THE PROPERTIES OF PARSLEY FERREDOXIN AND ITS SELENIUM-CONTAINING HOMOLOG

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(Received February 16th, 1971)

SUMMARY

The ferredoxin from parsley has been purified to a high degree. The protein contains 2.0 iron atoms per molecule, has an iron to labile sulfide ratio of unity and consumes 9 moles of mercurial, leading to calculated molecular weights of 10800, 10750 and 10960, respectively. These compare well with the results of amino acid analysis which gave a molecular weight of 10660. The dithionite-reduced material showed an EPR spectrum with the experimental g values: 1.899, 1.959, 2.061. Cl^- was found to have a specific broadening effect upon this spectrum.

Selenium has been substituted for the labile sulfur of the native protein to yield a biologically active homolog. A new method for this reaction has been developed. The optical and circular dichroism spectra of the oxidized and reduced forms of the selenium protein have been recorded and are compared with the corresponding state of the native protein.

The following isotopic derivatives have been prepared and studied by EPR spectroscopy: $^{32}S^{56}Fe$ (native), $^{32}S^{57}Fe$, $^{80}Se^{56}Fe$, $^{77}Se^{56}Fe$ and $^{80}Se^{57}Fe$. The $^{32}S^{57}Fe$ derivative shows a broadened EPR spectrum but no hyperfine structure is resolved, this is presumably due to the broad line of the native material. The $^{80}Se^{56}Fe$ spectrum tended toward axial symmetry with apparent g values of 1.937, 1.965, 2.062. This derivative also showed a substantially narrower line width in the z-region. Hyperfine structure is resolved in the z-region of the $^{77}Se^{56}Fe$ homolog, and the EPR spectrum has been analyzed to determine the number of $I = 1/2$ hyperfine centers contributing to the line widths. Hyperfine structure was not resolved in the $^{80}Se^{57}Fe$ species; analysis of the line shape of the low field resonance suggests that the broadening observed is consistent with two iron ($I = 1/2$) centers with similar effective hyperfine splitting constants.

INTRODUCTION

Ferredoxins isolated from the chloroplasts of green plants and algae are members of a subgroup of the general class of non-heme iron labile-sulfur containing proteins. This subgroup is characterized by its members having two atoms each of

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non-heme iron and labile sulfur, accepting a single electron from a suitable reducing system, and exhibiting an EPR spectrum in the reduced form with two principal resonances lying below the free-electron value of $g = 2$, *i.e.* most of the EPR absorption occurs at magnetic fields greater than observed with the free electron.

Proteins of this type have been characterized to varying degrees of completeness[1]. In spite of some differences, similarities in their EPR, optical and circular dichroism spectra suggest a fundamental uniformity between spinach ferredoxin and adrenodoxin[2]. In the cases of adrenodoxin and putidaredoxin the EPR spectra have been obtained of samples in which the magnetic nucleus ^{57}Fe ($I = 1/2$) has been substituted for the naturally occurring ^{56}Fe ($I = 0$) and for which ^{77}Se ($I = 1/2$) and ^{80}Se ($I = 0$) have been substituted for the labile sulfur[3-5]. The results of these studies demonstrated in an elegant and conclusive manner that both iron atoms and both labile-sulfur atoms reside in a single active center.

We would like to report here results of a study on an extensive characterization of parsley ferredoxin with particular emphasis on the EPR and optical properties of the native protein, and the iron-57- and selenium-containing derivatives.

EXPERIMENTAL PROCEDURE

Highest quality Tris base was purchased from Sigma Chemical Co., St. Louis, Mo.; dithiothreitol (99.3 % reduced form), NADPH, and cytochrome *c* from Calbiochem, Los Angeles, Calif.; $Na_2S \cdot 9H_2O$ (micro-analytical reagent) from British Drug Houses, Poole, England and; ^2H_2O (99.87 %) from Bio-Rad Labs, Richmond, Calif. The isotopes ^{77}Se (88 % enrichment), ^{80}Se (90 %) and ^{57}Fe (90 %) as elemental selenium and ferric oxide, respectively, were obtained from Oak Ridge National Lab., Oak Ridge, Tenn. $Na_2^{75}SeO_3$ was obtained in aqueous solution from Nuclear-Chicago, Des Plaines, Ill. Ferredoxin–NADP reductase was prepared from spinach as described by Foust *et al.*[6] and was the gift of G. Foust. All other chemicals were of highest commercial quality, and glass distilled water was used in all operations. The Tris–HCl buffers were made by appropriate dilution of 0.7 M Tris–HCl, pH 7.3 (25°).

Optical spectra were recorded with a Cary Model 15 or a Zeiss DMR 21 recording spectrophotometer. Circular dichroism spectra, obtained with a Durrum-Jasco Model ORD-UV recording spectropolarimeter, were taken at ambient temperatures.

The circular dichroism spectra were manually replotted as molar circular dichroism *vs.* cm^{-1} and these spectra were decomposed into a minimal number of gaussians in the usual manner. The "best" fit was determined by a visual comparison of experimental and computed spectra.

EPR spectra were obtained at X-band with a modified Varian V-4500 spectrometer; measurements were generally made in the 20–30° K temperature range[7]. Quantitation of EPR intensity was performed by manual double integration using Cu^{2+} in excess EDTA as a standard.

The preparation of parsley ferredoxin was accomplished by a minor modification of the procedure described by PETERING AND PALMER[8]. This modification is as follows: after the dialysis stage the protein is diluted 5 times with water and passed onto a 4 cm × 40 cm column of DEAE-cellulose equilibrated with 0.15 M Tris–HCl, pH 7.3. The protein collects as a dark brown band at the top of the column which is then developed with a gradient prepared from 1.5 l of 0.15 M Tris buffer containing 0.1 M

NaCl and 1.5 l of the same buffer containing 0.6 M NaCl. The red-brown eluate is diluted 2-fold with water and adsorbed on Whatman DE-52 anion exchanger. The remainder of the purification is as described previously[8].

The yield of ferredoxin from 20 kg of parsley is 100–200 mg protein with a critical ratio ($R = A_{422 \text{ nm}}/A_{277 \text{ nm}}$) greater than 0.6. The ferredoxin is stored as a concentrated solution (>1 mM) in 0.15 M Tris–HCl containing 1 M NaCl and under an oxygen-free atmosphere. The protein is indefinitely stable under these conditions.

The dry weight determination of protein concentration was done by the procedure by HUNTER[9] except that weighings were done on a Mettler Type H16 balance. Correction was made for the density of the KCl[10] in converting to volume units, but no correction was attempted for possible binding of salts to the protein. This weight was related to the optical properties of the original solution. The errors in the weight and absorbance measurements are both estimated at slightly over 1 %.

Iron analyses were carried out as described by MASSEY[11] and labile-sulfur measurements were made by the method of FOGO AND POPOWSKY[12] as modified by LOVENBERG et al.[13] and SIEGEL[14] using an extinction coefficient for methylene blue at 680 nm of $3 \cdot 10^4$ l·mole^{-1}·cm^{-1} (see ref. 15). It was necessary to carry out the base denaturation under anaerobic conditions in a two-sidearm modified Thunberg tube as aerobic denaturation led to values of S^{2-} significantly lower than anticipated for this protein.

Mercurial titrations were carried out as described by PETERING AND PALMER[8].

Apoprotein was obtained by treatment of the native protein with Mersalyl* (50-fold molar excess) and Tiron* (50-fold molar excess) followed by passage over a Bio-Gel P6 column[17] or by repeated precipitation of the native protein with 12 % trichloroacetic acid with re-solution in 0.7 M Tris–HCl. Reconstitution was affected by mixing in the following order, a 20-fold molar excess of dithiothreitol, a 3-fold molar excess of Na₂S, and a 3-fold molar excess of FeCl₃ (^{56}Fe or ^{57}Fe), dissolved in concentrated HCl. The mixture was strongly buffered near neutrality to offset the HCl. After standing for 1 h at room temperature the mixture was diluted with water and the protein adsorbed on Whatman DE-52 in a (2 cm × 5 cm) column. The resin was washed with several 100 ml of 0.15 M Tris–HCl and the ferredoxin eluted with similarly buffered 1 M NaCl. The eluted material was passed over a Bio-Gel P60 column (2 cm × 40 cm) equilibrated and developed with the 0.15 M Tris buffer. In this way it was possible to obtain reconstituted ferredoxin which by its optical spectrum is identical to native material.

Substitution of the labile sulfur with selenium

Apoprotein was prepared by trichloroacetic acid precipitation. Elemental selenium was converted to H₂SeO₃ by treatment with concentrated nitric acid[18], which was then removed by evaporation. The residue of solid H₂SeO₃ was dissolved in a known amount of water.

The reconstitution reaction with selenium was carried out under anaerobic conditions in a vessel having two sidearms. Apoprotein (2 μmoles) was buffered near neutrality with 0.7 M Tris–HCl and mixed with a 20-fold excess of freshly dissolved dithiothreitol. A 3-fold excess of H₂SeO₃ (in aqueous solution) along with a small

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* Abbreviations: Mersalyl, sodium o-[(3-hydroxymercuri-2-methoxypropyl)carbamyl]-phenoxyacetate; Tiron, disodium 2,3-dihydroxybenzene 3,5-disulfonate.

amount of radioactive $Na_2{}^{75}SeO_3$ was placed in one sidearm of the vessel and a similar quantity of ferric citrate was placed in the other sidearm. The system was thoroughly evacuated and flushed with oxygen-free helium. Finally the system was placed under a positive pressure of inert gas and the selenite solution mixed into the protein–dithiothreitol solution to give a final volume of 3.5 ml. A brown to golden-yellow color appeared immediately and generally disappeared after several minutes; the iron solution was then mixed in. A dark brown color formed immediately which, over a period of 1–2 h, gave way to a yellow-brown color. The solution was then opened to the air, diluted 10 times with water, and passed through a short column of Whatman DE-52. A red-brown band formed at the top of the column which was washed with several 100 ml of 0.15 M Tris–HCl buffer. The protein was eluted with buffered 1 M NaCl, and passed over a Sephadex G-75 column (2 cm × 40 cm) in the cold room. (Bio-Gel filtration materials could not be used to purify selenium-containing ferredoxin as the protein was bleached on passing into the matrix of the column.)

In certain experiments it was not important to remove the apoprotein but was important that no extraneous iron be present. In these cases the eluate from the DE-52 column was passed over a short Sephadex G-25 column.

Analysis of selenium was done by radioactive counting of samples containing a known quantity of iron and comparing the number of counts from a system containing a known quantity of ^{75}Se. The scintillation fluid was that described by GIBSON et al.[19], and the water content was equal in sample and standard vials. Counting was carried out on a Nuclear-Chicago Unilux I scintillation counter.

The activity of selenium-substituted parsley ferredoxin was measured by the ferredoxin-mediated reduction of cytochrome c by NADPH in the presence of ferredoxin–NADP reductase as described by LAZARINI AND SAN PIETRO (ref. 20, cf. also ref. 8). Native parsley ferredoxin and selenium-substituted parsley ferredoxin were compared and identical time courses were taken to indicate identical activities. The number of reducing equivalents necessary to reduce selenium-substituted parsley ferredoxin was determined using dithionite as the reductant, and the experiment was carried out in the apparatus described by FOUST et al.[21] as described previously[22].

In preparing samples of parsley ferredoxin and selenium-substituted parsley ferredoxin for EPR measurements it was often important to have the concentrated solutions of these proteins free of Cl⁻. The Tris–HCl buffer was exchanged by passing a protein solution over a short Bio-Gel P6 or Sephadex G-25 column equilibrated with the buffer of choice, usually Tris–H_2SO_4, the eluted protein was freeze–dried and the residue dissolved in a known quantity of water. The protein was reduced in the EPR tube with solid dithionite. No change in the optical spectrum occurs as a result of lyophilization.

For those measurements in which ^2H_2O was the desired solvent the above procedure was used with ^2H_2O substituted for H_2O in each solution.

RESULTS

Fig. 1 shows the disc-gel electrophoretic pattern of a ferredoxin solution having a critical ratio R, of 0.6. The minor band travelling with the bulk of the protein is red-brown colored and is a constant feature of the material obtained by the above procedure. It is presumed to be a ferredoxin and accounts for approx. 5–10 % of the

ferredoxin protein. The slower moving bands indicate a slight contamination but their intensity increases when the protein is exposed to air for long periods of time and they may be due to products of oxidative denaturation[23]. No attempt has been made to purify the protein further.

Our analytical data on the native protein are collected in Table I. The iron and sulfide contents, molecular weight, mercurial binding capacity and optical properties are all similar to those for the same protein isolated from spinach[8, 25].

Preparation of selenium-substituted parsley ferredoxin

The method used in preparing selenium-substituted parsley ferredoxin incorporated an *in situ* production of that form of selenium which replaces sulfur in the reconstitution reaction. Selenite anion reacts with thiols as is shown in Reaction I (ref. 28).
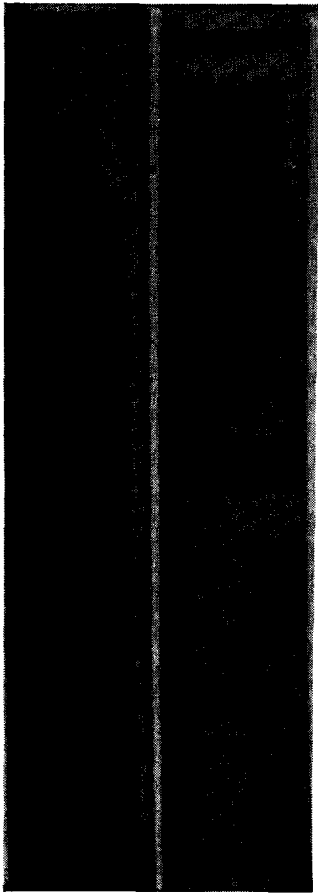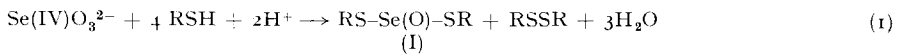
$$Se(IV)O_3{}^{2-} + 4 RSH + 2H^+ \longrightarrow RS\text{--}Se(O)\text{--}SR + RSSR + 3H_2O \qquad (1)$$
$$(I)$$

Fig. 1. Disc electrophoresis of ferredoxin on polyacrylamide gel (20 % left, 30 % right). The gels were prepared as described by BREWER AND ASHWORTH[60] and stained with amido black; 3.04 μg protein were applied. The ferredoxin travels slightly behind the front in both gels.

TABLE I

PROPERTIES OF NATIVE PARSLEY FERREDOXIN

Analysis	Result	Mol. wt., minimum	Mol. wt.
μatoms Fe per mg	0.185	5400	10 800 [*]
μatoms S^{2-} per mg	0.186	5380	10 760 [*]
Mercurial titration: μmoles mercurial bound per mg	0.822	1220	10 960 [**]
Amino acid analysis			10 658 [***]
			14 000 [§]
			19 000 [§]
Sedimentation diffusion			12 200 [§]

λ (nm)	Specific extinction $(ml \cdot mg^{-1} \cdot cm^{-1})$
255	1.147
260	1.175
277	1.39
294	0.909
330	1.134
390	0.692
422	0.865
448	0.759
463	0.779
690	0.090
ε_M at 422 nm	9.200 $l \cdot mole^{-1} \cdot cm^{-1}$ [§§]

[*] Assuming 2 atoms per mole.
[**] Assuming 5 cysteines and 2 labile sulfides.
[***] Personal communication (S. KESESZTES-NAGY, F. PERINI AND E. MARGOLIASH).
[§] Ref. 24.
[§§] Assuming mol. wt. = 10800.

The selenium which resides in (I) at a formal valence level of zero, is presumed to be reduced further to the level of Se(—II) in the presence of an excess of dithiothreitol. This contention is supported by the fact that cysteine trisulfide serves as a source of labile sulfur in the reconstitution of native ferredoxin under similar conditions (D. H. PETERING AND G. PALMER, unpublished results, cf. also ref. 23).

Properties of selenium-substituted parsley ferredoxin

As documented below the selenium-substituted protein is spectrally very similar to the native protein. The critical ratio $(A_{440 \text{ nm}}/A_{275 \text{ nm}})$ varied between 0.48 and 0.53 compared with 0.62 $(A_{422 \text{ nm}}/A_{275 \text{ nm}})$ for the native protein.

The selenium content is 0.9 ± 0.1 atom per atom iron as determined by radio-isotope analysis. Because of the limited amount of protein available we have not determined the iron content on a protein basis: for the remainder of this paper we assume that the reconstituted selenium protein contains 2 atoms of iron and therefore 2 atoms of selenium.

The biological activity of the selenium-substituted parsley ferredoxin was com-

pared with that of native parsley ferredoxin. The time course of cytochrome c reduction in the assay system is non-linear; therefore, the concentrations of selenium-substituted parsley ferredoxin and parsley ferredoxin were varied until two progress curves of similar curvature were obtained. This was found at a concentration of selenium-substituted parsley ferredoxin 1.27 times higher than the native protein and is taken to mean that the selenium-containing ferredoxin is approx. 80 % as active as native material.

The selenium-containing protein has been titrated with dithionite under anaerobic conditions to determine the number of electrons which it can accept. The titration is linear throughout most of the reaction and there is a well-defined end-point. After making a small correction for the initial lag phase (which is presumably due to residual oxygen[22]) the end-point is calculated to require 0.57 mole dithionite per 2 iron atoms, i.e. 1 electron per mole selenium-substituted parsley ferredoxin. This experiment clearly demonstrated that selenium-substituted parsley ferredoxin is a 1-electron accepting system as is the native protein[22]. This experiment was done in collaboration with Dr. S. G. Mayhew.
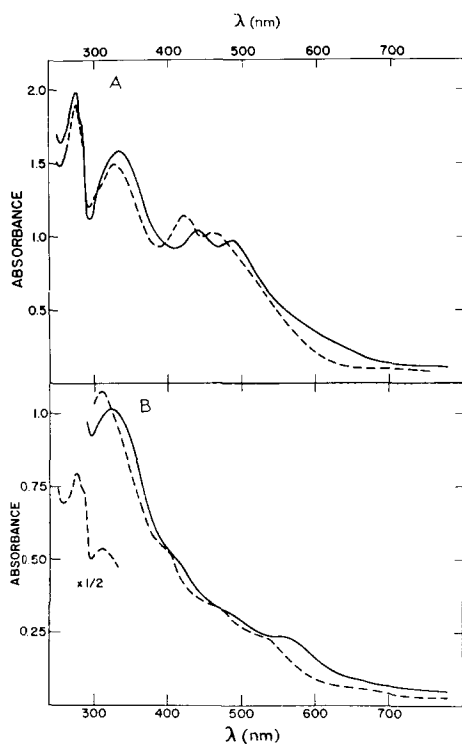

Fig. 2. A. Absorption spectra of the oxidized forms of parsley ferredoxin (-----), 0.125 mM, selenium-substituted parsley ferredoxin (————), 0.116 mM. The solutions were buffered at pH 7.5 with 0.15 M Tris–HCl in 1 M NaCl; light path, 1 cm. B. Absorption spectra of the fully reduced form of parsley ferredoxin (-----), 0.10 mM, and selenium-substituted parsley ferredoxin (————), 0.095 mM. The proteins were reduced by sodium dithionite in the titration apparatus of FOUST et al.[23], allowing the spectra to be taken in the presence of a very small excess of reductant.

Comparison of the optical properties of native and selenium-substituted parsley ferredoxin

In Fig. 2 we compare the optical absorption spectra of native sulfur-containing ferredoxin and its selenium-containing analog. The spectra are qualitatively similar both in intensity and number of maxima. However, with the exception of the peak at 275 nm, the main absorption bands are all shifted to longer wavelengths by 10–40 nm. Thus the two peaks which are characteristic of proteins of this class and which in parsley ferredoxin are located at 422 and 460 nm are shifted to 440 nm and 480 nm, respectively. Similarly the peak at 328 nm is shifted to 336 nm. The molar absorbance of the native protein at 422 nm is 9200 $l \cdot mole^{-1} \cdot cm^{-1}$. The molar absorption of the selenium analog at 440 nm varies somewhat, being 9000 $l \cdot mole^{-1} \cdot cm^{-1}$ in the best preparation (based on iron, assuming 2 atoms of iron per mole protein).

The bathochromic (red) shift is also observed in the reduced proteins (Fig. 2). There are no significant differences in intensity of the optical spectra of the reduced proteins.

The circular dichroism spectra of sulfur-containing parsley ferredoxin, 50 % reduced sulfur-containing parsley ferredoxin, and fully reduced sulfur-containing parsley ferredoxin; and selenium-substituted parsley ferredoxin, 50 % reduced selenium-substituted parsley ferredoxin, and fully reduced selenium-substituted parsley ferredoxin are shown in Fig. 3. As has been noted on several occasions[2, 28, 29] the circular dichroism spectra of these proteins exhibit considerably more detail than the absorption spectra and are thus much more valuable for comparative purposes. Table II contains the positions of Cotton effects, the corresponding intensities, and the wavelengths of the isosbestic points between the circular dichroism spectra of oxidized and reduced species.

The spectra are very similar to those described earlier for spinach ferredoxin[2] although there are differences. The circular dichroism spectrum of reduced parsley ferredoxin appears to be identical with the spinach protein. Because the spectrum was obtained on a sample which contained only a fractional molar excess of dithionite it was possible to record the data to 260 nm. Previously the great excess of dithionite absorbed all the available light in this region. Two new Cotton effects, both positive, were observed at 290 and 270 nm. The spectrum of the 50 % reduced sample establishes the existence of five isodichroic points at 312, 380, 401, 505 and 660 nm. Thus, during reduction, the spectra can be described by a linear combination of the completely oxidized and completely reduced proteins.

Selenium-substituted parsley ferredoxin is also optically active in its oxidized and reduced forms (Fig. 3), and indeed the circular dichroism spectra are qualitatively very similar to the natural protein. The principal difference between the two proteins is the general bathochromic shift of the entire spectrum including changes in the ultraviolet region which were not apparent in the optical spectra. In addition to this shift which is present in both oxidized and reduced proteins several specific differences between the native and selenium-substituted protein (Fig. 3), the most prominent being that oxidized selenium-substituted parsley ferredoxin exhibits a very pronounced shoulder at 485 nm which is present as an inflection at 470 nm in the native protein. This band appears to be more pronounced in oxidized alfalfa ferredoxin[30].

The spectrum taken at 50 % reduction establishes four isosbestic points at 316, 384, 412 and 526 nm. The isosbestic point at 660 nm in the native protein appears to be near 690 nm in selenium-substituted parsley ferredoxin. Thus as with the native

protein there appears to be no optically active intermediate present during reduction.

As an aid in the understanding of these spectra and in making a comparison between the two homologs sulfur-containing parsley ferredoxin and selenium-substituted parsley ferredoxin, the circular dichroism spectra of the four species have been resolved into a family of Gaussian components (*cf.* EXPERIMENTAL PROCEDURE), and the results presented in Table III. For this analysis the experimental spectra were replotted against cm^{-1} and the individual components assumed to be gaussian in these units rather than wavelength. The resulting changes in spectral shape required that
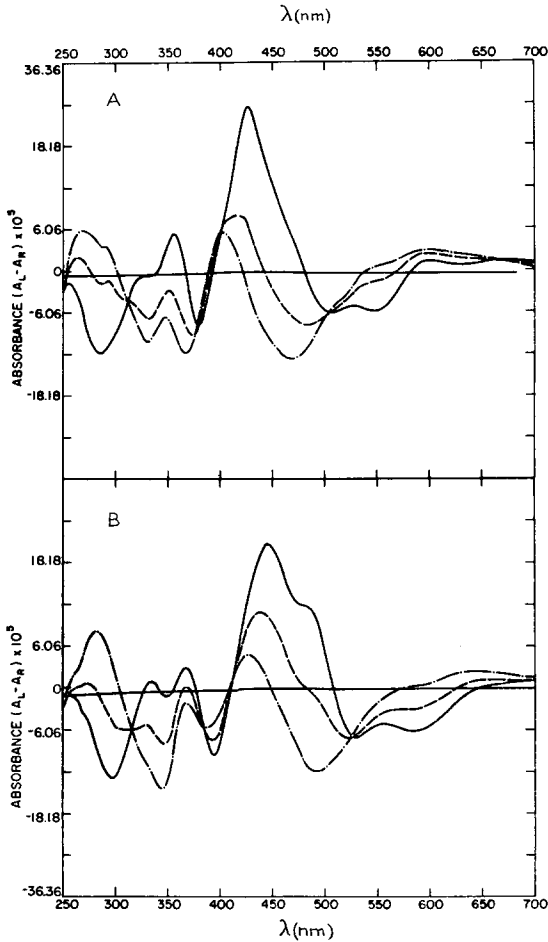

Fig. 3. A. Circular dichroism spectra of parsley ferredoxin in the fully oxidized (————), 50 % reduced (—————), and fully reduced (–·—·–) forms. The spectra were recorded during a dithionite titration of a 0.10 mM solution of parsley ferredoxin and no correction for volume change was made. The curves shown are direct tracings of the experimental lines, including the base line (————). B. Circular dichroism spectra of selenium-substituted parsley ferredoxin in the fully oxidized (————), 50 % reduced (—————), and fully reduced (–·—·–) forms. The spectra were recorded during a dithionite titration. The selenium-substituted parsley ferredoxin concentration was 0.095 mM at the beginning of the titration; the curves shown are direct tracings of the experimental lines, including the base line (————); no correction for volume change has been made. Conditions as in Fig. 2.

TABLE II

POSITIONS OF COTTON EFFECTS AND THEIR INTENSITIES AND ISOSBESTIC POINTS IN THE CIRCULAR DICHROISM SPECTRA

I = molar circular dichroism $(\varepsilon_L - \varepsilon_R)$, $l \cdot mole^{-1} \cdot cm^{-1}$.

Ferredoxin, oxidized	λ (nm)	287				356	380	427	508	550	600	675
	I	−11.2				5.3	8.0	23.0	6.1	5.5	1.5	1.?

Ferredoxin, reduced	λ (nm)	268	290(S)*	332	368	402	470	598
	I	4.7	4.4	9.7	11.5	5.8	−12.7	3.3

Isosbestic points λ (nm)   312, 380, 402, 506, 660

Selenium-substituted ferredoxin, oxidized	λ (nm)	298	334	350	367	395	445	530	586
	I	−13.7	1.1	−1.0	3.3	10.8	23.0	−7.7	−6.6

Selenium-substituted ferredoxin, reduced	λ (nm)		282	346	386	417	492	645
	I		9.3	14.8	5.8	5.4	12.5	2.5

Isosbestic points λ (nm)   316, 384, 412, 526

* Shoulder.

TABLE III

PARAMETERS NECESSARY TO FIT THE EXPERIMENTAL CIRCULAR DICHROISM SPECTRA BY SUPERPOSITION OF GAUSSIANS, $I \exp[(\nu_{max} - \nu)/\Delta_i]^2$

Intensities I in $l \cdot mole^{-1} \cdot cm^{-1}$ and rotatory strength R is in Debye units $\times$ Bohr magnetons[2,55].

Ferredoxin, oxidized	ν_{max} (cm^{-1})	37 000	35 600	34 000	32 500	29 500	29 350	28 100
	λ_{max} (nm)	270.2	280.8	294.1	307.6	338.9	340.7	355.8
	$\Delta\nu$ (cm^{-1})	900	1400	1200	950	600	800	1300
	I	−2.9	−8.9	−7.3	−4.7	3.8	−5.2	5.4
	R	0.0309	−0.159	0.132	−0.0693	0.0340	−0.0606	0.111
Selenium-substituted ferredoxin, oxidized	ν_{max} (cm^{-1})	35 900	34 600	33 200	31 300	30 000	28 300	27 100
	λ_{max} (nm)	278.5	289.0	301.2	319.4	333.3	353.3	369.0
	$\Delta\nu$ (cm^{-1})	1300	800	1550	700	900	800	900
	I	−5.4	−4.2	−13.2	−3.2	1.3	−1.6	3.67
	R	0.0858	0.0427	0.270	−0.0318	0.0171	0.0201	0.0535
Ferredoxin, reduced	ν_{max} (cm^{-1})	38 700	37 300	35 600	34 200	32 200	31 300	30 300
	λ_{max} (nm)	258.3	268.0	280.8	292.3	310.5	319.4	330.0
	$\Delta\nu$ (cm^{-1})	800	1300	1100	1100	900	500	1100
	I	2.7	6.3	3.4	3.2	−2.8	−3.0	−9.6
	R	0.0242	0.0963	0.0463	0.0452	−0.0319	−0.0216	−0.156
Selenium-substituted ferredoxin, reduced	ν_{max} (cm^{-1})	37 800	36 300	35 000	33 700	31 200	29 450	28 500
	λ_{max} (nm)	264.5	275.4	285.7	296.7	320.5	339.5	350.8
	$\Delta\nu$ (cm^{-1})	1300	1200	1450	700	1100	1050	700
	I	3.7	4.0	7.6	1.8	−6.7	−14.2	−7.2
	R	0.0561	0.0584	0.139	0.0160	0.102	−0.245	−0.0964

we use a larger number of gaussian components to obtain a good visual fit, than was anticipated from similar analyses performed on the direct spectra. With minor exceptions the data of Table III define the experimental spectra quite accurately and to that extent are a numerical expression of the spectra.
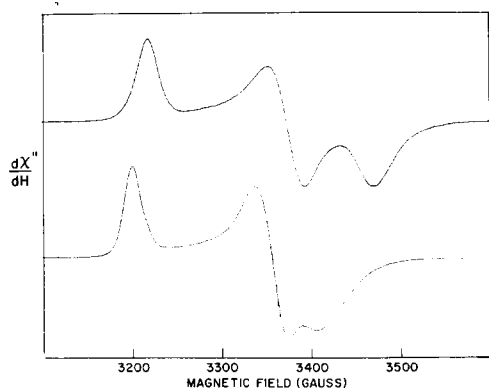

Fig. 4. EPR spectra of dithionite reduced parsley ferredoxin (upper) and $^{80}Se^{56}Fe$ parsley ferredoxin (lower). The parsley ferredoxin was present at 2.8 mM with 4 M Tris–HClO$_4$ in ^2H_2O, pH 7.5. The spectrum was recorded under the following conditions; microwave power, 3 mW; frequency, 9.230 GHz; modulation amplitude, 3.2 gauss; scanning rate, 100 gauss/min; time constant, 0.3 sec; temperature, 26° K. The selenium-substituted parsley ferredoxin was present at 1.62 mM with 3.3 M Tris–H$_2$SO$_4$ in water, pH 7.5. The spectrum was recorded under the same conditions as above except that frequency was 9.232 GHz.

50	23 900	23 300	22 100	19 900	18 900	17 600	17 100	14 800	
79.5	418.4	429.1	452.4	502.5	529.1	568.1	584.7	675.6	
00	1200	700	1000	700	1100	900	700	1100	
-8.7	10.9	12.3	9.5	—4.4	—3.5	—5.4	5.0	1.5	
-0.102	0.240	0.163	0.189	—0.0532	—0.0957	—0.135	0.0896	0.0503	
00	22 700	22 000	20 400	19 000	17 700	16 700	14 500		
95.2	440.5	454.5	490.1	526.3	564.9	598.8	689.6		
00	1350	1100	850	700	1500	800	1100		
11.0	16.7	8.0	11.2	—5.8	—4.5	—3.2	1.7		
0.172	0.437	0.175	0.205	—0.0943	—0.169	—0.0681	0.0575		
00	27 800	26 750	25 100	24 300	22 700	21 250	19 300	18 300	16 850	15 500
42.4	359.7	373.8	398.4	411.5	440.5	470.5	518.1	546.4	603.1	645.1
00	1000	900	600	700	800	1450	550	600	800	1000
-2.5	—7.0	—8.6	4.7	3.8	—3.4	—12.6	—2.4	0.9	2.9	2.0
-0.0265	—0.114	—0.141	0.0492	0.0485	—0.0504	—0.395	—0.0402	0.0123	0.0600	0.0574
00	25 600		23 500	22 600	21 000	20 250	18 800	15 900	14 900
74.5	390.6		425.5	424.4	476.1	493.8	531.9	628.9	671.1
00	800		800	600	400	1500	900	800	900
-3.0	—5.4		5.4	1.9	—0.8	—12.4	—1.0	1.6	2.0
-0.0377	—0.0763		0.0802	0.0217	—0.00696	—0.405	—0.0302	0.0342	0.0520

EPR spectra of native and isotopically substituted parsley ferredoxin

The following isotopically substituted derivatives of parsley ferredoxin have been examined by EPR spectroscopy: $^{32}S^{56}Fe$ (native), $^{32}S^{57}Fe$, $^{80}Se^{56}Fe$, $^{77}Se^{56}Fe$, $^{80}Se^{57}Fe$. In all the derivatives both atoms of iron and both atoms of chalcogen are substituted to a degree dictated by the abundance of the isotope and the statistics of random exchange (see DISCUSSION). The oxidized samples showed a variable, very small signal around $g = 4.3$. The intensity of this signal was generally a little larger in the selenium-substituted protein than in native or reconstituted material.

The EPR spectra of dithionite-reduced $^{32}S^{56}Fe$ and $^{80}Se^{56}Fe$ derivatives are shown in Fig. 4. The EPR spectrum of the native protein is almost exactly the same as that of spinach ferredoxin[7] being a typical powder spectrum of a single unpaired electron in rhombic symmetry: the experimental g values are 2.052, 1.959, 1.899. These compare with the values of 2.046, 1.957, 1.886 obtained with spinach ferredoxin under identical conditions in a single experiment. Apart from the differences in g values just noted the only other conspicuous difference is that the high-field resonance is considerably broader in the spinach protein. This has a half-width at half-height of 43 gauss in spinach ferredoxin compared to 30 gauss in parsley ferredoxin. The intensity of the EPR spectrum accounts for 1.08 unpaired electrons per molecule of parsley ferredoxin. The spectrum is very temperature sensitive and broadens rapidly at temperatures close to liquid nitrogen.

There appears to be a specific effect of Cl^- on the width of the EPR spectrum of this protein which is observed as a broadening of the low field peak (g_z) with increasing Cl^- (Fig. 5). In zero Cl^- the half-width of the peak is approx. 18 gauss, this
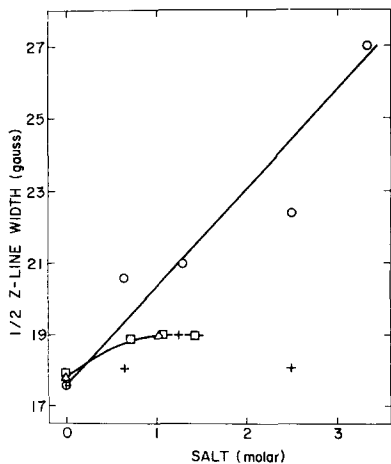

Fig. 5. The effect of various salts on the EPR spectrum of parsley ferredoxin and spinach ferredoxin. The ordinate is the half-width (in gauss) at half-height of the low-field resonance (z). Parsley ferredoxin: $\bigcirc$, NaCl; $\triangle$, NaF; $\square$, NaBr. Spinach ferredoxin: $+$, NaCl. Samples were prepared by diluting the protein into solutions of known ionic composition of pH (7.5), and reducing with solid dithionite. The data taken at zero "salt" concentration actually represents 3–5 M Tris–HClO$_4$ or Tris–H$_2$SO$_4$; salts which do not affect the spectrum. Conditions of EPR spectroscopy are generally as described in Fig. 6.

increases to 27 gauss in 3.3 M Cl^-. F^-, ClO_4^- and SO_4^{2-} have only a small effect on the width of the spectrum. The effect of Cl^- on the line width of spinach ferredoxin is small (Fig. 5). Because of this phenomenon all spectra shown in this paper were taken in Cl^--free buffers.

Samples of the native protein which have been soaked in ^2H_2O and also reconstituted in ^2H_2O have been examined by EPR. No change in EPR line width was observed.

The EPR spectrum of the $^{80}Se^{56}Fe$ protein (Fig. 4) shows significant differences from the native protein. The entire spectrum is moved to lower field (higher g) and the differences between g_x and g_y become smaller so that the spectrum tends towards axial symmetry. In addition the measured width of the resonance at low-field collapses to approx. 12 gauss: this proves to be vital in attempts to quantitate ^{57}Fe hyperfine interactions in these proteins (see below). The apparent g values are 1.937, 1.965, 2.061. The integrated EPR intensity accounts for 1.1 electrons per molecule of ferredoxin.

Substitution of ^{80}Se $(I = 0)$ with ^{77}Se $(I = 1/2)$ produced a marked broadening of the entire EPR spectrum of selenium-substituted parsley ferredoxin (Fig. 6). This is particularly evident in the g_z region where hyperfine splitting can be resolved (Fig. 6, inset) as clearly defined shoulders on both ascending and descending wings
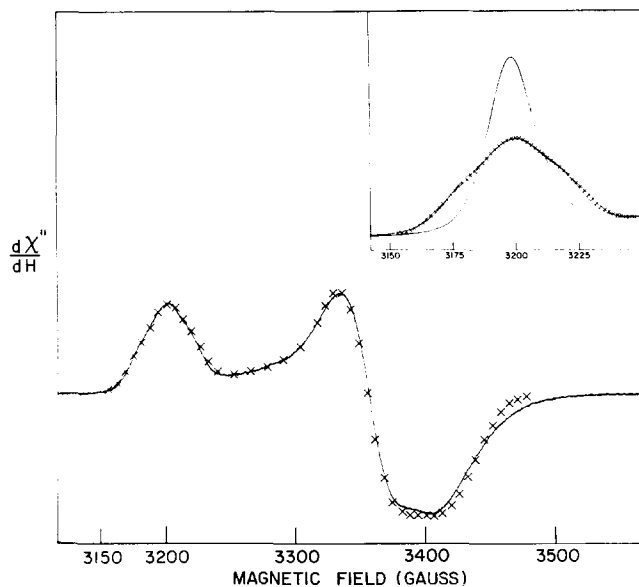

Fig. 6. The EPR spectrum of $^{77}Se^{56}Fe$ parsley ferredoxin (———), superpositioned with a computed spectrum ($\times$). The sample was 0.7 mM in protein, 3.3 M in Tris–H_2SO_4 at pH 7.5 in H_2O. The lower spectrum was recorded under the following conditions: microwave power, 3 mW; frequency, 9.232 GHz; modulation amplitude, 3 gauss; scanning rate, 100 gauss/min; time constant, 1 sec; temperature, 26° K. The inset shows an expansion of the low-field resonance superimposed with a calculated spectrum ($\times$), and also the low-field resonance of the $^{80}Se^{56}Fe$ derivative. The spectra were recorded under the same conditions as above except: $^{77}Se^{56}Fe$; microwave frequency, 9.229 GHz; scanning rate, 25 gauss/min; temperature, 27° K. For $^{80}Se^{56}Fe$ the conditions were the same except the frequency was 9.224 GHz. The $^{80}Se^{56}Fe$ parsley ferredoxin concentration was the same as in Fig. 4. The spectra were computed as described in the text using the appropriate parameters from Table IV.
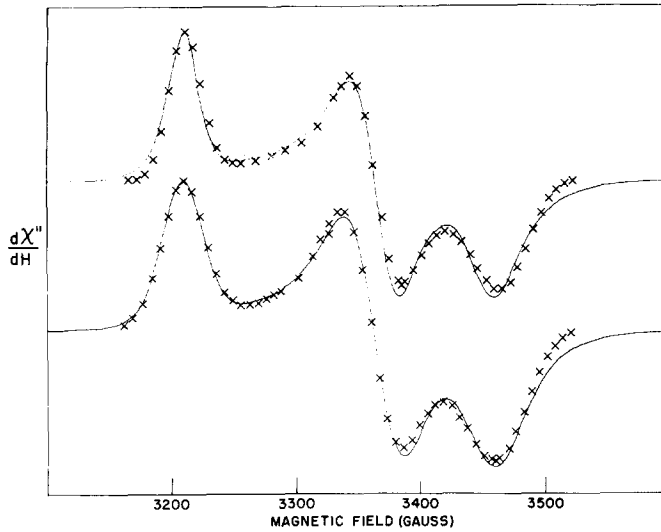
Fig. 7. EPR spectra of $^{32}S\ ^{57}Fe$ parsley ferredoxin (lower) and $^{32}S\ ^{56}Fe$ parsley ferredoxin (upper) and a superposition of computed spectra ($\times$). The native protein is the same as shown in Fig. 6, with conditions the same except: frequency, 9.199 GHz; temperature, 28° K. The $^{32}S\ ^{57}Fe$ parsley ferredoxin derivative was present at 2.06 mM with 1.37 M Tris–HClO$_4$ in ^2H_2O, pH 7.5. The spectrum was recorded under the same conditions as above, and the computed spectra were calculated using the appropriate parameters given in Table IV.
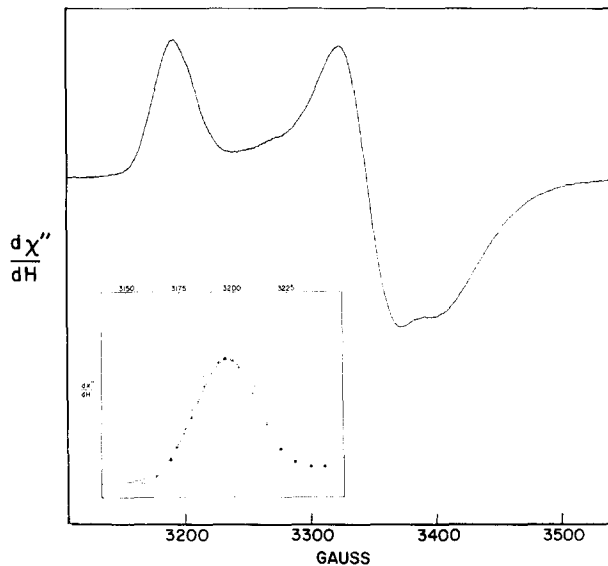

Fig. 8. EPR spectrum of $^{80}Se^{57}Fe$ parsley ferredoxin and (inset) computed shape of the low-field resonance assuming a single $I = 1/2$ hyperfine center ($\bullet$), $A_z = 22$ gauss, and two $I = 1/2$ hyperfine centers ($+$), $A_z = 15$ gauss. The derivative was present at 0.81 mM with 1.5 M Tris–H$_2$SO$_4$ in water. The conditions of recording were: microwave power, 3 mW; frequency, 9.198 GHz; modulation amplitude, 3 gauss; scanning rate, 100 gauss/min; time constant, 0.3 sec; temperature, 28° K.

of the low field line. The integrated intensity of this spectrum accounts for 0.8 electron per molecule of selenium-substituted parsley ferredoxin.

Substitution of ^{57}Fe ($I = 1/2$) for ^{56}Fe ($I = 0$) in sulfur-containing parsley ferredoxin produced only a slight broadening of the EPR spectrum (Fig. 7), with no resolution of hyperfine splitting as has previously been reported for the spinach protein[32]. Because of the narrower line width at g_z in the selenium derivatives we have also prepared the ^{80}Se^{57}Fe protein (Fig. 8). The EPR spectrum was again conspicuously broadened but no resolution of ^{57}Fe hyperfine splitting was obtained.

DISCUSSION

The data presented in this paper show that parsley ferredoxin is a typical chloroplast ferredoxin. BENDALL et al.[24] determined the molecular weight of the protein by ultracentrifugal methods and obtained a value of 12200. Their minimal molecular weight based on the iron content was 6400 and they concluded that the protein contained 2 atoms of iron per molecule. The protein we have prepared has an iron content of 0.186 μatom/mg protein corresponding to a minimal molecular weight of 5400 and a molecular weight of 10800 for the protein. The data from this study (Table I) support a molecular weight of near 10700, a value which is in agreement with the molecular weights of other chloroplast ferredoxins[25, 26, 32–35].

The sulfide to iron ratio is 1.0, consistent with observations on other proteins of this class, although there is a recent report that the protein from *Anacystis nidulans* contains only 0.5 mole S^{2-} per mole of iron[36]. Our experience with parsley ferredoxin indicates that with protein samples of indifferent quality (e.g. $R = 0.45$) the yield of labile sulfide is low and can be significantly improved by conducting the denaturing stage of the labile-sulfide assay under anaerobic conditions. In one experiment 1.1 moles of sulfide was found aerobically and 1.6 moles anaerobically. A phenomenon of this kind may be responsible for the apparent low yield obtained with the protein from Anacystis.

The protein does deteriorate when stored aerobically at $-1°$ in 1 M NaCl, e.g. for one sample, R had decreased from 0.62 to 0.45 in 7 weeks. However, when the protein is stored under inert gas it appears to be stable indefinitely. This has previously been observed for alfalfa ferredoxin[33] and seems to be due to the oxidation of the labile sulfide to a species at the formal oxidation state of zero, probably cysteine trisulfide[23].

The optical and circular dichroism spectra of the oxidized and reduced forms of parsley ferredoxin are very similar to those previously reported for spinach ferredoxin[2, 29]. The most conspicuous difference between the two proteins is the much higher ultraviolet absorption in spinach ferredoxin; a consequence of the larger number of aromatic amino acids in this protein, specifically the presence of a single tryptophan residue (personal communication, S. KERESZTES-NAGY, F. PERINI AND E. MARGOLIASH, cf. ref. 25 and Table I).

PADMANABHAM AND KIMURA[37, 38] have recently suggested that the optical activity of these proteins is not an inherent property of the iron–sulfur chromophore but that it arises from the interaction of this chromophore with the asymmetric environment of the protein, i.e. it is of extrinsic origin[39]. This would imply that not only are there similar sites in these protein but that the conformation of the poly-

peptide chain in the neighbourhood of these sites is also very similar; this despite anticipated differences in amino acid sequence. The observed dissimilarities in circular dichroism spectra could then reflect small differences in the conformation of the protein chain in the neighbourhood of the active center. However, the markedly similar circular dichroism spectra of such diverse non-heme iron proteins as xanthine oxidase[29,40], adrenodoxin[2], *Azotobacter vinelandii* proteins I and II[41]; and spinach[2,29] and parsley ferredoxins suggest that the optical activity may well be an intrinsic property of the iron–sulfur center of these proteins.

Selenium-substituted parsley ferredoxin is conveniently prepared by the method described in the experimental section, and no specialized apparatus is required. The method is based on two observations. First of all the inner sulfur of cysteine trisulfide (CysS–S–SCys) serves as a source of sulfide ion in reconstitution in the presence of dithiothreitol. Secondly, thiols react with selenious acid to produce a compound of structure RS–Se–SR analogous to cysteine trisulfide[27]. This latter reaction has recently been employed to insert selenium into the disulfide groups of ribonuclease[42]. Presumably, when present in excess, dithiothreitol reduced the RS–Se–SR to RS$^-$ and Se^{2-}; the latter is then incorporated into the protein.

In agreement with the earlier findings of TSIBRIS *et al.*[4] with putidaredoxin and of ORME-JOHNSON *et al.*[5] with adrenodoxin, the labile sulfide of parsley ferredoxin can be replaced by selenide to yield a product having properties which resemble those of the native protein in every respect.

First and foremost in the comparison of this protein with native parsley ferredoxin is that it is very active in the chosen assay *viz.* the ferredoxin mediated reduction of cytochrome *c* by NADPH and ferredoxin–NADP reductase; we find the protein to exhibit 80 % of the activity of a corresponding quantity of parsley ferredoxin in this assay. This is strong evidence that selenium-substituted parsley ferredoxin is a proper homolog of the native protein. The optical and circular dichroism spectra of both oxidized and reduced selenium-substituted parsley ferredoxin are also strikingly similar to the native protein, preserving both the general shape and intensity over the entire spectral range studied. With both phenomena the most obvious difference is a pronounced bathochromic shift throughout the ultraviolet and visible regions of the spectra. A similar observation has been made on the visible spectrum of putidaredoxin[1].

Relatively few comparative spectroscopic studies have been made of structurally homologous transition metal complexes containing higher member Group VIb elements. CHATT *et al.*[43] studied complexes of the type *trans*-(L, piperidine PtCl$_2$) with L being diethyl sulphur, diethyl selenium, and diethyl tellurium. They observed that the *d–d* transitions occurring in the 215–350-nm region were shifted some 5 nm to longer wavelengths with each increase in the atomic number of the chalcogen. The extinction coefficients increased several percent along the series. JØRGENSEN[44,45], in a more detailed investigation, compared the optical spectra of the diethyl diselenophosphates and diethyldithiophosphates of Cr(III), Rh(III), and Ir(III), where absorption envelopes of the selenium derivatives are shifted to lower energies compared with those of the sulfur compounds. Bathochromic shifts as large as 50 nm were seen. The transitions between molecular orbitals involving the *d*-orbitals of the metal ion were observed by JØRGENSEN to be more intense (by as much as a factor of 2) in the selenium complexes while the ligand to metal electron transfer bands were of comparable intensity in both compounds.

Other observations[46-49] suggest the generality of the bathochromic shift on replacing sulfur with selenium, and a quantitative expression of the shift is found in the optical electronegativities for these elements[50]. Shifts in these spectra do not imply structural alterations resulting from the substitution but only a lowering of the transition energies because of the higher polarizability of Se^{2-} (ref. 50).

From the above discussion it is clear that both d–d (pure metal) and ligand-to-metal transitions are shifted to lower energy when sulfur is substituted by selenium and thus it is not possible to use the bathochromic shift to distinguish between these two alternatives. However, the fact that the intrinsic visible absorption of this protein is very large, taken together with JØRGENSEN's observations that the intensity of electron-transfer bands is essentially unchanged when sulfur is substituted by selenium suggests that the color of this protein is due to sulfur (or selenium)-to-iron electron transfer bands. The d–d transitions for the ferredoxins appear to occur at longer wavelengths.

The previously published magnetic susceptibility results[52] on spinach and parsley ferredoxins show that in the oxidized form neither protein is paramagnetic in the region of 1–$4°$ K. One would therefore not expect an EPR signal from the oxidized protein, and a signal attributable to the native protein is absent. The magnetic susceptibility measurements showed that both spinach ferredoxin and parsley ferredoxin were paramagnetic in the reduced form with an absolute susceptibility and temperature dependence consistent with a single unpaired electron per molecule of protein. The reduced proteins do exhibit an EPR signal at low temperatures, the intensities of which correspond to one electron per molecule, $i.e.$ with an effective spin, $S' = 1/2$. The g tensor of the EPR spectrum of parsley ferredoxin is anisotropic as is that of spinach ferredoxin[7]. As indicated in RESULTS there are minor qualitative and quantitative differences between the two spectra.

All EPR spectra have been computed on an IBM 360 computer using a program written by I. Salmeen and H. Sachse. This program, which has been described in detail by SALMEEN[53], utilizes a line shape function which has an angular dependence similar to that of the g tensor. It may account for contributions from two $I = 1/2$ hyperfine centers having A tensors coincidental with the g tensor. In the present analysis two hyperfine centers in the $^{32}S^{57}Fe$, $^{77}Se^{56}Fe$, and $^{80}Se^{57}Fe$ derivatives are assumed equivalent. In general the fits were good except in the "tails" of the spectra where the line-shape function chosen (gaussian) appeared inadequate to account for the resonance. This was particularly true at the high field end of the spectra. A summary of the EPR data is given in Table IV.

The apparent line widths of the EPR spectrum of parsley ferredoxin are not affected by replacing all the exchangeable protons with deuterons. This observation excludes the possibility of strong magnetic interactions between the electron and exchangeable protons, and it suggests that the labile-sulfur atoms, which form an integral part of the active center ($vida$ $infra$), are not protonated at the pH (8) of the measurements.

The specific effect of chloride ion on the EPR spectrum of parsley ferredoxin was traced from large variations in line widths under differing conditions. The extent of the broadening is, by comparison to ^{57}Fe substitution ($vida$ $infra$) substantial, and would appear to be too large to be explained by electron-nuclear dipolar interaction at distances greater than the first coordination sphere[54]. If the broadening results

TABLE IV

SUMMARY OF EPR DATA

Sm, gm, Sm are the observed minimum slope, the crossover point, and the observed maximum slope, respectively, as defined by HASHIMOTO *et al.*[56]. They correspond to apparent g values. g_x, g_y, g_z are values required to best fit the EPR spectrum. L_x, L_y, L_z: defined as half-width at half-height in the first derivative spectrum. A_x, A_y, A_z: hyperfine constants, equivalent magnetic nuclei assumed[57,58]. Values in gauss.

Derivative			
$^{32}S^{56}Fe$ (native)			
Sm, gm, Sm	1.90	1.96	2.05
g_x, g_y, g_z	1.897	1.954	2.049
L_x, L_y, L_z	21	16	13
$^{32}S^{57}Fe$			
A_x, A_y, A_z	14	11.5	15
$^{80}Se^{56}Fe$			
Sm, gm, Sm	1.937	1.965	2.061
g_x, g_y, g_z	1.930	1.965	2.060
L_x, L_y, L_z	23	13	10
$^{77}Se^{56}Fe$			
A_x, A_y, A_z	6	15	23
$^{80}Se^{57}Fe$			
A_x, A_y, A_z	16	13	15

from a magnetic interaction between the unpaired electron and the nucleus ($I = 3/2$) of Cl^-, the latter would necessarily reside in the first coordination sphere of the active center iron. Alternatively, if the chloride is bound at site(s) far enough from the active center to proclude magnetic interaction it may influence the EPR spectrum by affecting small changes in the structure of the protein molecule. Line broadening of the $^{35}Cl^-$ NMR spectrum could yield a more definitive answer on this question. Other anions with magnetic nuclei, *e.g.* F^- and Br^- have essentially no effect on the spectrum (Fig. 5). The effect of Cl^- on the EPR spectrum of spinach ferredoxin is much smaller than found with parsley ferredoxin indicating a lack of generality of the phenomenon. A reproducible EPR spectrum of parsley ferredoxin was obtained in solutions containing no Cl^-; this in spite of widely varying concentrations of SO_4^{2-} and ClO_4^-.

The EPR spectrum of the ^{57}Fe-containing protein showed a general broadening, indicating a magnetic interaction between iron and the unpaired electron, the magnitude of which is comparable to the experimental line width. The low field region of the spectrum could be adequately computed assuming interaction with either one or two ^{57}Fe ($I = 1/2$) hyperfine centers.

The reduced form of the $^{80}Se^{56}Fe$ homolog shows a "$g = 1.94$" type spectrum at low temperatures which by integration is due to one unpaired electron per protein molecule. The shoulder apparent at field values slightly above 3200 gauss in this spectrum (Fig. 6) is most likely due to a small contamination with sulfur-containing parsley ferredoxin. The spectrum differs in some aspects from that seen with parsley ferredoxin. The average g value, $g_{av} = 1/3$ ($g_x + g_y + g_z$) $= 1.985$ is greater than

$g_{av} = 1.967$ for parsley ferredoxin. Also $\Delta g_{x-y} = 0.028$ for $^{80}Se^{56}Fe$ parsley ferredoxin is smaller than seen with the native protein ($\Delta g_{x-y} = 0.055$ (Table IV)). Of further note is the narrower line width in the z-region of the $^{80}Se^{56}Fe$ spectrum. The reason for this is not known.

Substitution of ^{77}Se for ^{80}Se results in a general broadening of the EPR spectrum of selenium-substituted parsley ferredoxin. The shoulders apparent in the z-region are indicative of an interaction between the unpaired spin and more than one $I = 1/2$ hyperfine center. Indeed it is impossible to compute such a line shape with a single ^{77}Se interaction. The calculated spectrum shown in Fig. 6 is based on the presence of two selenium nuclei with identical A_z values (Table IV).

The original selenium contained 88 % ^{77}Se, and the remainder is assumed to be composed of non-magnetic ($I = 0$) isotopes, e.g. ^{80}Se. The solution will therefore contain $0.88 \times 0.88 = 0.77$ $^{77}Se^{77}Se$, $2 \times 0.12 \times 0.88 = 0.21$ $^{80}Se^{77}Se$, and $0.12 \times 0.12 = 0.014$ $^{80}Se^{80}Se$, substituted proteins. This distribution has been accounted for in the computation.

In an attempt to resolve ^{57}Fe hyperfine structure we have taken advantage of the narrower line width of the selenium substituted protein, and have prepared the $^{80}Se^{57}Fe$ derivative. The spectrum (Fig. 8) shows a general broadening but, as with the $^{32}S^{57}Fe$ protein there is no resolved hyperfine structure. We have recorded the second-derivative spectrum in an attempt to locate any possible inflection point on the low-field side of the z-resonance position; none was detected.

Drawing on the earlier results of TSIBRIS et al.[3] who observed resolved hyperfine structure from ^{57}Fe in adrenodoxin and putidaredoxin in the form of a $1:2:1$ triplet, the triangular shape of the low-field line in ^{80}Se ^{57}Fe was suggestive of an unresolved hyperfine splitting similar to that found with the above proteins. Using the computer program and the parameters from the best fit of the $^{80}Se^{56}Fe$ spectrum we have attempted to reproduce the shape of the low-field resonance with a single $I = 1/2$ hyperfine center. This was unsuccessful. If the observed width of the lower half of this line was adhered to, a disparate broadness resulted in the upper portion. However, the line shape could be adequately reproduced with two $I = 1/2$ hyperfine centers having similar splitting constants (Table IV, Fig. 8, inset). This, in spite of a slight contamination of the sample with sulfur-containing parsley ferredoxin which explains the generally poor fit above 3200 gauss. While this analysis is not completely convincing, on balance the simulations are more consistent with the conclusion that both iron atoms also reside in a single active center.

The shape of the low field resonance of the $^{32}S^{57}Fe$ and $^{80}Se^{57}Fe$ derivatives is adequately fit with the parameters in Table IV assuming the A_z values of the two iron atoms to be identical and of an approximate absolute value of 15 gauss. The near identity of the A_z value required to fit the two spectra is circumstantial evidence for the suggestion that the active center is not greatly altered by the substitution of selenium for the labile sulfide.

The hyperfine interactions observed with chloroplast ferredoxins are not as visually striking as those observed with either putidaredoxin[3] or adrenodoxin[4], although the magnitudes of the interactions are comparable. This is because of the substantially narrower EPR line widths observed with the native proteins in the latter cases. Specifically why large line widths are associated with rhombic EPR spectra (chloroplast ferredoxin), while narrow line widths occur with axial EPR

spectra (adrenodoxin, putidaredoxin) is a question that remains to be answered. At this time the differences in EPR spectra are the most obvious discriminant between these two varieties of iron–sulfur proteins.

By contrast the body of data on the iron–sulfur proteins suggests a fundamental similarity between the chloroplast ferredoxins with adrenodoxin of mammalian origin and putidaredoxin from bacteria. Their characteristic "$g = 1.94$" EPR, absorption and circular dichroism spectra and their functional parallelism[1] suggest that the major features of the active center of these proteins are held in common. Thus, the earlier observations of TSIBRIS et al.[3], and ORME-JOHNSON et al.[4], and BEINERT AND ORME-JOHNSON[59] demonstrated that both atoms of iron and both atoms of labile sulfide were elements of the active centers of putidaredoxin and adrenodoxin. The data presented in this paper establish that both atoms of labile sulfide reside in the active center of a chloroplast ferredoxin and also suggest the presence of 2 iron atoms in this active center.

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