Properties of Crystalline Reduced Nicotinamide Adenine Dinucleotide Phosphate-adrenodoxin Reductase from Bovine Adrenocortical Mitochondria. I. Physicochemical Properties of Holo- and Apo-NADPH-adrenodoxin Reductase and Interaction between Non-heme Iron Proteins and the Reductase†

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ABSTRACT: A crystalline NADPH-adrenodoxin reductase was obtained from bovine adrenocortical mitochondria and its properties were investigated. Its molecular weight and isoelectric point were estimated to be 51 000 and 5.4, respectively. Amino acid and sugar contents and the interaction between the apo-reductase and flavin of NADPH-adrenodoxin reductase were investigated. Formation of a complex of bovine NADPH-adrenodoxin reductase with adrenodoxin, its apoadrenodoxin, or other non-heme iron proteins caused quenching of fluorescence of the tryptophanyl residue and bound FAD of the NADPH-adrenodoxin reductase. The results obtained suggest that adrenodoxin and apoadrenodoxin bind functionally to a site close to the tryptophanyl residue and the bound FAD of the reductase. The circular dichroism spectrum of oxidized NADPH-adrenodoxin reductase was measured in the ultraviolet and visible regions. This spectrum showed negative absorption in the visible region and was not appreciably influenced in either the ultraviolet or visible region by formation of a complex with adrenodoxin or apoadrenodoxin.

NADPH[1]-adrenodoxin reductase is a component of the NADPH-cytochrome P-450 linked monooxygenase system in adrenocortical mitochondria (Nakamura et al., 1966; Omura et al., 1966). The formation of a complex between NADPH-adrenodoxin reductase and adrenodoxin has been demonstrated spectrophotometrically and the effects of ionic strength and heat stability on formation of this complex have been reported (Chu and Kimura, 1973a). NADPH-adrenodoxin reductase was found to be a glycoprotein (Chu and Kimura, 1973b). It was obtained in a highly pure state, judged spectrophotometrically and electrophoretically by Suhara et al. (1972a) and Foster and Wilson (1975) and later it was crystallized by affinity chromatography (Sugiyama and Yamano, 1975).

In this work, we crystallized NADPH-adrenodoxin reductase from bovine adrenocortical mitochondria by a different procedure. We then investigated the chemical properties of the reductase, the binding site of adrenodoxin on the surface of the enzyme, and the interaction between the aporeductase and flavin.

Materials and Methods

Purification of NADPH-adrenodoxin Reductase. An NADPH-adrenodoxin reductase was crystallized from bovine adrenocortical mitochondria by the method of Omura et al. (1966) and Suhara et al. (1972a) with some modifications. Fresh bovine adrenal glands were obtained from a local slaughter house and stored whole in a desiccator at −20 °C for a week. Connective and fat tissues were removed from about 4 kg of whole adrenals with scissors. Then the adrenocortex was separated from the medulla and the capsule of the cortex was scraped with a razor. Unless otherwise indicated, all purification procedures were carried out at below 4 °C. The adrenocortex (2 kg) was homogenized with 4 volumes of ice-cold 0.25 M sucrose solution (adjusted to pH 7.4 with 0.1 M potassium phosphate, dibasic) in a Matsushita homogenizer, Model MX-140S, for about 2 min. The homogenate was passed through gauze to remove fat and pieces of tissue. The mitochondrial fraction was prepared from the homogenate of the adrenocortex by the method of Hatefi and Lester (1958). The mitochondrial pellet was suspended in 10 mM sodium phosphate buffer, pH 7.4, at a protein concentration of about 30 mg/ml and homogenized twice for 4-min periods in the homogenizer. The homogenate was centrifuged at 65 000*g* for 60 min in a refrigerated Hitachi 55P ultracentrifuge with a refrigerated barrel rotor. The yellow-brown supernatant fluid was fractionated between 35 and 60% saturation of ammonium sulfate and the precipitate obtained (about 40 g) was dissolved in 60 ml of 10 mM potassium phosphate buffer, pH 7.4, and dialyzed against the same buffer for 20 h using a magnetic stirrer, and changing the outer medium three times. The dialyzed solution was centrifuged at 9000*g* for 90 min and then the supernatant was applied to a DEAE-cellulose column (5 × 40 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4. The column was washed with 6 l. of 10 mM potassium phosphate buffer, pH 7.4. In this way a red pigment was removed. Then the yellow band of NADPH-adrenodoxin reductase was eluted with 50 mM potassium phosphate buffer, pH 7.4, while the brown pigment, adrenodoxin, remained at the top of the column. The eluted NADPH-adrenodoxin reductase (200 ml) was fractionated between 35 and 55% saturation of ammonium sulfate. The precipitate (about 3 g wet weight) was dissolved in 5 ml of 10 mM potassium phosphate buffer, pH 7.4, and dialyzed against 10 l. of the same buffer

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[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide.

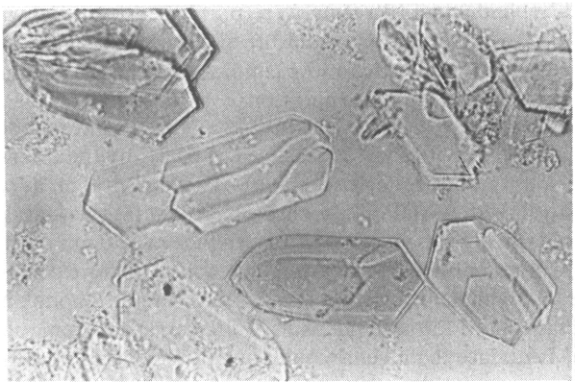

FIGURE 1: Microphotograph of crystals of NADPH-adrenodoxin reductase. Magnification: ×180.

for 20 h. The dialyzed enzyme was then applied to a second DEAE-cellulose column (2.5 × 30 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4. The column was eluted with a linear gradient of 10–50 mM potassium phosphate buffer, pH 7.4. The main fractions of NADPH-adrenodoxin reductase, with a 272 nm/450 nm absorption ratio of less than 18, were combined and fractionated between 40 and 55% saturation of ammonium sulfate. Then the material was passed through a Sephadex G-100 column (1.5 × 50 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 0.1 M potassium chloride. The main fractions, with an absorption ratio of 272 nm/450 nm of 8.0–8.8, were combined and adjusted to 60% saturation of ammonium sulfate. The precipitate was collected by centrifugation at 6000*g* for 20 min. The precipitate was added gradually to a small volume of 50 mM potassium phosphate buffer, pH 7.4, until the solution became slightly turbid. Most of the reductase crystallized out from this solution within a few days at 0 °C. The crystalline NADPH-adrenodoxin reductase (about 20 mg) was collected by centrifugation at 10 000*g* for 10 min and dissolved in 50 mM potassium phosphate buffer, pH 7.4. It was recrystallized with a fine powder of ammonium sulfate. The crystals of NADPH-adrenodoxin reductase were stored in the presence of ammonium sulfate at 0 °C. Under these conditions the enzyme was stable for at least 1 month. Just before use, the crystalline NADPH-adrenodoxin reductase was dialyzed against 0.1 M potassium phosphate buffer, pH 7.4, for 15 h at 0 °C. Figure 1 shows the appearance of crystals of bovine NADPH-adrenodoxin reductase: they were rhombic plates, often seen piled up on each other. Figure 2 shows that purified NADPH-adrenodoxin reductase solution had absorption peaks at 272, 377, and 450 nm and shoulders at 280, 366, 423, and 475 nm in oxidized form at 25 °C. The ratio of the heights of the peaks at 272 and 450 nm was 8.2, and the ratio of those at 450 and 377 nm was 1.18. In electrophoresis, the reductase was a single band of protein on gel containing 0.1% sodium dodecyl sulfate; it was homogeneous and highly pure, as shown in Figure 3.

Preparation of Apo-NADPH-adrenodoxin Reductase. Crystalline NADPH-adrenodoxin reductase (about 1.5 mg of protein) which had been stored at 0 °C as described above was put into seamless cellulose tubing, type $^{8}/_{32}$, and dialyzed against 0.1 M potassium phosphate buffer, pH 6.5, containing 2.2 M potassium bromide and 3 mM EDTA, following the method of Massey and Curti (1966). The dissolved reductase solution was dialyzed against 5 l. of the same buffer at 2 °C, changing the outer medium every 10 h for 60 h. During this period, the yellow color of the holoreductase disappeared. Then the reductase solution was dialyzed twice against 5-l. volumes of 0.1 M potassium phosphate buffer, pH 7.4 at 2 °C for 10 h, to remove the EDTA and potassium bromide completely. The apparent yield of apo-NADPH-adrenodoxin reductase from the holo-NADPH-adrenodoxin reductase was 85%. The optical absorption spectrum of apo-NADPH-adrenodoxin reductase is shown in Figure 2. This figure shows that the coenzyme FAD had been removed completely and the aporeductase (0.6 mg of protein per ml of 0.1 M potassium phosphate buffer, pH 7.4) was stable for at least a week at 0 °C. Before use the apo-

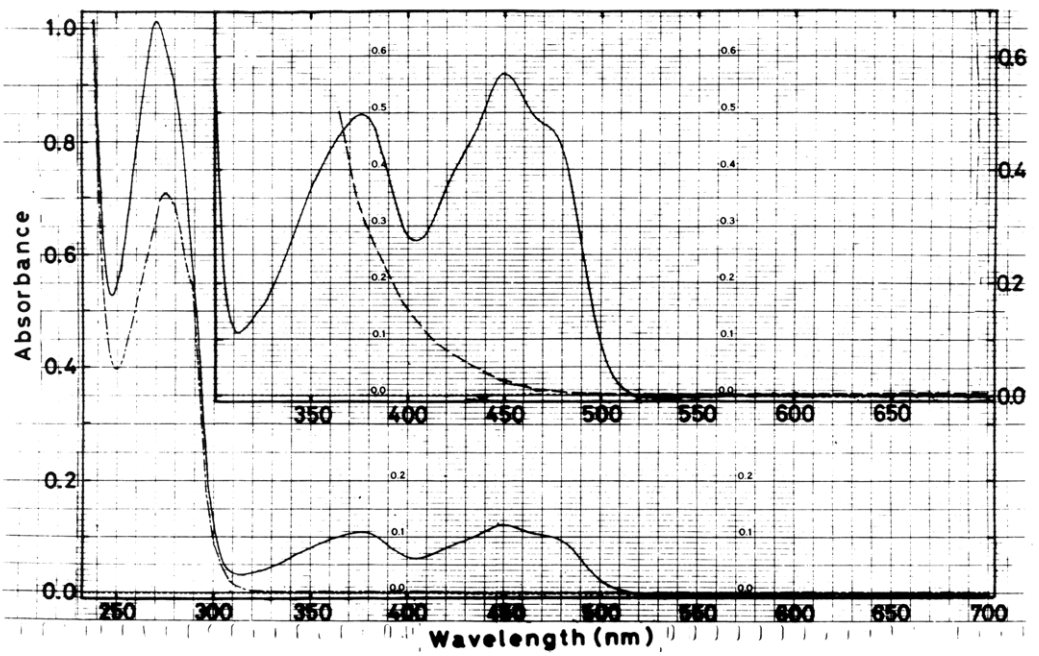

FIGURE 2: Optical absorption spectra of NADPH-adrenodoxin reductase. The cuvette contained 0.6 mg/ml protein (2.7 mg/ml protein for magnified spectra in the visible region) in 0.1 M potassium phosphate buffer, pH 7.4 (25 °C). (—) Oxidized form; (———) dithionite-reduced form; (— - —) apo-NADPH-adrenodoxin reductase; (- - -) baseline.

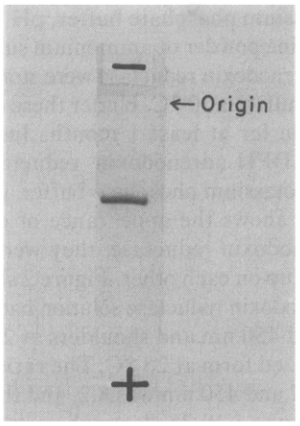

FIGURE 3: Electrophoretogram of NADPH-adrenodoxin reductase on sodium dodecyl sulfate–polyacrylamide gel. Amount of protein on the gel, 5 μg.

NADPH-adrenodoxin reductase solution was centrifuged at 8000*g* for 10 min at 2 °C, even if the insoluble aporeductase might have been present, and the supernatant was used for the experiments.

Purification of Other Enzymes and Analytical Procedures. Adrenodoxins were crystallized from bovine and pig adrenocortical mitochondria (Ichikawa, 1971; Estabrook et al., 1973; Suhara et al., 1972b) and apoadrenodoxin was obtained from bovine adrenodoxin (Bayer et al., 1965; Suhara et al., 1974). The concentration was estimated assuming a molar extinction coefficient of 9.8×10^3 M^{-1} cm^{-1} at 414 nm for adrenodoxin, and of 7.6×10^3 M^{-1} cm^{-1} at 276 nm for apoadrenodoxin.

Crystalline ferredoxin was obtained from spinach leaves (Tagawa and Arnon, 1962). Its concentration was estimated from the absorbance at 423 nm taking its molar extinction coefficient as 9.68×10^3 M^{-1} cm^{-1} (Tagawa and Arnon, 1968). Ovalbumin was crystallized by the method of Kekwick and Cannon (1936). Crystalline catalase was obtained from bovine liver (Shirakawa, 1953) and superoxide dismutase was purified from bovine erythrocytes (McCord and Fridovich, 1969).

Protein concentration was determined by the method of Lowry et al. (1951) or by the biuret reaction (Gornall et al., 1949), using bovine serum albumin as a standard.

Chemicals. NADPH, NADH, and their oxidized forms were purchased from Sigma Chemical Co. The molar extinction coefficients at 340 nm of their reduced forms and at 260 nm of their oxidized forms were taken as 6.22×10^3 and 17.8×10^3 M^{-1} cm^{-1}, respectively (Okunuki and Nozaki, 1956). Cytochrome *c* (type III) and horse spleen ferritin were purchased from Sigma Chemical Co. The cytochrome *c* and ferritin were both dissolved in 10 mM potassium phosphate buffer, pH 7.4. The cytochrome *c* was oxidized by adding 5 μM ferricyanide and then passed through a Sephadex G-100 column and stored at −20 °C until use. The molar extinction coefficient of reduced minus oxidized cytochrome *c* was taken as 18.5×10^3 M^{-1} cm^{-1} at 550 nm (Margoliash, 1954). The ferritin solution was passed through a Sephadex G-100 column before use. Commercial FAD was purified by the method of Massey and Swoboda (1963) to a stage giving only one spot on paper chromatography. Its molar extinction coefficient was taken as 11.3×10^3 M^{-1} cm^{-1} at 450 nm (Yagi and Matsuoka, 1956). Acrylamide for electrophoresis and *N,N'*-methylenebisacrylamide were purchased from Eastman Kodak Co. Standard proteins for sodium dodecyl sulfate electrophoresis and Sephadex gel filtration were purchased from Schwarz/Mann Co. All other reagents were of the highest purity available commercially. Seamless cellulose tubing was obtained from Union Carbide Corporation.

Assays of Enzymatic Activities. NADPH-adrenodoxin reductase activities were measured at 25 °C by the following methods unless otherwise stated. The activity of adrenodoxin-linked NADPH-cytochrome *c* reductase was assayed by measuring the increase of absorbance at 550 nm of the α-band of reduced cytochrome *c*. The reaction mixture contained the following components: 10 nM NADPH-adrenodoxin reductase; 3 μM ferriadrenodoxin; 25 μM ferricytochrome *c;* and 5 nM catalase to prevent reoxidation of ferrocytochrome *c* by hydrogen peroxide in 10 mM potassium phosphate buffer, pH 7.4, at 25 °C. A concentration of 2 μM superoxide dismutase was used. The reaction was started by adding 50 μM NADPH and continued for 5 min. The specific activity of NADPH-cytochrome *c* reductase was 8.8 μmol min^{-1} mg^{-1} protein at 25 °C.

NADPH-ferricyanide reductase activity was assayed by measuring the decrease in absorbance at 420 nm of ferricyanide. The reaction mixture contained 20 nM NADPH-adrenodoxin reductase and 600 μM ferricyanide in 0.1 M potassium phosphate buffer, pH 7.4, at 25 °C. The reaction was started by adding 50 μM NADPH and continued for 5 min. The specific activity of NADPH-ferricyanide reductase was 20.0 μmol min^{-1} mg^{-1} protein at 25 °C.

NADPH-oxidase activity was assayed by measuring the decrease in absorbance of NADPH at 340 nm. The reaction mixture contained 100 nM NADPH-adrenodoxin reductase, 3 μM ferriadrenodoxin and 5 nM catalase, to prevent inactivation of NADPH-adrenodoxin reductase by hydrogen peroxide, in 10 mM potassium phosphate buffer, pH 7.4 at 25 °C. The reaction was started by adding 50 μM NADPH and continued for 5 min. The specific activity of NADPH-oxidase was 1.01 nmol min^{-1} mg^{-1} protein at 25 °C.

Amino Acid Analysis. NADPH-adrenodoxin reductase solution was frozen and then lyophilized under anaerobic conditions. The residue was dissolved in 1 ml of 6 N HCl with or without 2% thioglycolic acid (Matsubara and Sasaki, 1969) and hydrolyzed at 110 °C for 24 h in an evacuated, sealed ampule. Then the material was dried in a desiccator and the residue was dissolved in 1.5 ml of 0.2 M sodium citrate-HCl buffer, pH 2.2. A 0.5-ml sample of the solution was examined in a Hitachi KLA-3B amino acid analyzer.

The tyrosine and tryptophan contents of the reductase were also estimated by the method of Beaven and Holiday (1952). Namely, the tyrosine content of the reductase was determined by measuring increase in absorbance of tyrosyl residues at 295 nm on increasing the pH from 7.5 to 13.0 with 1 N NaOH. The molar extinction coefficient of tyrosyl residues in alkali was assumed to be 2.33×10^3 M^{-1} cm^{-1}. The tryptophan content of the reductase was calculated by subtraction of the tyrosine absorbance calculated above and from the absorbance of the reductase at 278 nm in 0.1 M potassium phosphate buffer, pH 7.5. The molar extinction coefficients of tyrosine and tryptophan are 1.28×10^3 and 5.55×10^3 M^{-1} cm^{-1}, respectively, at this wavelength.

Content of Sulfhydryl Groups in NADPH-adrenodoxin Reductase. (1) The number of sulfhydryl groups in NADPH-adrenodoxin reductase in the presence and absence of 8 M guanidine hydrochloride was determined by the method of Ellman (1959). The molar extinction coefficient of absorbancy at 412 nm of sulfhydryl groups in the complex of the reductase with 5,5'-dithiobis(2-nitrobenzoate) was taken as

13.6×10^3 M^{-1} cm^{-1}, at pH 7.5. A correction was made for the absorbance of FAD in the reductase.

(2) Alternatively the content of sulfhydryl groups in the reductase was estimated by the method of Boyer (1954). The concentration of *p*-chloromercuribenzoate was determined spectrophotometrically, assuming that the molar extinction coefficient was 16.9×10^3 M^{-1} cm^{-1} at 232 nm at pH 7.0 and the sulfhydryl content of proteins was determined using a molar extinction coefficient of 7.6×10^3 M^{-1} cm^{-1} at 250 nm at pH 7.0 (Boyer, 1954). For this the reductase solution was incubated with *p*-chloromercuribenzoate for 10 h and then incubated with 0.1% trypsin to reduce the turbidity of the solution. A correction was made for the sulfhydryl group in the trypsin added to the reductase solution.

Sugar Analysis. Neutral sugars and amino sugars of NADPH-adrenodoxin reductase were separated by the method of Haberman et al. (1961) and their contents were determined by the method of Spiro (1972) with some modifications (Shinohara, unpublished method). Three buffer systems were used: 1st buffer, 0.15 M sodium borate at pH 7.5 for 0.5 h; 2nd buffer, 0.25 M sodium borate at pH 9.0 for 1.5 h; and 3rd buffer, 0.35 M sodium borate at pH 9.6 for 3 h. Sialic acid was determined by the method of Aminoff (1961).

Measurements of Optical Absorption Spectra. Optical absorption spectra were measured with a Cary, Model 17, spectrophotometer equipped with thermostatically controlled cell holders and a cuvette of 1-cm light path, using a neodymium glass filter as a standard of wavelength.

Measurements of Circular Dichroism Spectra. Circular dichroism spectra were measured with a Jouan Dichrograph II, Model CD 185, at 25 °C. Measurements were carried out at a sensitivity, $s = 1 \times 10^{-5}$/mm, using a sample cuvette with a light path of 10.0 mm.

Fluorescence Measurements. Fluorescence measurements were made in a Hitachi fluorescence spectrophotometer, Model MPF-2A and Model 204, equipped with a thermostatically controlled cell holder using a Coolnics, Model CTR-120 and circulator, Model CTE-120, Komatsu-Yamato Co., with the use of slits of 1.3 mm. Fluorescence intensity of tryptophanyl residues was measured at 337 nm with excitation at 285 nm and that of FAD was measured at 520 nm with excitation at 450 nm. Adrenodoxin or other non-heme iron protein was added from a syringe microburet (Jintan Terumo Co. Ltd.) to the sample solution. A correction was made for the effects on fluorescence of change in the sample volume. The most general equation introduced by Stern and Volmer (1919) was used for treating quenching data.

Determination of Molecular Weight. The molecular weight of NADPH-adrenodoxin reductase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Sephadex gel filtration and analytical ultracentrifugation.

Electrophoresis. Thin-layer sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (1969). Horse heart cytochrome *c* (13 500), beef pancreas chymotrypsinogen A (25 000), ovalbumin (43 000), bovine serum albumin (68 000) and rabbit muscle phosphorylase a (94 000) were used as standard proteins. These proteins and NADPH-adrenodoxin reductase were dissolved at concentrations of about 0.5 mg protein/ml in 10 mM sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol, and incubated at 50 °C for 2 h. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 0.1% sodium dodecyl sulfate in 10 mM sodium phosphate buffer, pH 7.0. The gels (2 mm × 10 cm × 15 cm) contained final concentrations of 7.5% acrylamide, 0.2% *N,N'*-methylenebisacrylamide, 0.1% sodium dodecyl sulfate, and 10 mM sodium phosphate buffer. Electrophoresis was carried out at 80 mA for 6 h at 25 °C. Gels were stained for 20 h in 0.25% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid, and destained with 7% acetic acid for 3 days.

Gel Filtration. Sephadex gel filtration was carried out by the method of Andrews (1964) with some modifications. Cytochrome *c*, ovalbumin, and bovine serum albumin were used as standard proteins. A Sephadex G-100 column (1.5 × 70 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.4, was used to estimate the molecular weight of NADPH-adrenodoxin reductase since the molecular weight of the reductase seemed to be below 130 000 judging from previous results (Suhara et al., 1972a; Chu and Kimura, 1973b).

Analytical Ultracentrifugation. Ultracentrifugation of NADPH-adrenodoxin reductase in 4 mM potassium phosphate buffer, pH 7.4, was carried out in short column cell in a Hitachi, Model 282, analytical centrifuge with absorption optics by sedimentation equilibrium. Equilibrium was obtained by centrifugation for 21 h in 10 000 rpm at 20 °C. The protein concentration was 736 μg of protein/ml.

Determination of Isoelectric Points. The isoelectric points of NADPH-adrenodoxin reductase, adrenodoxin, and their complex were determined by the method of Vesterberg and Svensson (1966). The temperature of the samples was controlled at 0 °C by circulating cold methanol through the methanol bath of a Kälte-Thermostat, type KT20S-2319, ColoraMesstechnik GmbHLorch/Württ.

Dissociation Constant of FAD for Apo-NADPH-adrenodoxin Reductase. The dissociation constant of FAD for the aporeductase was determined from the half-maximal velocity of NADPH-ferricyanide reductase activity, quenching of FAD fluorescence, and equilibrium dialysis of the aporeductase and FAD. The FAD used was purified as described above. These experiments were carried out in 0.1 M potassium phosphate buffer at pH 7.4 and 25 °C.

Results

Molecular Weight of NADPH-adrenodoxin Reductase. The molecular weight of NADPH-adrenodoxin reductase has been reported by several workers. However, this reductase is known to be a glycoprotein and judging from the previous results on the molecular weights of glycoproteins (Andrews, 1965), the molecular weight of NADPH-adrenodoxin reductase cannot be determined by Sephadex gel filtration alone.

Our result on determination of the molecular weight by Sephadex gel filtration was 39 000, and the molecular weight obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 52 000. Suhara et al. (1972a) reported that the molecular weight of the reductase might be 50 000 and 54 000 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration with a Sephadex G-100 column, respectively, while Chu and Kimura (1973b) estimated it to be 54 000 using the same methods. Foster and Wilson (1975) recently reported that the molecular weight of the reductase was 51 500 and 49 500 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and sedimentation equilibrium ultracentrifugation, respectively. The discrepancy is because the reductase is a glycoprotein and its sugar moiety may be present on the surface of the molecule so the value obtained by Sephadex gel filtration is probably inaccurate. The true molecular weight of the reductase seems to be estimated by the molecular weight of the protein molecule using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by

TABLE I: Amino Acid, Sugar, and Flavin Compositions of NADPH-adrenodoxin Reductase.

Amino Acid[a]	
Aspartate + Asparagine	38
Threonine	23
Serine	24
Glutamate + Glutamine	49
Proline	34
Glycine	41
Alanine	36
Cysteine	6[b]
Valine	27
Methionine	6
Isoleucine	14
Leucine	41
Tyrosine	8
Phenylalanine	14
Histidine	9
Lysine	19
Tryptophan	14[b] (11)
Arginine	30
Sugar[c]	
Mannose	13.5
Galactose	3.5
Fucose	1.6
Glucose	1.4
Glucosamine	6.2
Sialic acid	1.3
Flavin[d]	
FAD	1.0

[a] Values are amino acid residues per mole of NADPH-adrenodoxin reductase. [b] Values were obtained spectrophotometrically. Value in parentheses was obtained in the presence of 2% thioglycolic acid. [c] Values are moles of sugars per mole of NADPH-adrenodoxin reductase. [d] Value is mole of flavin per mole of NADPH-adrenodoxin reductase.

TABLE II: Molar Extinction Coefficient and $E_{1cm}^{1\%}$ at Selected Wavelengths of NADPH-adrenodoxin Reductase.

	$E_{1cm}^{1\%}$	ϵ (M^{-1} cm^{-1})	
Holo-NADPH-adrenodoxin reductase	17.9 (272 nm)	9.6×10^3 (377 nm)	11.3×10^3 (450 nm)
Apo-NADPH-adrenodoxin reductase	12.5 (278 nm)		

TABLE III: Effects of Non-heme Iron Proteins on the Activity of NADPH-cytochrome *c* Reductase.[a]

	NADPH-cytochrome *c* Reductase Act. (μmol min^{-1} mg^{-1} protein)	
Electron Carrier	In the Absence of Superoxide Dismutase	In the Presence of Superoxide Dismutase
None	Nil	Nil
Bovine adrenodoxin	8.80 ± 0.52	8.25 ± 0.58
Bovine apoadrenodoxin	Nil	Nil
Pig adrenodoxin	2.01 ± 0.18	1.83 ± 0.13
Spinach ferredoxin	0.11 ± 0.02	0.06 ± 0.02
Horse spleen ferritin	<0.003	<0.002

[a] Values are averages of five estimations ± standard deviations.

measuring the content of sugar of NADPH-adrenodoxin reductase. Assuming that the partial specific volume of the reductase is 0.728, which was estimated by the methods of Cohn and Edsall (1943) and Isemura and Fujita (1957), the molecular weight of the reductase was estimated to be 51 000 by sedimentation equilibrium.

Amino Acid and Sugar Compositions of NADPH-adrenodoxin Reductase. Table I shows the amino acid and sugar contents of NADPH-adrenodoxin reductase. Amino acid residues were examined in an amino acid analyzer and the tryptophan content was also measured spectrophotometrically. Values for the content of sulfhydryl groups determined by the methods of Ellman (1959) and Boyer (1954) were similar. Although Foster and Wilson (1975) reported that the reductase contained 1.7% carbohydrate by weight, we found that it contained 9% sugar by weight. Table I gives the sugar composition.

Molar Extinction Coefficient and $E_{1cm}^{1\%}$ at Selected Wavelengths. The molecular weight of NADPH-adrenodoxin reductase was determined to be 56 000 from the sum of the total amounts of sugars, and protein of NADPH-adrenodoxin reductase, estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Values for the molar extinction coefficient and $E_{1cm}^{1\%}$ of NADPH-adrenodoxin reductase of bovine adrenocortical mitochondria calculated from the molecular weight are shown in Table II.

Activities of NADPH-adrenodoxin Reductase. Table III shows the NADPH-cytochrome *c* reductase activity from NADPH to cytochrome *c* via NADPH-adrenodoxin reductase and various non-heme iron proteins. The interaction between NADPH-adrenodoxin reductase and adrenodoxin or the non-heme iron proteins could be measured indirectly as the activity of NADPH-cytochrome *c* reductase. Superoxide dismutase and/or catalase was added to the test system to prevent effects of the superoxide and hydrogen peroxide on NADPH-cytochrome *c* reductase activity. This table shows that spinach ferredoxin and horse spleen ferritin were slightly effective in place of adrenodoxin for NADPH-cytochrome *c* reductase activity. NADPH-cytochrome *c* reductase activity via ferredoxin or ferritin was influenced by superoxide dismutase.

Circular Dichroism Spectra of NADPH-adrenodoxin Reductase and of the Complex of the Reductase with Adrenodoxin. The circular dichroism spectra of NADPH-adrenodoxin reductase and the complex are shown in Figure 4. The circular dichroism spectrum of oxidized NADPH-adrenodoxin reductase in the visible region, unlike that of D-amino acid oxidase (Edmondson and Tollin, 1971), shows negative absorption. This negative absorption of the circular dichroism spectra may be a specific characteristic of the reductase group of flavoproteins, as reported by Edmondson and Tollin (1971). The circular dichroism spectrum of the complex of NADPH-adrenodoxin reductase with adrenodoxin or apoadrenodoxin can be explained as representing the superposition of the individual circular dichroism spectra of NADPH-adrenodoxin reductase and adrenodoxin. No specific change in the circular dichroism spectra was observed on formation of the complex. No large conformational change which might affect the circular dichroism spectrum was observed on formation of a complex between NADPH-adrenodoxin reductase and adrenodoxin. The circular dichroism was measured over the wide range of 230 to 600 nm and over the expanded range of 220 to 230 nm to detect possible conformational changes of the proteins. However, no significant changes in the circular

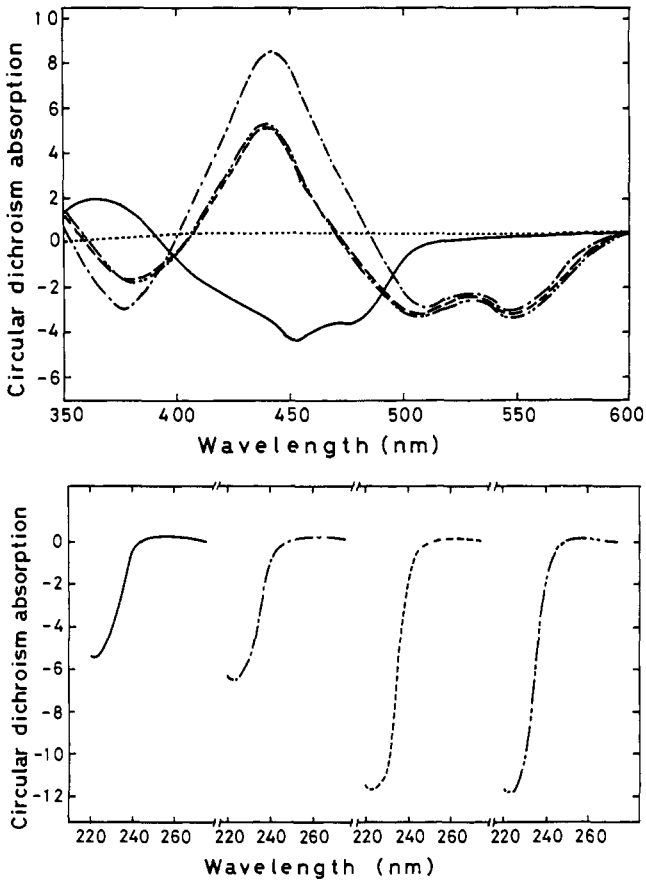

FIGURE 4: Circular dichroism spectra of NADPH-adrenodoxin reductase, adrenodoxin, and their complex. The proteins were in 10 mM potassium phosphate buffer, pH 7.4 (25 °C). Measurements were performed at a sensitivity of $s = 1 \times 10^{-5}$/mm, using a sample cuvette of 10.0-mm light path. The concentration of NADPH-adrenodoxin reductase or adrenodoxin used was 40 μM in the visible region and 1.5 μM in the ultraviolet region. (—) NADPH-adrenodoxin reductase; (— · —) adrenodoxin; (— — —) complex of NADPH-adrenodoxin reductase with adrenodoxin; (— · · —) addition spectrum of individual circular dichroism absorption of NADPH-adrenodoxin reductase and adrenodoxin; (· · ·) baseline.

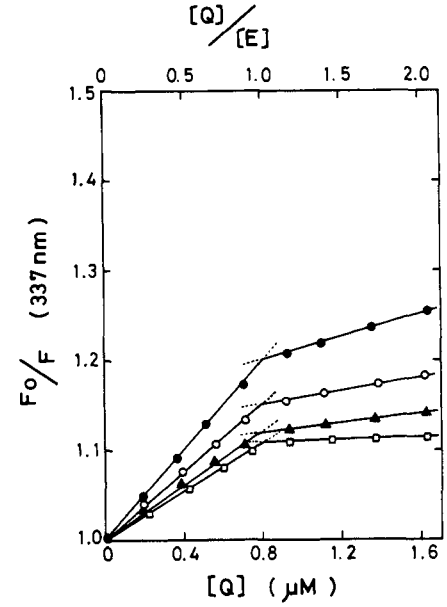

FIGURE 5: Stern-Volmer plots for the quenching of the tryptophan fluorescence of NADPH-adrenodoxin reductase during formation of a complex between the reductase and adrenodoxin or other non-heme iron proteins. NADPH-adrenodoxin reductase (0.8 μM) in 10 mM potassium phosphate buffer, pH 7.4, was used. The adrenodoxin concentration was varied by adding various amounts of buffer containing 20 μM adrenodoxin to the reductase solution. A correction was made for the effect of dilution due to addition of adrenodoxin solution. Fluorescence was measured after incubation at 25 °C for 15 min. The ordinate shows the inverse fractional fluorescence intensity, F_0/F. The abscissa shows the concentration of non-heme iron protein [(Q)] and the ratio of non-heme iron protein to the reductase (Q)/(E). (●) Bovine adrenodoxin; (○) bovine apoadrenodoxin; (□) pig adrenodoxin; (▲) spinach ferredoxin.

dichroism spectra in the visible and ultraviolet regions were observed under the experimental conditions employed.

Isoelectric Points of NADPH-adrenodoxin Reductase, Adrenodoxin, and the Complex of the Reductase with Adrenodoxin. The isoelectric points of NADPH-adrenodoxin reductase, adrenodoxin of bovine adrenocortical mitochondria, and the complex were 5.4, 4.0, and 4.7, respectively. The isoelectric point of the complex of the reductase and adrenodoxin was affected by the respective values of the two proteins but was different from either, being 4.7. This shows that the two proteins are tightly combined in the complex.

Effects of Non-heme Iron Proteins on the Fluorescence of Tryptophanyl Residues of NADPH-adrenodoxin Reductase. The fluorescence intensity at 337 nm of the tryptophanyl residue of NADPH-adrenodoxin reductase was quenched by bovine or pig adrenodoxin, bovine apoadrenodoxin, and spinach ferredoxin, but not by ovalbumin, as shown in Figure 5. The quenching of the tryptophanyl fluorescence in the complex of the reductase and adrenodoxin could not be accounted for by the quenching of fluorescence of adrenodoxin at 337 nm. This observation can be explained by the fact that bovine adrenodoxin has no tryptophanyl residue (Tanaka et al., 1970). The fluorescence change required 15 min to complete the complex of the reductase with adrenodoxin. The intensity of fluorescence of free FAD at 337 nm with excitation at 285 nm was 2% of the intensity of fluorescence of free FAD at 520 nm, and the fluorescence of FAD at 520 nm of the reductase with excitation at 285 nm was negligible. Accordingly, we can conclude that the quenching of the fluorescence at 337 nm of the reductase was not due to quenching of the fluorescence of FAD at 337 nm. On titration of NADPH-adrenodoxin reductase with a solution of adrenodoxin or related proteins, the quenching of the fluorescence intensity at 337 nm of NADPH-adrenodoxin reductase was observed up to a molar ratio of one to one. Thus this figure shows biphasic curves. A similar result was observed with apoadrenodoxin. This shows that the chromophore group of adrenodoxin is not involved in formation of the complex between NADPH-adrenodoxin reductase and adrenodoxin.

Effects of Non-heme Iron Proteins on the Fluorescence of FAD and NADPH-adrenodoxin Reductase. It has been found that the fluorescence of bound FAD in NADPH-adrenodoxin reductase was 12% of that of free FAD. This value differs from the 98.5% reported by Chu and Kimura (1973b) and Foster and Wilson (1975).

Figure 6 shows the effects of bovine and pig adrenodoxins, bovine apoadrenodoxin, and spinach ferredoxin on the fluorescence intensity at 520 nm of FAD of NADPH-adrenodoxin reductase on excitation at 450 nm. The FAD fluorescence at 520 nm, like the fluorescence of tryptophanyl residues in NADPH-adrenodoxin reductase, was quenched by increasing the amount of adrenodoxin or apoadrenodoxin added up to an equimolar ratio, and the quenching effect was diminished by addition of more than an equimolar amount of adrenodoxin or apoadrenodoxin. Bovine adrenodoxin could be partially

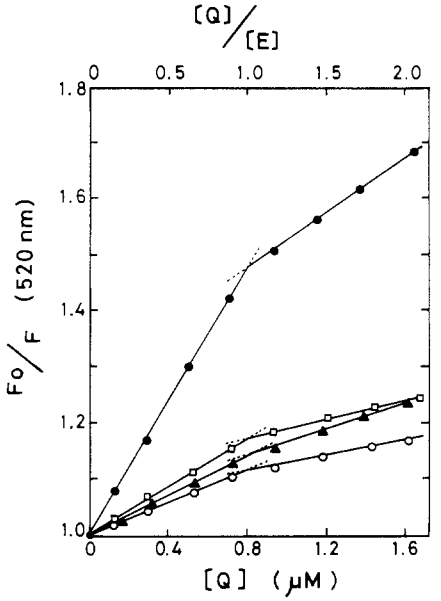

FIGURE 6: Stern-Volmer plots for the quenching of fluorescence of flavin of NADPH-adrenodoxin reductase during formation of a complex between the reductase and adrenodoxin or other non-heme iron proteins. NADPH-adrenodoxin reductase (0.8 μM) was used. The experimental conditions and symbols used are the same as for Figure 5.

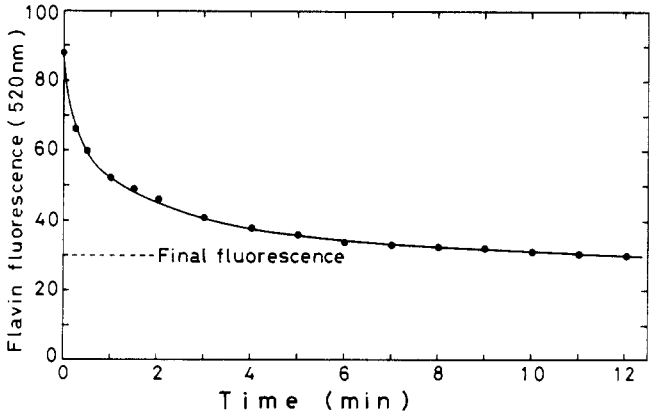

FIGURE 7: Change in fluorescence of flavin on mixing 0.43 μM FAD with 4.4 μM apo-NADPH-adrenodoxin reductase in 0.1 M potassium phosphate buffer, pH 7.4 (22 °C). Fluorescence was measured at 520 nm with excitation at 450 nm.

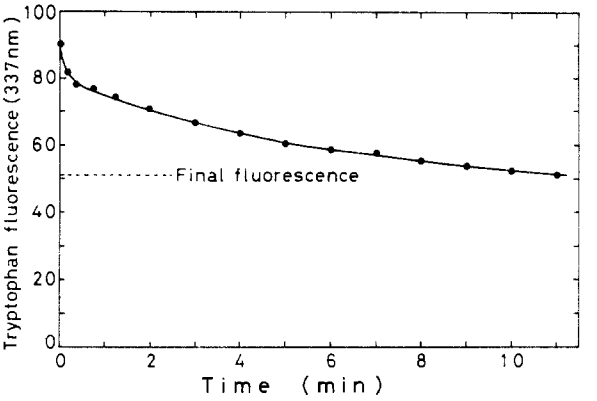

FIGURE 8: Change in fluorescence of tryptophan on mixing 0.88 μM apo-NADPH-adrenodoxin reductase with 1.8 μM FAD in 0.1 M potassium phosphate buffer, pH 7.4 (22 °C). Fluorescence was measured at 337 nm with excitation at 285 nm. A correction was made for the absorbance of added FAD at 337 nm.

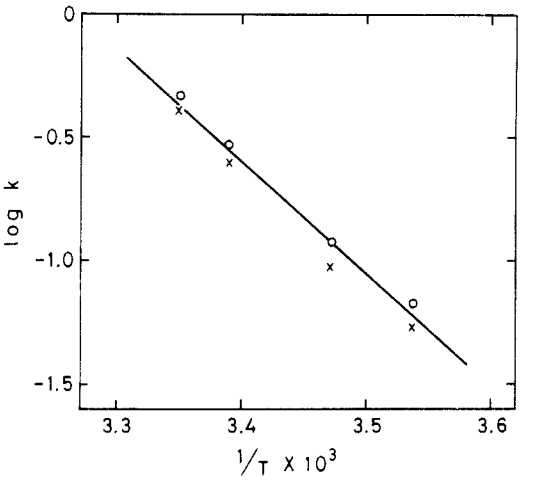

FIGURE 9: Arrhenius plot of the rate constant of the slow phase as a function of the reciprocal of the absolute temperature. (O) Calculated from changes in fluorescence of tryptophan; (X) calculated from changes in fluorescence of flavin.

replaced by spinach ferredoxin. Figures 5 and 6 show that formation of a complex affects the fluorescence of the prosthetic group and tryptophanyl residues of NADPH-adrenodoxin reductase. Therefore, adrenodoxin is probably closely bound to a tryptophanyl residue near the FAD of NADPH-adrenodoxin reductase.

Quenching of FAD Fluorescence on Addition of Apo-NADPH-adrenodoxin Reductase. From the studies on fluorescence changes on addition of FAD to the apoproteins of flavoproteins, it has been proposed that formation of a complex is a multistep process (Massey and Curti, 1966; Mayhew, 1971). The association between apo-NADPH-adrenodoxin reductase and FAD also seemed to be a multistep process: a rapid decrease in FAD fluorescence was observed, followed by a much slower decrease in fluorescence of FAD. These results are shown in Figure 7. Judging from a plot of the logarithm of the intensity of FAD fluorescence against time and the slow secondary change, the rate constant is 0.30 min^{-1}. This constant is independent of the concentration of aporeductase. However, the time required for the rapid fluorescence change with a pseudo-first-order rate constant of 2.3 min^{-1} is completely dependent on the concentration of aporeductase. As the concentration was 4.4 μM, this is equivalent to a second-order rate constant of 5.2×10^5 M^{-1} min^{-1}. The specific activities of the reconstituted NADPH-adrenodoxin reductase from the aporeductase and FAD were 7.8 and 18.8 μmol min^{-1} mg^{-1} protein in 0.1 M potassium phosphate buffer at pH 7.4 and 25 °C for NADPH-cytochrome *c* reductase and NADPH-ferricyanide reductase activities, respectively.

The dissociation constant of the FAD-aporeductase complex determined from the titration curve was 1.1×10^{-8} M at pH 7.4. The concentration of FAD was determined for the half-maximal velocity of NADPH-ferricyanide reductase activity of NADPH-adrenodoxin reductase. The aporeductase and various concentrations of FAD were incubated for 2 h in the dark at 25 °C. The dissociation constant obtained from the equilibrium dialysis experiment was 5.5×10^{-8} M. When an excess of the aporeductase was added to FAD, quenching of FAD fluorescence was completed within 15 min at 25 °C. Although the dissociation constant obtained was different for each method, the FAD seems to be tightly bound to the aporeductase.

Quenching of the Tryptophanyl Fluorescence of Apo-NADPH-adrenodoxin Reductase. Next the fluorescence of tryptophanyl residues of the aporeductase was used to monitor the effect of formation of holoreductase by combination of FAD with the aporeductase. The fluorescence of tryptophanyl residues of the aporeductase, like the fluorescence of FAD, was quenched by formation of the holoreductase. The modes of quenching of the fluorescence of FAD and tryptophanyl residues of the aporeductase were similar, as shown in Figure 8. The time courses of change in the fluorescence of FAD and tryptophanyl residues were affected by temperature, as shown in Figure 9. The activation energy was 25 000 cal mol^{-1} from this figure.

Discussion

Different values for the molecular weight of NADPH-adrenodoxin reductase were obtained using different methods. This is because the reductase is a glycoprotein (Chu and Kimura, 1973b). In disagreement with previous reports (Suhara et al., 1972a; Chu and Kimura, 1973b), the molecular weight of the reductase estimated by Sephadex gel filtration method was smaller than those estimated by other methods. This is probably because the glycoprotein has affinity for carbohydrate. The low value obtained by Sephadex gel filtration suggests that the carbohydrate is present on the surface of NADPH-adrenodoxin reductase. NADPH-adrenodoxin reductase was found to contain 9% by weight of sugar. This reductase contained a larger amount of neutral sugar than other known glycoproteins (Winzler, 1970). Glucosamine was detected in the reductase, but not galactosamine or mannosamine. Attempts were made to determine the molecular weight and partial specific volume of NADPH-adrenodoxin reductase by sedimentation equilibrium (Edelstein and Schachman, 1967). However, this was unsuccessful, owing to the interaction of the molecules of reductase in D_2O solution (Henderson and Henderson, 1970; Baghurst et al., 1972).

The amino acid composition of NADPH-adrenodoxin reductase is given in Table I, but the results do not agree with those of other workers (Chu and Kimura, 1973b; Wickramasinghe, 1974). This is probably due to a difference in the purities of the samples of reductase used for estimations, although it could be due to species or strain differences in the animal sources and/or proteolytic modification of the reductase by lysozomal enzymes.

Edmondson and Tollin (1971) distinguished two types of circular dichroism spectra of flavoproteins. The oxidase and dehydrogenase type shows positive absorption in the visible region whereas the reductase type shows negative absorption. The NADPH-adrenodoxin reductase from bovine adrenocortical mitochondria showed a typical circular dichroism spectra of the reductase type. The reason for the difference in the types is not clear. Formation of a complex between NADPH-adrenodoxin reductase and adrenodoxin quenched the fluorescences of both FAD and tryptophanyl residues of the reductase since the fluorescence intensity at 337 nm of tryptophanyl residues did not decrease with a change of pH from 8.0 to 11.0, although the fluorescence intensity of free tyrosine diminishes with increasing pH (Kimura et al., 1972). The complex could also be formed with spinach ferredoxin instead of adrenodoxin or apoadrenodoxin, but not with ovalbumin. However, the complex of ferredoxin with NADPH-adrenodoxin reductase was not tight, judging from the NADPH-cytochrome *c* reductase activity and the extent of quenching of fluorescence of FAD and tryptophanyl residues of the reductase in this complex. The quenchings of the fluorescence of tryptophanyl residues and FAD by different non-heme iron proteins were different. The quenching may be affected by the amount of iron and the structure of protein and iron in the molecules of non-heme iron proteins. The quenchings of the fluorescence of FAD and tryptophan of the reductase on formation of a complex with adrenodoxin suggest that adrenodoxin probably binds in the neighborhood of a tryptophanyl residue near the prosthetic group of NADPH-adrenodoxin reductase. The complex of the reductase and adrenodoxin seemed to be formed in the same way as that of spinach ferredoxin-$NADP^+$ reductase and ferredoxin, which was first investigated by Shin and San Pietro (1968) and Shin (1973).

Before use, the NADPH-adrenodoxin reductase solution was passed through a Sephadex G-25 column under dim light to remove free FAD and other possible contaminants. The FAD fluorescence of the resulting preparation was very weak, being 12% of that of free FAD at 520 nm. Under similar conditions, Chu and Kimura (1973b) and Foster and Wilson (1975) reported that the FAD fluorescence of the reductase was 98.5% of that of free FAD. Other flavoproteins are known to have a fluorescence of less than 20% of that of free FAD: for instance, that of hepatic NADPH-cytochrome *c* reductase is 12% of that of free FAD (Ichikawa, unpublished result), that of spinach ferredoxin-$NADP^+$ reductase is 0.6% (Shin, 1973), and that of D-amino acid oxidase is 17% (Massey et al., 1966). The FAD fluorescence of crystalline NADPH-adrenodoxin reductase in the presence of about 45% saturation of ammonium sulfate was stable for at least 3 weeks at 0 °C in the dark.

Massey et al. (1969) have reported the effects of superoxide dismutase on various flavoproteins. The effect of superoxide dismutase on NADPH-adrenodoxin reductase in the presence of various non-heme iron proteins was studied to examine the formation of a complex between the reductase and non-heme iron proteins and Table III shows the results.

Formation of holo-NADPH-adrenodoxin reductase from FAD and aporeductase involves at least two stages: first rapid binding of FAD and then slower secondary changes. No enzymatic activity was detected in the first rapid stage with quenching of fluorescence of FAD and tryptophanyl residues of apo-reductase. The activity appeared in the second slow phase. A slow conformational change of protein may occur after the rapid binding of FAD to the aporeductase.

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References

Aminoff, D. (1961), *Biochem. J. 81,* 384–392.
Andrew, P. (1964), *Biochem. J. 91,* 222–233.
Andrew, P. (1965), *Biochem. J. 96,* 595–606.
Baghurst, P. A., Nichol, L. W., and Sawyer, W. H. (1972), *J. Biol. Chem. 247,* 3199–3204.
Bayer, E., Parr, W., and Kazmaier, B. (1965), *Arch. Pharm. Ber. Dtsch. Pharm. Ges. 298,* 196–206.
Beaven, G. H., and Holiday, E. R. (1952), *Adv. Protein Chem. 7,* 319–386.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76,* 4331–4337.
Chu, J. W., and Kimura, T. (1973a), *J. Biol. Chem. 248,* 5183–5187.
Chu, J. W., and Kimura, T. (1973b), *J. Biol. Chem. 248,*

2089–2094.
Cohn, E. J., and Edsall, J. T. (1943), in Proteins, Amino Acids and Peptides, New York, N.Y., Reinhold, pp 370–381.
Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242,* 306–311.
Edmondson, D. E., and Tollin, G. (1971), *Biochemistry 10,* 113–124.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70–77.
Estabrook, R. W., Suzuki, K., Mason, J. I., Baron, J., Taylor, W. E., Simpson, E. R., Purvis, J., and McCarthy, J. (1973), in Iron Sulfur Proteins, Vol. 1, Lovenberg, W., Ed., New York, N.Y., Academic Press, pp 193–223.
Foster, R. P., and Wilson, L. D. (1975), *Biochemistry 14,* 1477–1484.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177,* 751–760.
Haberman, W., Mattenheimer, H., Sky-Peck, H., and Shinohara, H. (1961), *Chimia 15,* 339–341.
Hatefi, Y., and Lester, R. L. (1958), *Biochim. Biophys. Acta 27,* 83–88.
Henderson, R. F., and Henderson, T. R. (1970), *J. Biol. Chem. 245,* 3733–3737.
Hiwatashi, A., Ichikawa, Y., and Yamano, T. (1975), 26th Annual Meeting of Protein Structure, Nagasaki, Japan, pp 93–96.
Ichikawa, Y. (1971), in Mitochondria, Hagihara, B. Ed., Tokyo, Asakura Publishing Co., pp 266–291.
Isemura, T., and Fujita, S. (1957), *J. Biochem. (Tokyo) 44,* 797–805.
Kekwick, R. A., and Cannon, R. K. (1936), *Biochem. J. 30,* 227–234.
Kimura, T., Ting, J. J., and Huang, J. J. (1972), *J. Biol. Chem. 247,* 4476–4479.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.
Margoliash, E. (1954), *Biochem. J. 56,* 535–543.
Massey, V., and Curti, B. (1966), *J. Biol. Chem. 241,* 3417–3423.
Massey, V., Curti, B., and Ganther, H. (1966), *J. Biol. Chem. 241,* 2347–2357.
Massey, V., Strickland, S., and Mayhew, S. G., Howell, L. G., Engel, P. C., Mathews, R. G., Schuman, M., and Sullivan, P. A. (1969), *Biochem. Biophys. Res. Commun. 36,* 891–897.
Massey, V., and Swoboda, B. E. P. (1963), *Biochem. Z. 338,* 474–484.
Matsubara, H., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35,* 175–181.
Mayhew, S. G. (1971), in Flavins and Flavoproteins, Kamin, H., Ed., Baltimore, Md., University Park Press, pp 185–209.
McCord, J. M., and Fridovich, I. (1969), *J. Biol. Chem. 244,* 6049–6055.
Nakamura, Y., Otsuka, H., and Tamaoki, B. (1966), *Biochim. Biophys. Acta 122,* 34–42.
Okunuki, K., and Nozaki, M. (1956), in Koso Kenkyuho, Vol. I, Akabori, S., Ed., Tokyo, Asakura Publishing Co., pp 670–684.
Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y., and Rosenthal, O. (1966), *Arch. Biochem. Biophys. 117,* 660–673.
Shin, M. (1973), *Biochim. Biophys. Acta 292,* 13–19.
Shin, M., and San Pietro, A. (1968), *Biochem. Biophys. Res. Commun. 33,* 38–42.
Shirakawa, M. (1953), in Hyojun Seikagaku Jikken, Egami, F., Ishimoto, M., Maruo, B., Miura, Y., Sekine, Y., Suda, M., and Tamiya, N., Tokyo, Bunkodo, pp 321–327.
Spiro, R. G. (1972), *Methods Enzymol. 28,* 3–43.
Stern, O., and Volmer, M. (1919), *Phys. Z. 20,* 183–188.
Sugiyama, T., and Yamano, T. (1975), *FEBS Lett. 52,* 145–148.
Suhara, K., Ikeda, Y., Takemori, S., and Katagiri, M. (1972a), *FEBS Lett. 28,* 45–47.
Suhara, K., Kanayama, K., Takemori, S., and Katagiri, M. (1974), *Biochim. Biophys. Acta 336,* 309–317.
Suhara, K., Takemori, S., and Katagiri, M. (1972b), *Biochim. Biophys. Acta 263,* 272–278.
Tagawa, K., and Arnon, D. I. (1962), *Nature (London) 195,* 537–543.
Tagawa, K., and Arnon, D. I. (1968), *Biochim. Biophys. Acta 153,* 602–613.
Tanaka, M., Haniu, M., and Yasunobu, K. T. (1970), *Biochem. Biophys. Res. Commun. 39,* 1182–1188.
Vesterberg, O., and Svensson, H. (1966), *Acta Chem. Scand. 20,* 820–834.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406–4412.
Wickramasinghe, R. H. (1974), *Int. J. Pept. Protein Res. 6,* 187–193.
Winzler, R. J. (1970), in Handbook of Biochemistry, Sober, H. A., Ed., Cleveland, Ohio, The Chemical Rubber Co., pp c–42.
Yagi, K., and Matsuoka, Y. (1956), in Koso Kenkyuho, Vol. I, Akabori, S., Ed., Tokyo, Asakura Publishing Co., pp 687–698.