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Studies on Steroid Hydroxylase. Molecular Properties of Adrenal Iron–Sulfur Protein*

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ABSTRACT: The molecular weight of adrenal iron–sulfur protein (adrenodoxin) is approximately 12,000 as determined by sedimentation–diffusion, by sedimentation–equilibrium, and from amino acid composition of the protein. The partial specific volume is 0.70 ml/g at 20°, and the diffusion coefficient, $D_{20,w}$, is 11.2 × 10^{-7} cm^2 sec^{-1}. As described previously, the sedimentation coefficient, $s_{20,w}$, is 1.55 S. The frictional ratio, f/f_0, was calculated to be 1.29. The intrinsic viscosity is 3.0 ml/g at 22°. Therefore, the protein is a globular protein with the value of a/b (for major to minor axis of an ellipsoid) = about 5.

The adrenal iron–sulfur protein contains approximately 100 amino acid residues with an abundance of acidic amino acids and a paucity of aromatic amino acids. From the ultraviolet optical rotatory dispersion, circular dichroism, and infrared absorption measurements, a gross difference in protein conformation between adrenal and spinach iron–sulfur proteins was found.

Adrenal iron–sulfur protein (adrenodoxin)[1] containing both nonheme iron and labile sulfur has been isolated from mitochondria of the adrenal cortex and found to serve as an oxidation–reduction component in the electron transport system of steroid hydroxylation reactions at the 11β, 18, 20, and 22 positions (Suzuki and Kimura, 1965; Omura *et al.*, 1965; Nakamura *et al.*, 1966; Ichii *et al.*, 1967).

Investigation of those physical and chemical properties of adrenal iron–sulfur protein which are influenced by the nonheme iron and its environment revealed marked similarities to plant ferredoxins and distinct difference from bacterial ferredoxins. Thus, both adrenal iron–sulfur protein and plant ferredoxins appear to have an identical iron–sulfur linkage as an essential entity for catalysis, as judged from optical absorption (Kimura and Suzuki, 1967), optical rotatory dispersion (Kimura and Suzuki, 1967), circular dichroism (Palmer *et al.*, 1967; Kimura and Ohno, 1968), electron paramagnetism (Watari and Kimura, 1966), and magnetic susceptibility[2] measurements. However, in spite of fundamentally identical features of the iron coordination structure of both adrenal and plant proteins, spinach ferredoxin cannot substitute for adrenal iron–sulfur protein in the steroid 11β-hydroxylation reaction, and conversely, adrenal iron–sulfur protein cannot replace spinach ferredoxin in the photosynthetic pyridine nucleotide reduction mediated by chloroplasts (Suzuki and Kimura, 1965; Kimura and Ohno, 1968). These experimental facts led us to investigate the detailed properties besides the iron coordination structure of adrenal iron–sulfur protein in comparison with those of plant ferredoxins.

Experimental Procedure

Materials. Adrenal iron–sulfur protein was prepared from beef adrenal cortex tissue by the previously described pro-

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[1] A trivial name for adrenal iron–sulfur protein in the steroid hydroxylases.

[2] These data were presented by T. Kimura at the 3rd International Conference of Magnetic Resonance, Va., Oct 1968.

cedure (Kimura, 1968). Spinach ferredoxin was prepared according to Tagawa and Arnon (1962). The samples of adrenal iron–sulfur protein used in this investigation had 103–109 mμatoms of iron/mg of protein on a biuret basis and the $A_{414}:A_{276}$ ratio was 0.76. The preparation of spinach ferredoxin had 149 mμatoms of iron/mg of protein and an $A_{420}:A_{276}$ ratio of 0.43. Upon ultracentrifugation, all preparations were found to be homogeneous.

All chemicals were of reagent grade and were used without purification.

Methods. Iron was determined by the ferrous–*o*-phenanthroline method previously described (Kimura and Suzuki, 1967). Protein concentration was determined by a biuret reaction and a correction factor of 0.70 was used for conversion into the dry weight of protein.

Sedimentation experiments were carried out in a Spinco Model E ultracentrifuge equipped with both schlieren and Rayleigh interference optical systems. Since adrenal iron–sulfur protein has a deep reddish brown color, a Kodak No. 29 filter was used instead of the conventional Kodak Wratten No. 77A filter to obtain a photograph suitable for analysis.

Diffusion measurements were performed in a synthetic boundary cell at 20° using schlieren optics.

The partial specific volume of adrenal iron–sulfur protein was determined by measuring the densities of solutions at various concentrations of protein in 0.2 M NaCl and 0.001 M phosphate buffer (pH 7.4). The value of the partial specific volume can be calculated by the equation, $\rho = \rho_0 + (1 - V\rho_0)w$, where ρ is the density of a solution containing w grams of solute in 1.0 ml, ρ_0 is the density of the solvent, and V is the partial specific volume.

The viscosity of a solution of adrenal iron–sulfur protein in 0.2 M NaCl and 0.01 M phosphate buffer (pH 7.4) was measured with an Ostwald-type viscometer at 22.00 ± 0.01°.

Amino acid analysis was carried out as follows: adrenal iron–sulfur protein was precipitated in 5% trichloroacetic acid. The precipitates were washed with the above acid, alcohol, and ether successively, and dried to constant weight. The protein was hydrolyzed in 6 N HCl at 110° for 20, 40, or 80 hr. The hydrolysates were subjected to amino acid analysis by the use of an automatic analyzer. Cysteine was determined as cysteic acid in the acid hydrolysate of the performic acid oxidized protein. The content of tryptophan was determined by the spectrophotometric method.

Optical rotatory dispersion and circular dichroism measurements were made with a Jasco Model ORD/UV-5 spectropolarimeter with a circular dichroism attachment. The absence of optical artifacts was confirmed by determinations at different concentrations of the protein and with different optical path lengths (10, 1, and 0.1 mm). The absorbance at near 200 mμ was confirmed to be below 2.00.

The molar rotation, $[M]$, was calculated by the conventional way: $[M] = [\alpha] \times M/100$, where $[\alpha]$ is specific rotation and M is molecular weight (the molecular weight of both adrenal iron–sulfur protein and spinach ferredoxin is 12,000). The reduced mean residue rotation was calculated by the equation: $[m']_\lambda = 3/(n^2 + 2) \times [\alpha] \times M_0/100$, where monomer residue weights, M_0, of 129 and 133 are used for adrenal iron–sulfur protein and spinach ferredoxin, respectively. The molar ellipticity, $[\theta]$, was calculated from the equation, $[\theta] = 3300 \times \Delta\epsilon_{tr}$ ((deg cm²)/dmole). The reduced mean residue ellipticity, $[\theta']$, was calculated from the equation: $[\theta'] = [\theta] \times 3/(n^2 + 2) \times M_0/M$. The experimental errors in the molar rotation measurements were approximately 5% at 250 mμ and 15% at 200 mμ, and in the molar ellipticity 5% at 250 mμ and 20% at 200 mμ. The reproducibility of both measurements was satisfactory for the conclusions discussed below.

Results

Partial Specific Volume. The value for the partial specific volume of adrenal iron–sulfur protein was 0.70 ml/g at 20°. This is close to the value of 0.72 ml/g which is computed from the amino acid composition obtained in this investigation.

Diffusion Coefficient. Free diffusion of adrenal iron–sulfur protein was measured in a synthetic boundary cell. The concentration of adrenal protein was 9.68 mg/ml, the solvent used was 0.2 M NaCl and 0.001 M phosphate buffer (pH 7.4), and photographs were taken at 62, 121, and 180 min after formation of the boundary between the solution and the solvent at 20°. The diffusion coefficient, $D_{20,w}$, was computed as 11.2×10^{-7} cm² sec^{-1}.

We have previously reported the value of the sedimentation coefficient of adrenal protein to be 1.55 S (Kimura and Suzuki, 1967). The molecular weight, calculated from the values of the partial specific volume, the diffusion coefficient, and the sedimentation coefficient, is 11,200.

Sedimentation equilibrium experiments were performed by three different methods described under Experimental Procedure. In the case of runs either by the meniscus depletion methods or by the short-column (3 mm) method, plots of the logarithm of fringe displacement as a function of the cell coordinate or as a function of the square of the distance from the axis of rotation were always satisfactorily linear, indicating homogeneity of the preparation. There was no indication of dissociation or aggregation of the molecule. Molecular weight values were calculated from the slopes of the curves in these two methods, while in the multichannel method the concentration gradient at the midpoint of the solution column was used. The results of the above experiments are summarized in Table I. The average molecular weight of adrenal protein was found to be approximately 12,000. No tendency of variation of the molecular weight with concentration was observed.

When *p*-mercuribenzoate-treated adrenal iron–sulfur protein, which had no color, was subjected to sedimentation equilibrium, a molecular weight of 12,000 was obtained, in agreement with the value for untreated adrenal protein.

Amino Acid Composition. The results of the amino acid analyses of adrenal iron–sulfur protein are shown in Table II. Maximal value (4.0 residues/molecule) for the cysteine content was obtained by the *p*-mercuribenzoate titration method of Boyer (1954) after a prolonged reaction period (24 hr) at room temperature. When the cysteine was determined as cysteic acid, following oxidation with performic acid, about 4 residues/molecule were recovered. The tryptophan was found to be 0.06 residue/molecule of protein from estimations of the absorbance at 294.4 mμ, after the sample had stood overnight in 0.1 N NaOH at room temperature.

Adrenal iron–sulfur protein contains about 100 amino acid residues with an abundance of aspartic acid and glutamic acid, accounting for its strong affinity for DEAE-cellulose. A paucity of tyrosine and tryptophan is reflected in the unusual low absorbance in the region of 280 mμ. The molecular

TABLE I: Molecular Weight of Adrenal Iron–Sulfur Protein by Sedimentation Equilibrium.[a]

Expt	Initial Concn (mg/ml)	Mol Wt × 1000	Av
I	7.0	12.0	
	5.0	11.4	11.5
	3.0	11.0	
II	1.7	12.4	
	0.4	12.3	12.9
	0.1	14.0	
III	3.0		11.9
IV	8.5	12.5	
	5.7	12.3	12.1
	2.8	11.4	

[a] For the determination of molecular weight by sedimentation equilibrium, the following three methods were employed: (1) the multichannel method of Yphantis (1960), (2) the meniscus depletion method of Yphantis (1964), and (3) the short-column sedimentation–equilibrium method described by Van Holde and Baldwin (1958). All measurements were carried out in a buffer containing 0.2 M Tris at pH 7.4 and the solution of adrenal protein was dialyzed against the buffer prior to the experiments. Fluorocarbon FC-43 was used as base for the solution column. In expt I, the solution of the untreated adrenal protein was centrifuged by the multichannel method using the schlieren optics at 25,980 rpm. In expt II, the solution of the untreated adrenal protein was centrifuged by the meniscus depletion method using the interference optics at 44,770 rpm. In expt III, a solution of the untreated protein was centrifuged by the short-column method using the interference optics at 17,250 rpm. In expt IV, the solution of adrenal protein treated by the addition of 4 moles of *p*-mercuribenzoate/g-atom of iron in the protein was centrifuged by the multichannel method using the schlieren optics at 25,980 rpm.

TABLE II: Proposed Amino Acid Composition of Adrenal Iron–Sulfur Protein.

Amino Acid	Averaged or Extrapolated Values[a] μmoles/mg of Protein	moles/mole of Proline	moles/12,000 g of Protein	Proposed Nearest Interger
Lysine	0.380	4.63	4.56	5
Histidine	0.214	2.61	2.57	3
Ammonia	0.456	5.56	5.47	6
Arginine	0.234	2.85	2.81	3
Aspartic acid	1.139	13.89	13.67	14
Threonine	0.551	6.72	6.61	7
Serine	0.475	5.79	5.70	6
Glutamic acid	0.836	10.20	10.03	10
Proline	0.082	1	0.98	1
Glycine	0.545	6.65	6.54	7
Alanine	0.487	5.94	5.84	6
Half-cystine[b]				4
Valine	0.385	4.70	4.62	5
Methionine[c]	0.335	4.08	4.02	4
Isoleucine	0.548	6.68	6.58	7
Leucine	0.755	9.21	9.06	9
Tyrosine	0.062	0.76	0.74	1
Phenylalanine	0.225	2.74	2.70	3
Tryptophan[d]	0.005	0.06	0.06	0
Total				95

[a] The values were obtained from six different samples hydrolyzed for 20, 40, and 80 hr, respectively. [b] The value was determined by titration with *p*-mercuribenzoate or by amino acid analysis of hydrolysates of performic acid oxidized protein. [c] This value is calculated from the total amounts of methionine and methionine sulfoxide. [d] Tryptophan was determined spectrophotometrically.

weight calculated from the amino acid composition, including 2 atoms of iron and 2 atoms of sulfur, is 11,000, while that calculated from the iron analysis is 13,600 on the assumption of 2 iron atoms/molecule. The agreement in values of the molecular weight determined by four methods, is quite satisfactory.

Intrinsic Viscosity. From the results of viscometry of adrenal iron–sulfur protein, the reduced viscosity, $\eta_{sp}/c = (\eta/\eta_0 - 1)/c$, is plotted against the square of the concentration of the protein, where η is the viscosity of the solution, η_0 is that of the solvent, and c is the concentration of the protein. The intrinsic viscosity, $[\eta]$,which is η_{sp}/c at $c = 0$, was computed to be 3.0 ml/g.

Optical Rotatory Dispersion. As illustrated in Figure 1, the optical rotatory dispersion spectrum of adrenal iron–sulfur protein shows multiple Cotton effects in the region between 190 and 300 mμ. The troughs appear at 233 and 211 mμ. The curve is not similar to that exhibited by a protein containing typical α helix. Nevertheless, the clear trough at 233 mμ and the peak at 198 mμ suggest the presence of some α helix, and the deep trough at 211 mμ may be an indication of the presence of random-coiled structures in this protein.

In Figure 1, the optical rotatory dispersion of spinach ferredoxin is also presented. There is a trough at 227 mμ and a peak at 203 mμ. The cross-over point is at 215 mμ. Although the curve is not typical for any ordered structure, a trough at 227 mμ and a peak at 203 mμ may suggest the presence of some β structure.

In three different preparations of adrenal iron–sulfur protein the average values of $[m']$ were found to be -3.7×10^3 at 233 mμ and $+16.0 \times 10^3$ at 198 mμ. For spinach ferredoxin the value was -3.7×10^3 at 227 mμ.

Circular Dichroism. The circular dichroism spectrum of adrenal iron–sulfur protein is shown in Figure 2. A minimum appears at 228 mμ which is at a longer wavelength than a minimum at 222 mμ of a typical α-helical protein. The values of $[\theta']$ can be computed as -2.3×10^3 and -11.0×10^3 (deg cm²)/dmole at 228 and 205 mμ, respectively. In Figure 2, the dichroism spectrum of spinach ferredoxin is also presented: the negative peak occurs at 219 mμ and the cross-over points are 210 and 246 mμ. This may suggest the presence of some

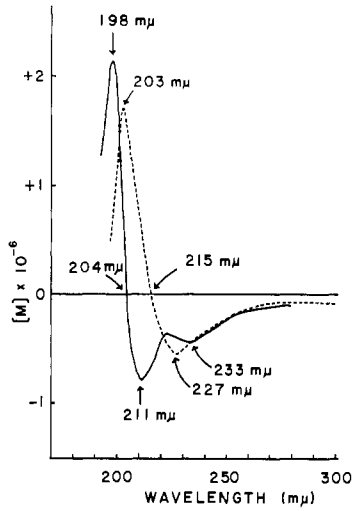

FIGURE 1: Ultraviolet rotatory dispersion of adrenal iron–sulfur protein and spinach ferredoxin. The solid curve is the dispersion of adrenal protein. The protein concentration was varied from 8.9×10^{-8} to 3.0×10^{-8} M in 0.01 M phosphate buffer (pH 7.4). The dotted curve is the dispersion of spinach ferredoxin. The protein concentration was varied from 2.6×10^{-7} to 3.2×10^{-8} M in 0.01 M phosphate buffer (pH 7.4). The optical paths used were 10, 1, and 0.1 mm.

β structure in spinach ferredoxin. The value of $[\theta']$ is -2.3×10^3 at 219 mμ.

Infrared Absorption. Infrared absorption spectroscopy of adrenal iron–sulfur protein and spinach ferredoxin was utilized to investigate the conformation of these proteins. The measurements were performed on three types of samples.

Table III shows wave numbers of the absorption due to amide I. From data on amide I absorption in globular proteins of known structure (Susi *et al.*, 1967; Timasheff *et al.*, 1967), it is possible to say that adrenal iron–sulfur protein contains some α-helical structure, whereas spinach ferredoxin has some β structure, although the observed wave number at amide I in the protein is not exactly identical with those of typical ordered forms.

In addition to this, upon removal of the iron and labile sulfur from adrenal protein, it appears that the conformation was not grossly affected.

TABLE III: Wave Numbers at Amide I Absorption of Adrenal Iron–Sulfur Protein and Spinach Ferredoxin.[a]

Sample	Wave Number in KBr (cm^{-1})	Wave Number in D_2O (cm^{-1})	Wave Number in Nujol (cm^{-1})
Adrenal iron–sulfur protein	1651 1659	1656 1650 1641	1657 1649
Adrenal iron–sulfur apoprotein	1656 1647	1655 1649 1638	1650
Spinach ferredoxin	1640 1621	1632 1641	

[a] Lyophilized adrenal iron–sulfur protein (2.7 mg) and KBr (400 mg), lyophilized adrenal iron–sulfur apoprotein (3.0 mg) and KBr (450 mg), or lyophilized spinach ferredoxin (3.0 mg) and KBr (450 mg) were pressed into disks by hydrostatic pressure (180 kg/cm²). Deuterated protein was prepared by repeated lyophilization and subsequent dissolution in D_2O, the solution being kept at 0° for 48 hr or more. D_2O solutions of samples were 4% (w/v) in adrenal protein, 12% in the apoprotein, or 9% in spinach ferredoxin. D_2O was run as a reference for the spectroscopy, and a special precaution was taken for atmospheric moisture. The cell length was 50 μ. Nujol suspension of samples were about 5% protein (w/v). Nujol was run as a reference. The cell length was 50 μ. The wavelength was calibrated with a standard polystyrene film. The region between 1500 and 1700 cm^{-1} was measured on an expanded scale.

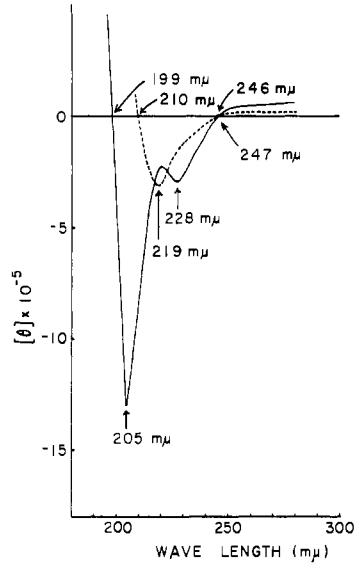

FIGURE 2: Ultraviolet circular dichroism spectra of adrenal iron–sulfur protein and spinach ferredoxin. The solid curve is the dispersion of adrenal protein. The protein concentration was varied from 8.9×10^{-5} to 3.6×10^{-4} M in 0.01 M phosphate buffer (pH 7.4). The dotted curve is the dispersion of spinach ferredoxin. The protein concentration was varied from 2.6×10^{-4} to 8.7×10^{-5} M in 0.01 M phosphate buffer (pH 7.4). The optical paths used were 10, 1, and 0.1 mm.

Discussion

The present reinvestigation of the molecular weight of adrenal iron–sulfur protein reveals it to be approximately 12,000 by three methods: sedimentation–diffusion with direct measurement of the partial specific volume (11,000), sedimentation–equilibrium (11,900), and total amino acid composition (11,000). Previous estimate of the molecular weight as about 15,000–20,000 (Kimura and Suzuki, 1967) is incorrect. This discrepancy resulted from the determination of protein by a biuret reaction without using a correction factor for conversion into the dry weight of protein and also from the erroneous assumption of diffusion coefficient and partial specific volume. The correct molecular weight is, thus, approximately 12,000, which is close to the values for spinach,

alfalfa, and green algae ferredoxins reported recently (Tagawa and Arnon, 1968; Keresztes-Nagy and Margoliash, 1966; Matsubara, 1968).

The values of the partial specific volume and the diffusion coefficient of adrenal protein are similar to those of plant ferredoxins (0.71 ml/g) (Keresztes-Nagy and Margoliash, 1966; Bendall *et al.*, 1963). Measurement of the intrinsic viscosity and calculation of the frictional ratio (f/f_0 = 1.29) and the axial ratio (a/b = about 5) of adrenal protein suggests that the protein is a globular molecule.

The striking similarity of the iron–sulfur linkage between adrenal iron–sulfur protein and spinach ferredoxin has been pointed out previously (Kimura and Suzuki, 1967; Kimura, 1968). The comparative study can be further extended from this investigation to the number and variety of amino acid residues. Both proteins show an abundance of acidic amino acid in approximately equal numbers of the residues. However, it should be noted that the number of basic amino acids in adrenal protein is greater than that of plant ferredoxins. The number of tyrosyl residue in adrenal protein is lower than the four residues per molecule present in plant ferredoxins (Keresztes-Nagy and Margoliash, 1966; Matsubara *et al.*, 1967; Matsubara, 1968). Tryptophan appears to be absent in adrenal protein as it is in ferredoxin from green algae (Matsubara, 1968), but present in spinach (Matsubara *et al.*, 1968) and alfalfa (Keresztes-Nagy and Margoliash, 1966) ferredoxins. Adrenal protein is similar to the ferredoxin from green algae as both contain methionine, whereas it differs from spinach and alfalfa ferredoxins which have no methionine.

Although the conformational study on the iron–sulfur proteins must await the X-ray crystallographic data, the present optical rotatory dispersion, circular dichroism, and infrared absorption studies are likely to suggest that adrenal iron–sulfur protein contains some α helix, whereas spinach ferredoxin has some β structure. This may partially be due to the difference in the number of prolyl residues in these two types of proteins. In this connection, it is of interest to note again that spinach, euglena ferredoxins, and putidaredoxin cannot substitute for adrenal iron–sulfur protein in the steroid hydroxylation reaction as previously described (Suzuki and Kimura, 1965; Kimura and Ohno, 1968).

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