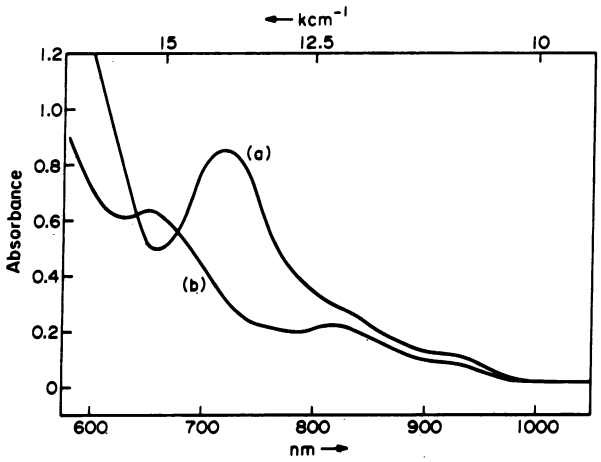

FIG. 1. Electronic absorption spectra of oxidized (*a*) and reduced (*b*) spinach ferredoxins in 1:1 D_2O ethylene glycol at 77°K. Protein concentration is 1.03×10^{-3} M (1-cm pathlength).

tained by slowly evaporating a concentrated solution of protein on quartz plates at 6°C.

Reduction was accomplished by adding a three-fold excess of sodium dithionite to a 1:1 D_2O/ethylene glycol solution of oxidized protein. Prior to reduction, nitrogen was slowly bubbled through the solution, utilizing standard syringe techniques.

All spectra were measured on a Cary 14RI spectrophotometer equipped with a low temperature dewar. Magnetic susceptibility data were taken on a Princeton Applied Research FM-1 vibrating-sample magnetometer. System calibrations were performed with $HgCo(SCN)_4$.

RESULTS AND DISCUSSION

The absorption spectra of Fd_{ox} and Fd_{red} in the region 1200–600 nm at 77°K in a 1:1 ethylene glycol/D_2O glass are shown in Fig. 1. Band positions, molar extinction coefficients, and assignments are set out in Table 1. Relatively weak bands are resolved for Fd_{ox} at 720, 820, and 920 nm. These three features have been observed previously in spectra of Fd_{ox} taken in sucrose solution at 77°K (15). In addition, the band positions in the thin-film spectrum of Fd_{ox} are in close agreement with those obtained in the low-temperature glass.

The only major change in the 1200- to 600-nm region which occurs upon reduction of the protein is the disappearance of the 720-nm band and the development of a new band at 652 nm. Interestingly, the weak features at 820 and 920 nm are still present in Fd_{red}, clearly indicating that the responsible chromophore has not been chemically altered in the electron-transfer process.

The spectral properties outlined above establish that nonequivalent Fe(III) sites are present in Fd_{ox}. The data are nicely accommodated if the site undergoing reduction is formulated as having a tetrahedral $Fe(III)S_4$ core structure, as proposed by Eaton, *et al.* (4). The 13.9-kcm^{-1} peak in Fd_{ox}, which disappears upon reduction, strongly suggests tetrahedral $Fe(III)S_4$, as oxidized clostridial rubredoxin exhibits a similar band at 13.4 kcm^{-1} (ϵ 360) (ref. 4). The assignment of the 13.9-kcm^{-1} band to a tetrahedral $Fe(III)S_4$ center is supported by spectral results obtained for $KFeS_2$. The latter compound, which features S^{2-}-bridged $Fe(III)S_4$ tetrahedra (16), displays a broad absorption system in the 13- to 16-kcm^{-1} region in a TlCl disk at 77°K, with no absorption attributable to electronic transitions at lower energies. Thus, a band at about 14 kcm^{-1} appears to be characteristic of Fe^{3+} coordinated tetrahedrally by S^{2-} (or $-CH_2S^-$) donor atoms.

TABLE 1. *Electronic absorption spectra (1200–600 nm) of oxidized and reduced spinach ferredoxins in 1:1 D_2O/ethylene glycol at 77°K*

	λ_{max}, nm	$\bar{\nu}$, kcm^{-1}	ϵ, M^{-1} cm^{-1}	Assignment
Fd_{ox}	920	10.9	80 ± 10	Nonreducible Fe(III)
	820	12.2	260 ± 30	Nonreducible Fe(III)
	720	13.9	800 ± 80	$Fe(III)S_4$ $^6A_1 \rightarrow {}^4T_1$
Fd_{red}	920	10.9	70 ± 10	Nonreducible Fe(III)
	820	12.2	200 ± 20	Nonreducible Fe(III)
	652	15.3	600 ± 60	$Fe(II)S_4$ $^5E \rightarrow {}^3T_1$ or Fe(II) → Fe(III)

Energetic considerations favor assignment of the 14-kcm^{-1} band in tetrahedral $Fe(III)S_4$ to the spin-forbidden $^6A_1 \rightarrow {}^4T_1$ *d–d* transition. Reasonable values for $Fe(III)S_4$ ligand field parameters (−10 Dq = 6–8, B = 0.6 kcm^{-1}, C/B = 4.5), for example, place the $^6A_1 \rightarrow {}^4T_1$ transition in the 13.5- to 15-kcm^{-1} range. Additional support for a *d–d* transition is provided by a spectral comparison of $KFeS_2$ and $KFeSe_2$. The lowest absorption system of $KFeSe_2$ at 77°K in a TlCl disk is observed in the 11- to 13-kcm^{-1} region, which represents a much smaller red shift from $KFeS_2$ than would be expected for a transition of the S(Se) → Fe(III) charge transfer type. The moderate red shift, however, is consistent with an excitation of substantial *d–d* character.

It is interesting that the ϵ value for the 13.4-kcm^{-1} band in clostridial rubredoxin is not very much smaller than that for the analogous absorption peak of Fd_{ox}. This observation makes it clear that there is very little intensity enhancement of the $^6A_1 \rightarrow {}^4T_1$ transition attributable to spin-spin coupling in the binuclear unit of Fd_{ox}. It is probable, therefore, that relaxation of the $^6A_1 \rightarrow {}^4T_1$ spin-forbiddenness results primarily from mixing of the excited $^4T_1$ state with nearby $^4T_2$ S → Fe(III) charge transfer states through spin-orbit coupling.

A band at 652 nm (15.3 kcm^{-1}) in Fd_{red} is entirely consistent with the presence of a tetrahedral $Fe(II)S_4$ center. In addition to a spin-allowed $^5E \rightarrow {}^5T_2$ system, tetrahedral $Fe(II)S_4$ should exhibit a large number of quintet → triplet transitions. Taking a reasonable B range of 0.7–0.9 kcm^{-1} (C/B = 4.6), the lowest spin-forbidden transition, $^5E \rightarrow {}^3T_1$, is calculated to fall between 13 and 18 kcm^{-1} for a −10 Dq of 5 kcm^{-1} (ref. 4). One attractive possibility, therefore, is that the band at 15.3 kcm^{-1} in Fd_{red} is due to an intensity-enhanced $^5E \rightarrow {}^3T_1$ transition of the $Fe(II)S_4$ unit of the binuclear site. The proposed assignment derives some support from single-crystal absorption spectral measurements (Siiman, O., and Gray, H. B., manuscript in preparation) on $Fe[S_2(PPh_2)_2N_2]$ (ref. 17), which is known to contain a tetrahedral $Fe(II)S_4$ core (18). The position of the $^5E \rightarrow {}^3T_1$ band in the model complex, 15.4 kcm^{-1} (ϵ 10), is virtually the same

as that of the peak under discussion in Fd_{red}. However, another very reasonable candidate assignment for the 15.3-kcm^{-1} band in Fd_{red} is an Fe(II) → Fe(III) intervalence transition. It is not possible from the limited information available to make a definite choice between the two proposals.

The absorption peaks at 10.9 and 12.2 kcm^{-1} attributable to the nonreducible Fe(III) site in the protein fall between the $^6A_1 \rightarrow {}^4T_1$ position (about 7 kcm^{-1}) observed for $^6A_1$ $Fe(III)S_6$ complexes (19) and the 13.5 to 15-kcm^{-1} range predicted for tetrahedral $Fe(III)S_4$. The relatively large band splitting indicates that the structure is significantly distorted from cubic symmetry. Distorted octahedral coordination of the type $Fe(III)S_4X_2$ (X = O or N) appears to be ruled out from the band positions. Even with three sulfur-donor atoms, as in the high-spin (μ_{eff} 5.91, 300°K) $Fe(III)S_3O_3$ complex $Fe(OPPh_2NPPh_2S)_3$, the $^6A_1 \rightarrow {}^4T_1$ band peaks at 8.9 kcm^{-1} (77°K, TlCl disk). Considering the evidence available, then, the most reasonable possibility for the Fe(III) site in Fd_{red} is either a highly distorted (squashed toward D_{2d}) tetrahedral structure or perhaps an $Fe(III)S_4$ unit involved in additional weak coordination to an available nitrogen or oxygen donor atom. The near infrared spectra of a variety of Fe(III) complexes containing sulfur-donor ligands are now being investigated in an effort to provide a satisfactory model for the nonreducible site.

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