Sulfhydryl Groups of Iron Environment in Non-Heme Iron Protein (Adrenodoxin) as an Oxidation–Reduction Component of Steroid 11β-Hydroxylase from Adrenal Mitochondria*

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ABSTRACT: Equimolar amounts of non-heme iron and labile sulfide in a non-heme iron protein (adrenodoxin) isolated in a homogeneous state from pig adrenal mitochondria are catalytic and essential entities of the protein. There are two atoms of iron in adrenodoxin, and the environment of each one shows a strong absorbance in the visible region and contains four sulfhydryl groups per iron atom, as determined by *p*-mercuribenzoate (PMB) and amperometric titrations. Accordingly, the two atoms of iron coordinate to eight sulfhydryl groups, assuming that 2 moles of labile sulfide in the molecule combine with 4 moles of PMB. The spectrum and activity of the PMB-treated adrenodoxin can be restored by adding 2-mercaptoethanol.

A non-heme iron protein (adrenodoxin) has been recently demonstrated to be an essential oxidation–reduction component for the 11β-hydroxylation of deoxycorticosterone in adrenal mitochondria; and further, a flavoprotein which catalyzes reduction of adrenodoxin by NADPH[1] and a heme protein called cytochrome P_{450} have also been shown to be the intermediates of the electron-transfer mechanism of hydroxylation (Suzuki and Kimura, 1965; Kimura and Suzuki, 1965; Watari and Kimura, 1966; Omura *et al.*, 1965a,b).

We have reported elsewhere (Suzuki and Kimura, 1965; Kimura and Suzuki, 1967) that adrenodoxin contains an equimolar amount of labile sulfide and non-heme iron and shows the characteristic absorption spectrum of non-heme iron protein. The marked similarity of adrenodoxin to spinach ferredoxin in terms of physical and chemical properties was emphasized, although the catalytic functions of these two proteins were found to be not exchangeable.

That non-heme iron is a functional entity of the oxidation–reduction reaction is postulated by many other investigators. We decided to investigate whether the non-heme iron in adrenodoxin is an essential catalytic site of the reaction or not. In view of the function of non-heme iron, the present paper deals with the relationship between the catalytic activity and the non-heme iron or labile sulfide content in adrenodoxin; and we shall demonstrate that the iron binding site of adrenodoxin includes cysteinyl sulfhydryl groups and labile sulfide.

Experimental Procedure

Materials. All of the chemicals used in these experiments were obtained commercially, as follows: Sephadex G-25 from Pharmacia Fine Chemicals, Inc.; NADPH, NADP, and G-6-P from Sigma Chemical Co.; PMB, phenylmercuric acetate, and silver nitrate from Tokyo Kasei Kogyo Co., Ltd.; urea from Junsei Pure Chemicals Co., Ltd.; G-6-P dehydrogenase from C. F. Boehringer and Soehne. The PMB was recrystallized three times by the method of Boyer (1954); and the urea was recrystallized twice.

Methods. The adrenodoxin which was prepared was purified by the method we have reported elsewhere (Suzuki and Kimura, 1965; Kimura and Suzuki, 1967). Purified adrenodoxins used throughout this investigation had an iron content between 103 mμ-atoms of iron/mg of protein and 70 mμatoms of iron/mg of protein, which corresponds to 100–68% purity.

The determinations of non-heme iron and labile sulfide were carried out as have been described in a report published elsewhere (Kimura and Suzuki, 1967). The method of analysis for iron was essentially the same as that described by Massey (1957), and of labile sulfide determination as that described by Fogo and Popowsky (1949).

The procedure for titration with PMB was in accordance with the method of Boyer (1954), with minor modifications. For the amperometric titration of sulfhydryl groups, the method of Allison and Cecil (1958) was employed.

The protein concentrations were determined by

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[1] Abbreviations used: PMB, *p*-mercuribenzoate; G-6-P, glucose 6-phosphate; G-6-P dehydrogenase, glucose 6-phosphate dehydrogenase; NADPH, reduced nicotinamide–adenine dinucleotide phosphate; NADP, nicotinamide–adenine dinucleotide phosphate; sce, saturated calomel electrode.

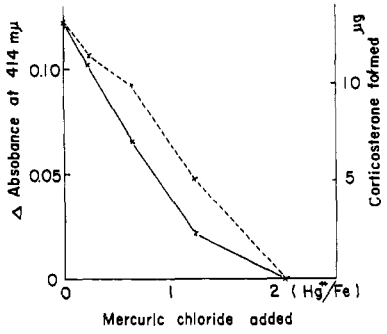

FIGURE 1: Relationship between enzyme activity and absorbance of mercuric chloride treated adrenodoxin. The reaction mixture contained adrenodoxin equivalent to 107 mμatoms of iron and 35 μmoles of phosphate buffer (pH 7.4) in 3.5 ml. After each addition of mercuric chloride, absorbance at 414 mμ was measured, and then 0.3 ml of duplicated aliquots was removed and subjected to determination of the activity of 11β-hydroxylation. The 11β-hydroxylation was determined as follows. The reaction mixture contained 1 μmole of deoxycorticosterone in propylene glycol, 60 μmoles of Tris buffer (pH 7.4), 10 μmoles of $MgCl_2$, 50 μmoles of nicotinamide, 1 μmole of mercaptoethanol, 0.38 μmole of NADPH, 2.4 mg of fraction A, and mercuric chloride treated adrenodoxin equivalent to 9 mμatoms of iron. After shaking for 30 min at 37° in air, the corticosterone formed was extracted with dichloromethane and the quantity was estimated by the fluorometric method of Mattingly (1962). Fraction A was prepared by the method previously reported (Suzuki and Kimura, 1965). The acetone powder of the mitochondrial fraction was extracted with 0.15 M KCl and fractionated with ammonium sulfate. The fraction precipitating at 20–40% saturation was dialyzed against 0.01 M phosphate buffer (pH 7.4). This fraction is called fraction A. (×——×) Absorbance at 414 mμ. (×– – –×) Corticoid formed (micrograms).

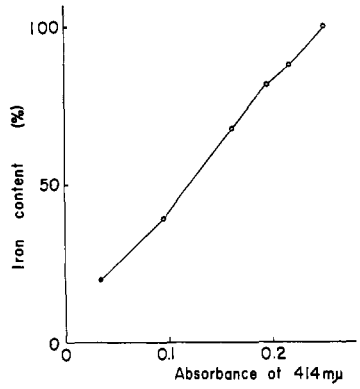

FIGURE 2: Relationship between absorbance and iron content of *o*-phenanthroline-treated adrenodoxin. The reaction mixture contained adrenodoxin equivalent to 750 mμatoms of iron, 60 μmoles of phosphate buffer (pH 7.4), and 6 μmoles of *o*-phenanthroline in 3.3 ml. Aliquots of 0.55 ml were used for Sephadex G-25 column filtration (1 × 24 cm) after 40 min, 80 min, 2 hr, 6.5 hr, and 11 hr at 25°. The values in percentage were calculated on the basis of the amount of protein by biuret estimation, and the mixture of the control experiment contained the same components except *o*-phenanthroline.

biuret reaction. The spectrophotometric measurement was carried out with an automatic recording spectrophotometer (Hitachi EPS-3), and the amperometric titration with an automatic recording polarograph with a dropping mercury electrode (Yanagimoto PA-102).

The enzyme activity of steroid 11β-hydroxylation was measured as previously reported (Suzuki and Kimura, 1965). The corticosterone formed was estimated by the fluorescent method of Mattingly (1962).

Results

Relationship between the Visible Absorbance and the Catalytic Activity. Since adrenodoxin has strong absorption maxima at 414 and 455 mμ, we attempted to find out whether a relationship between the visible absorption and the 11β-hydroxylation exists or not. As has been previously reported, mercurials can prominently bleach the entire region of the visible absorption in adrenodoxin (Suzuki and Kimura, 1965). Adrenodoxin treated with various amounts of mercuric chloride was used for the tests of the 11β-hydroxylation in the presence of partially purified fraction A (see legend of Figure 1). The loss of visible color was measured separately by the absorbance at 414 mμ. The results are shown in Figure 1. It is fairly well indicated that there is a close relationship between the decrease of the catalytic activity and the loss of the absorbance.

Iron Chromophore. We have already reported that purified adrenodoxin shows a homogeneous pattern in ultracentrifugation, and has 2 moles of labile sulfide and two atoms of non-heme iron/mole with a molecular weight of approximately 15,000–20,000; and further, that in the preparation of adrenodoxin no significant amounts of flavin, heme, or metals other than iron can be detected. Adrenodoxin has a molar extinction coefficient of 4900 cm^{-1} at 414 mμ and 4200 cm^{-1} at 455 mμ. Spinach ferredoxin and iron flavoprotein also have similar values of extinction coefficient per iron atom (Whatley *et al.*, 1963; Rajagopalan and Handler, 1964). The foregoing evidence suggests that the chromophore of adrenodoxin is due to coordination of iron and labile sulfide to the protein moiety.

Figure 2 shows the relationship between the visible absorbance and the content of non-heme iron in adrenodoxin treated with *o*-phenanthroline after passage through Sephadex G-25. The results apparently indicate that there is a linear relationship between absorbance and iron content.

TABLE I: Changes of Absorbance, Non-Heme Iron, Labile Sulfide, and 11β-Hydroxylation in Urea-Treated Adrenodoxin

Expt[a]	Sample −Urea	Sample +Urea	Reaction Time (hr)	Absorbance at 414 mμ	%	Non-Heme Iron mμ-atoms	%	Labile Sulfide mμ-moles	%	Act. Corticosterone Formed μg	%
	1		0	0.198	100	181	100	90	100	9.0	100
1		2	1.5	0.133	67	188	103	57	63	6.1	68
		3	2.5	0.125	63	188	103	52	58	5.6	62
		4	20	0.045	23	170	94	5	6	0	0
	1		0	0.366	100	252	100	162	100	20.8	100
2		2	0.5	0.308	84	208	83	148	91	15.6	75
		3	4.5	0.137	37	90	36	44	27	6.4	31
	1		0	0.346	100	208	100	182	100	23.0	100
		2	0.5	0.232	67	173	83	120	66	15.4	67
3		3	1	0.230	67	180	86	117	64	16.0	70
		4	3	0.210	61	166	80	123	68	12.3	54
		5	5	0.184	53	154	74	89	49	11.4	50

[a] The reaction mixtures contained, for expt 1, adrenodoxin equivalent to 684 mμatoms of iron (9.8 mg of protein), and 21 μmoles of phosphate buffer (pH 7.4) in 2.55 ml of 5 M urea; for expt 2, adrenodoxin equivalent to 660 mμatoms of iron (9.3 mg of protein), and 21 μmoles of phosphate buffer (pH 7.4) in 2.5 ml of 5 M urea; for expt 3, adrenodoxin equivalent to 1120 mμatoms of iron (15.1 mg of protein), and 46 μmoles of phosphate buffer (pH 7.4) in 4.2 ml of 5 M urea and 0.4 M potassium chloride. These mixtures were kept at 0° for various periods as indicated. Aliquots of 0.85 (expt 1) and 1.05 ml (expt 2 and 3) were subjected to filtration in a Sephadex G-25 column (1 × 25 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.4). The control mixtures were of similar composition as described in expt 1, 2, and 3, respectively, but did not contain urea. Determination of activity for 11β-hydroxylation was made as described in Figure 1 except that the following were used: 2.9 mg of fraction A and 0.1 mg of adrenodoxin (expt 1), 4 mg of fraction A and 0.09 mg of adrenodoxin (expt 2), and 4 mg of fraction A and 0.11 mg of adrenodoxin (expt 3) and an NADPH-generating system consisting of 5 μmoles of G-6-P, 2.0 μmoles of NADP, and an excess of G-6-P dehydrogenase. The values were calculated on the basis of equal amounts of protein on a biuret basis: expt 1, 3.0 mg in 3.0 ml; expt 2, 3.5 mg in 3.0 ml; and expt 3, 3.3 mg in 3.0 ml.

A similar relationship between the visible absorbance and the content of labile sulfide was also examined. The adrenodoxin used was passed through Sephadex G-25 after treatment with 5 M urea for various reaction times at 0°. As shown in Table I, the visible color was greatly bleached, and analysis for labile sulfide content showed a marked loss. It could be further demonstrated that there is a closely linear relationship between the visible absorbance or labile sulfide content and the catalytic activity.

Iron atoms present in the urea-treated samples were sometimes liberated, depending on the experimental conditions used. This phenomenon must be explained by further experiments.

Iron Binding Site of Adrenodoxin. In order to investigate the iron binding site, adrenodoxin was titrated with various amounts of PMB or mercuric chloride. The spectra of the PMB-treated adrenodoxin are shown in Figure 3. It is to be noted that the disappearance of visible color and the appearance of the absorption maximum at 250 mμ due to the formation of mercaptide were observed simultaneously.

By the measurement of increase of absorbance at 250 mμ and decrease at 414 mμ, it was found that, for reaction times up to 30 min and temperatures in the range 15–37°, the reaction with PMB depends neither on reaction time (Figure 4) nor on temperature. Therefore, the sulfhydryl groups coordinated with iron do not seem to be affected by the steric configuration of the iron environment, and appear to be exposed to the protein surface. From the titration curves with PMB at 15° as illustrated in Figure 5, it can be seen that 4 moles of PMB/iron atom were required to produce minimum absorbance of visible color and maximum absorbance of ultraviolet. Similar titrations were also performed with mercuric chloride and cupric sulfate. The data essentially substantiate the conclusion obtained with PMB.

The sulfhydryl groups in the protein were also measured by the amperometric titration method of Allison and Cecil (1958). At 25°, adrenodoxin with 200 mμatoms of iron was titrated both with phenylmercuric acetate and with silver nitrate (Figure 6). The data indicate the same conclusion as that obtained from the titration with PMB.

Since adrenodoxin contains equimolar amounts of labile sulfide and iron atoms, it must be considered that the labile sulfide may react with PMB. It was

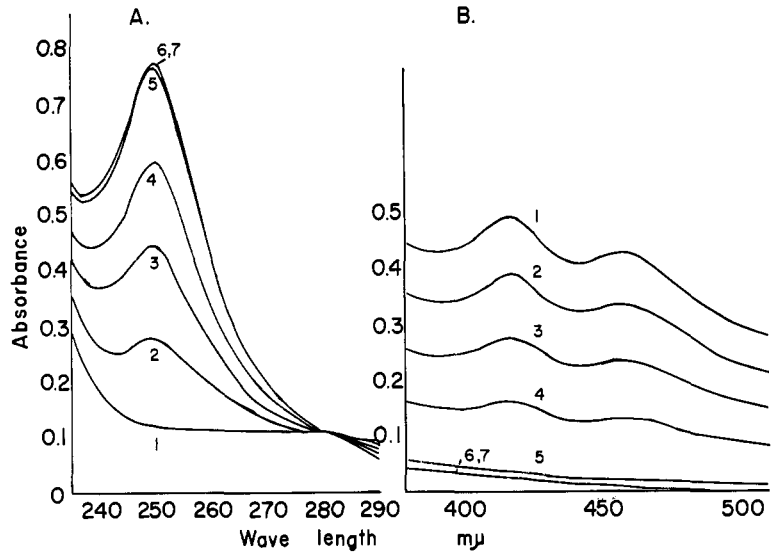

FIGURE 3: Spectral changes of adrenodoxin with the addition of PMB. The cuvet contained adrenodoxin equivalent to 50 mμatoms of iron and 60 μmoles of Tris buffer (pH 7.4) in 3.0 ml (A) and adrenodoxin equivalent to 300 mμatoms of iron and 60 μmoles of Tris buffer (pH 7.4) in 3.0 ml (B). PMB (0–6 moles)/iron atom in adrenodoxin was added to the solution and the control also contained the same amount of PMB and Tris buffer (pH 7.4) as the experimental cuvet. After the addition of PMB, spectra were immediately measured by an automatic recording spectrophotometer. (curve 1) Original adrenodoxin; (curve 2) adrenodoxin plus PMB (1:1, mole/atom); (curve 3) adrenodoxin plus PMB (2:1); (curve 4) adrenodoxin plus PMB (3:1); (curve 5) adrenodoxin plus PMB (4:1); (curve 6) adrenodoxin plus PMB (5:1); and (curve 7) adrenodoxin plus PMB (6:1).

found that 1 mole of hydrogen sulfide can combine with 2 moles of PMB with the appearance of absorbance at 250 mμ. The molar extinction coefficient of the compound of 1 mole of hydrogen sulfide with 2 moles of PMB is 10.5×10^3 cm^{-1}/mole of PMB at 250 mμ (pH 8.1). Adrenodoxin combined with 4 moles of PMB/iron atom had a molar extinction coefficient value of 9.5×10^3 cm^{-1} (pH 7.4). In this regard, Boyer (1954) reported that PMB–cysteine mercaptide has a molar extinction coefficient of 7.6×10^3 cm^{-1} (pH 7.0), and also Katoh and Takamiya (1963) reported that spinach ferredoxin bound with PMB has a value of 10.3×10^3 cm^{-1} (pH 7.0). These results add evidence to our findings, and permit us to conclude that adrenodoxin has four sulfhydryl groups per iron atom. Therefore, the two atoms of iron coordinate to eight sulfhydryl groups. If one can assume that 1 mole of labile sulfide combines with 2 moles of PMB, the other 2 moles of PMB should combine with the sulfhydryl groups of the protein, which can reasonably be assumed to be cysteine. Accordingly, in adrenodoxin, the two atoms of iron combine with four sulfhydryl groups which can be assumed to be those of cysteine, and four sulfhydryl groups which can be assumed to be those of 2 moles of labile sulfide. However, since the nature of labile sulfide in the protein has not yet been clarified, the assumption that 1 mole of labile sulfide combines with 2 moles of PMB must remain tentative.

Reactivation of PMB-Treated Adrenodoxin. It is interesting to note that all the iron atoms in adrenodoxin could be extracted as ferric iron by 5% trichloroacetic acid in the presence of 4 moles of PMB/iron atom. On the other hand, iron atoms in about one-half of the PMB-treated sample could be liberated as iron mercaptide upon the addition of 2-mercaptoethanol. Of importance is the fact that partial restoration of the other half of the PMB-treated sample after the addition of 2-mercaptoethanol was observed simul-

TABLE II: Comparison of Untreated and Restored Adrenodoxin.[a]

	Untreated Adrenodoxin	Restored Adrenodoxin
E_{455}/E_{414}	0.87	0.84
E_{414}/E_{280}	0.62	0.40
E_{455}/E_{280}	0.54	0.33
E_{414}/E_{320}	0.76	0.71
E_{455}/E_{320}	0.66	0.60
E_{320}/E_{280}	0.82	0.56
Activity (μg/mμatom of iron)	2.6	2.4

[a] Activity for 11β-hydroxylation was determined as described in Figure 1, except that 0.32 μmole of NADPH and 1.7 mg of protein from fraction A were used.

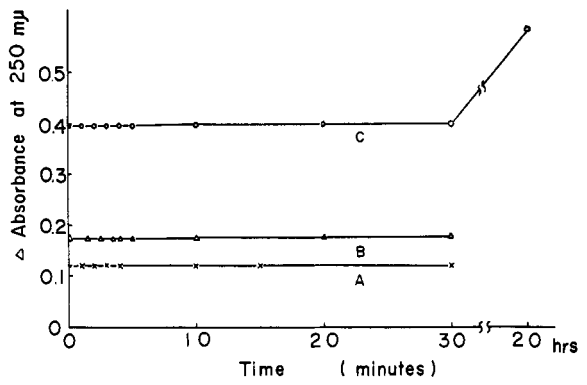

FIGURE 4: Effect of reaction time on the formation of mercaptide. The reaction mixtures contained adrenodoxin equivalent to 32 mμatoms of iron and 120 μmoles of Tris buffer (pH 7.4) in 3.0 ml. PMB (40 (curve A), 60 (curve B), and 200 (curve C) mμmoles) was added to each reaction mixture, which was further treated at the indicated temperature for various reaction periods. The measurement of absorbance at 250 mμ was performed at 20°. The data were corrected for the dilution with the addition of PMB. Control experiments were run by using mixtures which contained, respectively, 40, 60, and 200 mμmoles of PMB and Tris buffer.

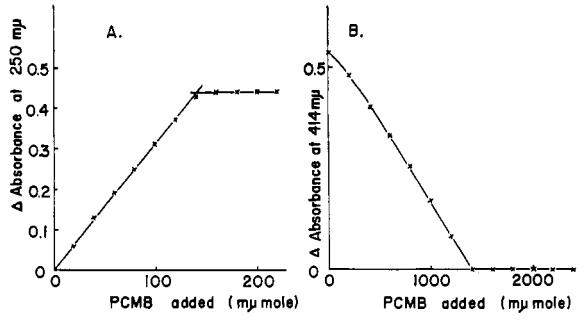

FIGURE 5: Titration of sulfhydryl groups with PMB. The reaction mixture contained adrenodoxin equivalent to 35 mμatoms of iron and 90 μmoles of Tris buffer (pH 7.4) in 3.0 ml (A) and adrenodoxin equivalent to 350 mμatoms of iron and 90 μmoles of Tris buffer (pH 7.4) in 3.0 ml (B). To the reaction mixture, various amounts of PMB were added and the changes of absorbance at 250 (A) and 414 mμ (B) were measured at 15°, immediately after the addition of PMB. The control had the same amount of PMB and Tris buffer. Correction for the dilution with the addition of PMB was made.

taneously with the liberation. In Figure 7, the spectra of the original, the PMB-treated and the restored samples are shown. The ratios of absorbances at various wavelengths were calculated as indicated in Table II. From the data, the restored adrenodoxin has lower values of $E_{414\,m\mu}/E_{280\,m\mu}$, $E_{455\,m\mu}/E_{280\,m\mu}$, $E_{320\,m\mu}/E_{280\,m\mu}$.

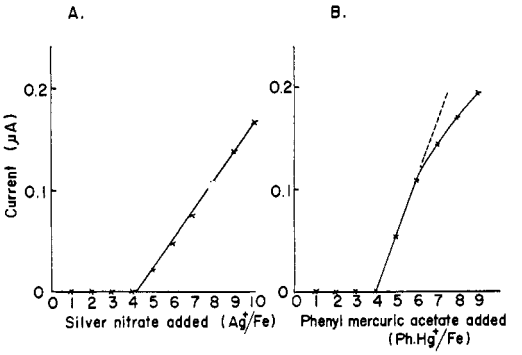

FIGURE 6: Amperometric titration of sulfhydryl groups in adrenodoxin. (A) The reaction mixture contained adrenodoxin equivalent to 200 mμatoms of iron and 80 μmoles of borate buffer (pH 9.0) and 10 μmoles of EDTA and 1 drop of octyl alcohol in 4.0 ml. Amperometric titration was performed after bubbling with oxygen-free nitrogen gas for 15 min at 25°. After each addition of silver nitrate from a micropipet, nitrogen gas was passed for 3 min. The titrations were carried out at −0.3 v *vs.* sce. (B) The reaction mixture was the same as A, except EDTA was not added, and the titration was performed with phenylmercuric acetate. The entire procedure was the same as A. The titrations were carried out at −0.4 v *vs.* sce.

This discrepancy may be explained by the assumption that either the restored sample contained some 50% of the colorless iron-free protein or it consisted of the protein with one atom of iron per mole. However, the molar extinction coefficient per iron atom of both samples showed an identical value of 4900 cm^{-1}. Furthermore, the restored adrenodoxin had the same specific activity (corticosterone formed/30 min per iron atom) as that of the original adrenodoxin. Thus, the restored adrenodoxin has the perfect structure of the chromophore, which can display the catalytic function.

Discussion

It has already been discovered that several non-heme iron proteins serve as intermediary electron carriers in bacteria, plants, and animals. Among these, clostridial ferredoxin and spinach ferredoxins are most well chemically investigated (Fry and San Pietro, 1962; Lovenberg *et al.*, 1963; Malkin and Rabinowitz, 1966; Sobel and Lovenberg, 1966).

The iron of these non-heme iron proteins is thought to be the functional group of their catalysis. The investigations concerned with the iron binding site of the proteins revealed that the iron atoms are coordinated with sulfhydryl groups including cysteine and labile sulfide.

It is our main interest to compare adrenodoxin with clostridial and spinach ferredoxins. In clostridial ferredoxin, the amino acid sequence determination

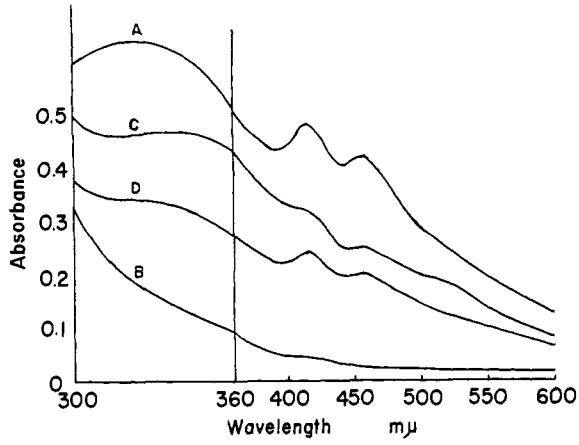

FIGURE 7: Absorption spectrum of restored adrenodoxin. The cuvet contained adrenodoxin equivalent to 300 mμatoms of iron and 60 μmoles of Tris buffer (pH 7.4) in 3.0 ml. The spectrum of this sample was then measured (curve A). Next, to this solution, 4 moles of PMB/iron atom in adrenodoxin was added and then the sample was measured spectrophotometrically (curve B). To the PMB-treated sample, 288 μmoles of 2-mercaptoethanol was added. The spectrum was again measured after 20 min at room temperature (curve C). The restored adrenodoxin was prepared as follows. The solution contained adrenodoxin equivalent to 300 mμatoms of iron, 20 μmoles of Tris buffer (pH 7.4), and 1.8 μmoles of PMB in 1.0 ml. To this solution, 576 μmoles of 2-mercaptoethanol was added and allowed to stand for 15 min at 37°. The restored sample was passed through a Sephadex G-25 column (1 × 25 cm) to remove excess mercaptoethanol. Then the spectrum was measured with correction for the dilution on the basis of protein concentration (curve D).

indicated that 7 iron atoms in the molecule are coordinated with 7 moles of cysteinyl sulfhydryl groups and 7 moles of labile sulfide. The evidence led to postulation of the linear array arrangement of 7 iron atoms (Tanaka *et al.*, 1966), although the results of the investigation on ferredoxin from *Micrococcus aerogenes* by X-ray analysis (Sieker and Jensen, 1965) were not perfectly consistent with the structure proposed by the amino acid sequence. In spinach ferredoxin, cysteine sulfhydryl groups were found to be coordinated with the iron atoms (Katoh and Takamiya, 1963). Iron flavoproteins, which have iron and labile sulfide in the molecule, were also thought to have iron–sulfur bonds (Brumby *et al.*, 1965; Handler *et al.*, 1964). The results of the present work indicate that the iron environment of adrenodoxin is very closely related to that of the nonheme iron proteins. Similarities are also noted with respect to properties of the spectrum, optical rotatory dispersion (Kimura and Suzuki, 1967), and electron spin resonance (Watari and Kimura, 1966). However, as has been demonstrated previously (Suzuki and Kimura, 1965), functions of adrenodoxin and spinach ferredoxin are not interchangeable; and also an NADPH dehydrogenase from liver microsomes as prepared by the method of Horecker (1950) does not reduce adrenodoxin (Kimura and Suzuki, 1965). It seems reasonable to assume that the iron environment of adrenodoxin is slightly different from that of spinach ferredoxin.

As for the structure of the iron environment in adrenodoxin, although it has not yet been determined in detail, the instability of the iron coordination structure on storage and on treatment with various reagents suggests a highly distorted octahedral or tetrahedral structure.

Acknowledgment

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Conformation of Ferritin and Apoferritin in Solution. Optical Rotatory Dispersion Properties*

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ABSTRACT: Ferritin exhibits a plain optical rotatory dispersion (ORD) curve in the spectral region from 600 to 250 mμ. The curve obtained in the far-ultraviolet region with a trough at 233 mμ and peak at 198 mμ is typical of a protein containing an appreciable content of helical segments. The rotatory parameters which were obtained using the Moffitt–Yang and Shechter–Blout treatments of the data in the visible region of the spectrum suggest that nearly half of the native protein exists in the helical form. Ferritin fractions of varying iron content were obtained by ammonium sulfate fractionation or by density gradient centrifugation. The rotatory properties were independent of the iron content. Apoferritin prepared after chemical reduction of ferritin at pH 4.7, however, showed rotational changes consistent with additional folding in the molecule. The optical rotatory properties of ferritin and apoferritin were unaffected by 10 M urea. In concentrations as great as 3 M guanidinium chloride, the rotatory properties of apoferritin were unchanged. However, between 3 and 6 M guanidinium chloride, the rotations changed in a manner compatible with an unfolding of the molecule. The optical rotatory properties remained constant over a wide pH range, but significant changes were observed at pH values of 2.5 and 11. The rotations of ferritin and apoferritin at 233 mμ in several organic solvents were similar to, although somewhat lower than, the rotations in aqueous solutions. Therefore disruption of hydrophobic interactions did not induce additional α-helix formation.

Horse spleen ferritin is a well-characterized protein, uniquely suited for its function of storing iron in an amount equivalent to over 20% of its weight (Granick, 1942). The iron occurs as a hydrated ferric oxide–phosphate micelle core surrounded by a protein shell (Farrant, 1954). The iron micelles were originally thought to be composed of four subunits in a square-planar arrangement or of six subunits situated at the corners of a trilateral prism (Farrant, 1954; van Bruggen *et al.*, 1960). More recently, high-resolution electron micrographs suggest a polyhedral-type structure of variable shape (Haggis, 1965). The quantity of iron in highly purified preparations of native ferritin is variable, and the protein can be fractionated on the basis of its iron content by means of ammonium sulfate precipitation (Mazur *et al.*, 1950) or by high-speed density gradient centrifugation (Fischbach and Anderegg, 1965). The electrophoretic heterogeneity (one major and two or three minor components) has been ascribed to different states of molecular aggregation rather than to different iron contents (Harrison and Gregory, 1965; Suran and Tarver, 1965).

The molecular dimensions of ferritin and apoferritin have been estimated from data obtained by X-ray diffraction and electron microscopy studies. From X-ray diffraction measurements on "wet" ferritin crystals, Harrison (1963) concluded that the protein moiety is a hollow sphere with an external diameter of approximately 122 A and an internal diameter of 76 A. Dry ferritin crystals visualized in electron micrographs have somewhat smaller dimensions (Farrant, 1954; Kuff and Dalton, 1957; Labaw and Wykoff, 1957). Low-angle, X-ray-scattering studies of ferritin in solution (Fischbach and Anderegg, 1965; Bielig *et al.*, 1966) disclose dimensions that correspond to those obtained by X-ray diffraction of the wet crystals. Scattering from iron micelles of ferritin molecules containing a full complement of iron show they are uniform, nearly spherical aggregates having a diameter very close to that of the center cavity of apoferritin. Similar dimensions for the micelles were computed from studies of denatured ferritin by X-ray scattering (Kleinwachter, 1964).

Apoferritin, prepared after chemical reduction of the iron to facilitate its removal from the protein

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