Purification and Characterization of Adrenodoxin Reductase from Bovine Adrenal Cortex†

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ABSTRACT: NADPH-adrenodoxin reductase from steer adrenal cortex mitochrondria has been purified to homogeneity (on sodium dodecyl sulfate polyacrylamide gel electrophoresis) by chromatography on DEAE-cellulose, Sephadex, and hydroxylapatite. A molecular weight of 51,500 was determined from sodium dodecyl sulfate polyacrylamide gel electrophoresis, while sedimentation equilibrium ultracentrifugation gave a value of 49,500. All of the flavine present was identified as FAD; 1 mol/52,000 g of protein. The reductase contained 1.7% carbohydrate (using glucose as standard) by weight. Homogeneous adrenodoxin reductase exhibited a typical oxidized flavoprotein absorbance spectrum, with maxima at 270, 376, and 450 nm, and gave an absorbance ratio A_{450}/A_{270} of 0.122–0.128 (depending on the preparation). Reduction of the flavoprotein with NADPH or dithionite gave progressive bleaching of the 450-nm peak. The reductase was absolutely required, in the presence of adrenodoxin, for electron transfer from NADPH to cytochrome *c* or to particulate cytochrome P450. Adrenodoxin reductase is obligatory for reconstitution of 11β-hydroxylation activity using deoxycorticosterone as substrate, and for the side-chain cleavage of 20α-hydroxycholesterol or cholesterol. The specific activity of the homogeneous preparation in cytochrome *c* reduction is at least 17,000 nmol min^{-1} mg of $protein^{-1}$, corresponding to a turnover number of 850 min^{-1}. No evidence for the existence of multiple forms or subunits was obtained.

Adrenodoxin reductase (AR)[1] is the name given to an FAD-containing flavoprotein first identified by Nakamura et al. (1966) in rat and porcine adrenal cortex mitochondria, by Omura et al. (1965, 1966) in steer adrenals and by Kimura and Suzuki (1965). This flavoprotein was shown to function in an electron transport sequence, separate from the respiratory chain, which consisted of NADPH, reductase, non-heme iron protein (adrenodoxin), and cytochrome P450. Reconstitution of cytochrome P450-dependent steroid 11β- and 18-hydroxylase activity was achieved with the partly purified components (Nakamura et al., 1966; Omura et al., 1965; Kimura and Suzuki, 1967).

Success in elucidating the mechanism of mitochondrial cytochrome P450 dependent reactions depends on the purification and characterization of each component of the electron transporting pathway for hydroxylation. The preparation of homogeneous and crystalline adrenodoxin has been described (Kimura and Suzuki, 1967; Suhara et al., 1972a). The goal of the present study was to purify and characterize the reductase, which had not yet been examined in depth. During the course of this work, reports appeared from the laboratories of Katagiri (Suhara et al., 1972b) and Kimura (Chu and Kimura, 1973a,b) in which the preparation and properties of the purified reductase were described. We have confirmed and extended much of their data and wish to report here a procedure for the preparation of homogeneous adrenodoxin reductase (and adrenodoxin) in reasonable yield and further characterization of the reductase.

Materials and Methods

Chemicals. NADP, NADPH, NADH, FAD, FMN, 2,6-dichlorophenolindophenol (DPIP), morpholinopropanesulfonic acid (Mops), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 2-amino-2-(hydroxymethyl-1,3-propandiol (Tris, reagent grade), *N*-tris(hydroxymethyl)-methylglycine (Tricine), sodium dodecyl sulfate (SDS), D-glucose 6-phosphate, glucose-6-phosphate dehydrogenase (from yeast), and bovine serum albumin (crystallized and lyophilized) for protein determination standard, were all purchased from Sigma Chemical Co.

Equine heart cytochrome *c* (cyt *c*) was obtained from Calbiochem. For molecular weight standards, bovine serum albumin, ovalbumin, and human γ-globulin were purchased from Schwartz/Mann; pepsin (from swine stomach mucosa) was the product of Worthington Biochemical Corporation. Acrylamide, *N',N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylendiamine were manufactured by Eastman Kodak Co. Whatman DE-52 DEAE-cellulose was obtained from H. Reeve Angel and Co., Inc. Sephadex G-75 and G-100 gels were purchased from Pharmacia Fine Chemicals. Hydroxylapatite slurry (Bio-Gel HT) was produced by Bio-Rad Laboratories.

Cholesterol was obtained from Applied Science Laboratories, Inc.; 11-deoxycorticosterone, from Mann Research Laboratories; 20α-hydroxycholesterol, from Ikapharm Ltd.; [$^{14}C_4$]cholesterol, [$^{14}C_4$]deoxycorticosterone, and [$^{3}H_7$]-20α-hydroxycholesterol were from New England Nuclear Corp. (specific radioactivities were 0.14 Ci/g, 0.17 Ci/μg, and 23 Ci/g, respectively).

Electrophoresis of adrenodoxin reductase was performed

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[1] Abbreviations used are: FAD, flavine adenine dinucleotide; FMN, flavine mononucleotide; DPIP, 2-6-dichlorophenolindophenol; Mops, morpholinopropanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Tricine, *N*-tris(hydroxymethyl)methylglycine; SDS, sodium dodecyl sulfate; DOC, deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; 20α-hydroxycholesterol, cholest-5-ene-3β,20α-diol; AR, adrenodoxin reductase; cyt *c*, cytochrome *c*.

in 7.5% polyacrylamide gels essentially according to the procedure of Weber and Osborn (1969). Samples were incubated at 65° for 1 hr in buffer containing SDS and 2% β-mercaptoethanol and applied to 5- or 6-mm diameter gels. Electrophoresis was carried out in 0.166 *M* sodium phosphate buffer (pH 7.4) containing 1% β-mercaptoethanol and 0.1% SDS, at 7 mA/gel for 3–6 hr. Protein was stained with Coomassie Blue.

Analytical sedimentation experiments were performed in a Beckman Model E ultracentrifuge equipped with a temperature control unit and schlieren and Rayleigh interference optics. Photographic plates were measured with a Nikon Model 6C profile projector. The sedimentation coefficient of adrenodoxin reductase in 0.1 *M* NaH_2PO_4 buffer (pH 7.0) containing 1 m*M* each EDTA and β-mercaptoethanol was determined by centrifugation at 56,000 rpm and 20° in a double-sector synthetic boundary cell.

The molecular weight of the reductase was determined by the sedimentation equilibrium method according to the meniscus depletion technique of Yphantis (1964). A Beckman 12-mm six-channel Kel-F centerpiece was used. Sedimentation equilibrium was performed in 0.1 *M* NaH_2PO_4 (pH 7.0) containing mercaptoethanol and EDTA (1 m*M* each), at 30,000 rpm for 18–48 hr at 5 or 10°. Molecular weights were determined as the average of those obtained from each cell sector (0.1, 0.2, and 0.4 mg of protein/ml at several times after equilibrium was attained; a partial specific volume of 0.730 ml/g was assumed).

The absorption spectrum of adrenodoxin reductase was obtained with a Cary 14 recording spectrophotometer. The fluorescence spectrum was obtained with a Perkin-Elmer (Hitachi) MPF-2A recording fluorescence spectrophotometer. Prior to taking spectra, the flavoprotein, in 0.1 *M* Mops or 0.2 *M* phosphate (pH 7.4), was dialyzed overnight against fresh buffer and centrifuged to sediment any particulate matter which may have formed during storage or dialysis.

Assays. Protein was determined by the biuret method (Gornall et al., 1949) or by the method of Sutherland et al. (1949), using bovine serum albumin as standard. Carbohydrate was determined according to the method of Dubois et al. (1956), using glucose as standard.

The content of cytochrome P450 in mitochondrial membrane preparations was measured as described under cytochrome P450 reduction, except that complete reduction was chemically produced with sodium dithionite (Omura and Sato, 1964).

Commercially available FAD and FMN were purified by the method of Massey and Swoboda (1963) and used as standards for fluorimetry and thin-layer chromatography. Quantitation of each flavine nucleotide and mixtures of these was achieved employing the method of Faeder and Siegel (1973). Fluorescence was measured in a Turner spectrofluorimeter; the exciting wavelength was 450 nm and the emitted light was measured at 530 nm. In addition to 0.1 *M* potassium phosphate buffer (pH 7.70) and 0.1 m*M* neutralized EDTA, the reaction mixture contained 1 m*M* Mops (pH 7.4) in a total volume of 4.0 ml. After separation of flavine from denatured protein (boiling water bath for 3 min in foil-wrapped Corex tubes, then centrifugation at 4°), 3.0-ml aliquots were taken for fluorimetry. Fluorescence of samples and standards were read before and after addition of 0.35 ml of 1.0 *N* HCl to the cuvet; final pH was 2.8. The temperature of the reaction mixture was 24 ± 1°. The assay was linear over the ranges of concentrations of 0.2×10^{-7} to 2.0×10^{-7} *M* for FMN and 0.8×10^{-7} to 10×10^{-7} *M* for FAD.

Thin-layer chromatography of flavines was done on glass or plastic sheets precoated with silica gel G. These were activated at 110° for 60 min and cooled; then 5–25 μl of the aqueous sample was spotted. Standard solutions of purified FMN and FAD were always run on channels adjacent to the unknown sample. The plates were developed with 0.7 *M* phosphate (pH 6.8) or with 0.2 *M* sodium acetate (pH 7.0). Both systems gave reproducible results and, with both, a minor and more polar impurity was observed with the standard flavine nucleotide samples. FMN had a mobility of 0.71 compared with FAD using either developing system and the plastic sheets, while the relative mobility of FMN on the precoated glass slides was 0.65.

The reaction mixture for measuring diaphorase activity contained 0.1 *M* Mops buffer (pH 7.4), 1 m*M* NADH or NADPH, and 0.6 m*M* potassium ferricyanide (FeCN) or 30 μ*M* DPIP. The reaction was initiated by the addition of the reductase. The rates of DPIP reduction at 590 nm and FeCN reduction at 420 nm were determined with a Beckman spectrophotometer, utilizing a millimolar absorption coefficient of 19 for DPIP (Savage, 1957) and 1.0 for FeCN (Lee et al., 1967).

Samples to be assayed for cytochrome *c* (cyt *c*) reductase activity were added to a cuvet containing the following reagents (3-ml volume): 0.1 *M* Mops buffer (pH 7.4), 20 μ*M* horse heart cytochrome *c*, 0.1 m*M* NADH or NADPH, and 0.3 μ*M* adrenodoxin. For routine measurements, a generator consisting of NADP (20 μ*M*), glucose 6-phosphate (1 m*M*), and glucose-6-phosphate dehydrogenase (0.3 unit/ml) was substituted for NADPH. The NADPH generator gave linear reaction rates, whereas use of NADPH did not. Concentrations of all reaction mixture components and pH were selected to give optimal rates of cyt *c* reduction. The rate in 0.1 *M* Mops buffer was several times greater than in phosphate buffer of the same concentration. The rate of cyt *c* reduction was determined by following the absorbance increase at 550 nm relative to 540 nm using a Phoenix dual-wavelength recording spectrophotometer. The initial linear reaction velocity is expressed as nanomoles of cyt *c* reduced per minute employing a difference absorption coefficient of 19.1 mM^{-1} cm^{-1} (Chance and Williams, 1956).

For measurement of cyt P450 reductase and hydroxylation activities, crude cytochrome P450 depleted of adrenodoxin reductase and adrenodoxin was prepared from the 150,000*g* pellet obtained in the preparation of extract from sonicated mitochondria. The sediment derived from sonicated mitochondria equivalent to about 2 g of protein was suspended in 20 ml of 0.1 *M* phosphate buffer (pH 7.4) by sonication. Sonication was applied for 15 min at 4° with a Branson W140 sonifier at power setting 7. The brown suspension was diluted to 80 ml and centrifuged for 2 hr at 150,000*g* (4°). Supernatant was completely drained from the resulting pellets, which were frozen until use. When needed, a pellet was homogeneously suspended in 5 ml of 0.1 *M* phosphate buffer (pH 7.4) by sonication, the protein and cytochrome P450 content were determined, and the material was used immediately. Loss of cytochrome P450 occurred after several hours, even at −20°, once these membrane preparations were suspended in phosphate buffer.

A 3-ml reaction cuvet was prepared containing 0.1 *M* Mops buffer (pH 7.4), NADPH generator, 0.3 μ*M* adrenodoxin, and sonicated mitochondria equivalent to 1 mg of

protein/ml. The mixture was bubbled with carbon monoxide for 2 min at a rate of 1 bubble/sec. Cytochrome P450 (cyt P45) reduction was monitored by the absorbance difference at 450 nm minus 490 nm in the presence of saturating carbon monoxide using a Phoenix dual-wavelength spectrophotometer, at room temperature. The initial velocity and extent of cyt P450 reduction were calculated employing a difference absorption coefficient of 91 mM^{-1} cm^{-1} for the reduced CO complex minus the reduced form (Omura and Sato, 1964). To initiate the reaction, adrenodoxin reductase was added.

Reconstitution of hydroxylase activities was carried out with crude cytochrome P450 preparations depleted of adrenodoxin reductase and adrenodoxin as described earlier. Measurement of 11β-hydroxylation of deoxycorticosterone was done as previously described (Harding et al., 1965), as were the side-chain cleavage of cholesterol (Wilson and Harding, 1970) and 20α-hydroxycholesterol (Wilson and Harding, 1973). A typical reaction mixture (3-ml volume) contained 0.1 *M* Mops buffer (pH 7.4), NADPH generator, 50 μM steroid substrate, sonicated mitochondria equivalent to 0.3 μM cytochrome P450, 2.3 μM adrenodoxin, and 0.22 μM reductase. The reductase used in these experiments had an A_{450}/A_{270} ratio of 0.126. The reaction was initiated with 20 μM NADPH after a 5-min preincubation at 37° and 0.5-ml aliquots were removed at intervals, quenched in 5 ml of ethyl acetate, and processed as described previously. The ^{14}C-labeled substrates added contained 1.5×10^5 dpm while the [$^3H_7$]-20α-hydroxycholesterol contained 19×10^5 dpm.

Purification Procedures. Intact adrenal glands were collected at a local packing plant from freshly killed steers and chilled in ice. Adhering fat was removed, the glands were bissected, and after the medulla was removed, the cortex was scraped from the capsule. The scrapings were added to 9 volumes of cold 0.25 *M* sucrose solution containing 0.01 *M* Hepes buffer and 0.001 *M* EDTA at pH 7.4.

The suspended cortex scrapings were homogenized at 4° with a 200-ml Potter-Elvehjem type glass motor-driven homogenizer fitted with a Teflon pestle machined to 0.004 in. clearance at 25° (Glenco Scientific, Inc.). Subsequent passage of the homogenate through a standard clearance 55-ml ground-glass (Tenbroek type) manual homogenizer was found to improve the yield of mitochondria and this step was routinely included.

All preparative operations were carried out at 0–4°.

The homogenate was centrifuged at 800*g* for 10 min and the pellet was discarded. Mitochondria were sedimented by centrifugation at 20,000*g* for 10 min, resuspended in homogenization medium in one-half the original volume, and centrifuged at 24,000*g* for 10 min. The pellet of washed mitochondria was stored frozen until use.

Mitochondria equivalent to about 20 g of protein were thawed and suspended in 10 m*M* Mops buffer (pH 7.4) to give a protein concentration of 25 mg/ml. The mitochondrial suspension was passed through the continuous flow cell of a Branson W185 sonifier at power setting 6. The flow rate through the cell was adjusted to 10 ml/min. Temperature of the sonicate was maintained at 4° by circulating a 0° methanol–water mixture through the cooling jacket of the flow cell.

The sonicated mitochondria were centrifuged at 105,000*g* for 30 min, yielding a brown pellet. The resulting supernatant was centrifuged at 150,000*g* overnight to give a compact, red translucent pellet and a clear straw-colored liquid. This supernatant material is designated as mitochondrial extract.

Cold saturated ammonium sulfate solution, buffered with Tris (pH 7.4 at room temperature), was added to the mitochondrial extract to give 35% saturation. After the mixture was stirred for 1 hr, the slight precipitate which formed was removed by centrifugation. The supernatant was then brought to 65% saturation with buffered ammonium sulfate and stirred for 1 hr. The resulting cream-colored precipitate was collected by centrifugation, dissolved in 100 ml of 10 m*M* phosphate buffer (pH 7.4), and dialyzed overnight against 7 l. of this buffer.

The dialyzed 35–65% ammonium sulfate fraction, containing over 90% of the adrenodoxin-dependent NADPH–cytochrome *c* reductase activity, was clarified by centrifugation and applied to a 2.5 × 25 cm column of Whatman DE-52 resin equilibrated with 10 m*M* phosphate buffer (pH 7.4). An intense orange-brown band collected at the top of the bed and a 1.5-cm bright yellow band formed several centimeters below. Washing with 200 ml of 10 m*M* phosphate buffer did not move the colored bands, but considerable protein passed through the column at this step.

A linear gradient of phosphate buffer (pH 7.4) from 10 to 100 m*M* concentration, 1200 ml total volume, was then applied to the DE-52 column, at a flow rate of 100 ml/hr. The yellow band spread and resolved into at least three zones, each one eluting as a distinct shade of yellow. A correspondence was noted between the ratio of absorbance at 450 to 280 nm (A_{450}/A_{280}) and NADPH–cytochrome *c* reductase activity (based on A_{450}) of the fractions, so that both measurements were used as preliminary criteria for purification of this flavoprotein. The fractions with highest specific activity and best absorbance ratio (bright yellow in color) were combined.

The combined fractions from DEAE-cellulose chromatography were concentrated by adding solid ammonium sulfate to 80% saturation (buffered to pH 7.4 by including 0.29 g of Tris for each 10 g of salt) and collecting the yellow precipitate by centrifugation. The pellet was dissolved in 5 ml of 10 m*M* phosphate buffer. Recovery of protein and NADPH–cytochrome *c* activity was complete.

The concentrated material was applied directly to a 2.5 × 80 cm Sephadex G-100 column equilibrated with 10 m*M* phosphate buffer (pH 7.4) containing 1 m*M* neutralized EDTA and 1 m*M* β-mercaptoethanol. Elution was carried out at a flow rate of 20 ml/hr, using this mixture as running buffer. Inclusion of EDTA and mercaptoethanol retarded denaturation of the reductase in phosphate buffer alone during storage and lengthy preparative steps such as dialysis and chromatography.

A single yellow band, coincident with the major protein peak, was eluted from the Sephadex column, following a much smaller peak of colorless protein. The fractions with greatest specific activity and best ratio were combined and applied without further treatment to a 1.5 × 5.5 cm Bio-Gel HT (hydroxylapatite) column equilibrated with 10 m*M* phosphate buffer (pH 7.4). EDTA and β-mercaptoethanol (1 m*M* each) were included in all buffers used for this chromatography. A bright yellow band developed in the top centimeters of the column, and spread somewhat when washed with 50 ml of 50 m*M* phosphate buffer (pH 7.4). With less purified materials, a golden-yellow component was rapidly eluted at this phosphate concentration and additional similarly colored material was eluted with 100 m*M* phosphate, a concentration at which the bright yellow AR band spread

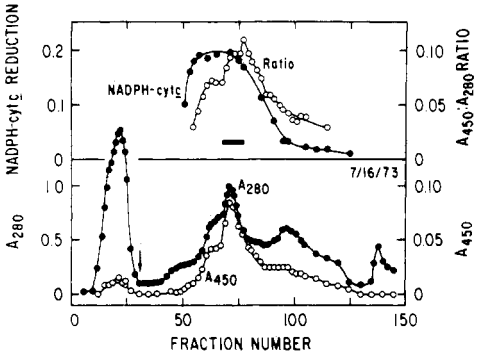

FIGURE 1: DEAE-cellulose chromatography of mitochondrial extract precipitated with ammonium sulfate between 35 and 65% saturation. Bed volume of the column was 120 ml; 10-ml fractions were collected beginning with application of sample (100 ml) to the column. After washing with 10 m*M* phosphate buffer, a 1200-ml linear gradient was applied (arrow), 10–100 m*M* phosphate, pH 7.4. NADPH-cyt *c* reduction is expressed as ΔA ($A_{550} - A_{540}$) per minute in the standard reaction mixture as described in assays. The volume of each fraction used was varied so that the absorbance at 450 nm was constant in all assays. Therefore, this is a specific activity, relative to the 450-nm absorbance of the sample. Fractions 68–77 (indicated by the bar) were combined to give 100 ml of partially purified reductase at A_{450} = 0.067 and with an average A_{450}/A_{280} ratio of 0.089.

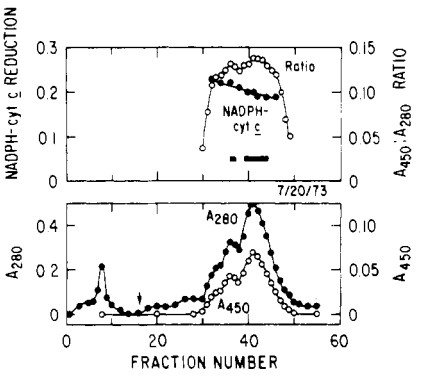

FIGURE 2: Elution pattern of purified adrenodoxin reductase on hydroxylapatite gel. The peak fractions from chromatography on Sephadex G-100 were applied to a 1.5 × 5.5 cm bed of Bio-Gel HT equilibrated with 10 m*M* phosphate (pH 7.4). Fractions (5 ml) were collected beginning with sample application. After washing with 50 m*M* phosphate, a linear gradient in phosphate, 50–250 m*M*, 200 ml total volume, was applied (arrow). All buffers contained mercaptoethanol and EDTA (1 m*M* each). NADPH-cyt *c* reduction is expressed in units of specific activity (relative to A_{450}) as in Figure 1. Fractions indicated by bars were combined to yield 40 ml of highly purified reductase having A_{450} = 0.053 and an A_{450}/A_{280} ratio of 0.14.

and moved very slowly.

After washing the hydroxylapatite column with 50 m*M* phosphate, the bright yellow band was eluted using a linear gradient of phosphate buffer (pH 7.4) from 50 to 250 m*M* concentration, 200-ml total volume, at a flow rate of 30 ml/hr. The fractions with the best A_{450}/A_{280} ratios were combined in dialysis tubing, concentrated to several milligrams per milliliter using Ficoll powder or by stirring with sucrose syrup (5 lb of sucrose dissolved with 1200 ml of water), and dialyzed overnight against 0.1 *M* Mops buffer (pH 7.4) with a buffer change.

Adrenodoxin. Adrenodoxin was purified from the brown band remaining on the DEAE-cellulose column after elution of the yellow AR band. The 65–85% ammonium sulfate fraction of the mitochondrial extract was also taken and, after dialysis, added to the column. After equilibration of the column with 0.1 *M* Tris buffer (pH 8.0), a linear NaCl gradient, from 0 to 0.3 *M* in 0.1 *M* Tris buffer, 1200 ml total volume, was passed through the column, at a flow rate of 100 ml/hr.

Fractions with A_{415}/A_{280} ratios greater than 0.4 were combined and further purified by fractionation with solid ammonium sulfate (buffered to pH 8.0 by including 0.57 g of Tris with each g of salt). First, 56.0 g of ammonium sulfate was added to each 100-ml adrenodoxin solution (giving approximately 85% saturation at 4°) and the resulting brown precipitate removed by centrifugation. The supernatant was then taken to about 95% saturation by adding an additional 10.1 g of ammonium sulfate per original 100 ml. The dark brown precipitate was sedimented and dissolved in 5 ml of 10 m*M* Tris (pH 8) and then applied to a 2.5 × 80 cm bed of Sephadex G-75 or G-100 equilibrated with 10 m*M* Tris (pH 8.0). The brown band was chromatographed with the same buffer and eluted as a single colored peak.

Adrenodoxin with an A_{415}/A_{280} ratio of 0.88–0.90 was prepared by this procedure, even from mitochondria which had been frozen at −20° for 1 year. The yield was about 35 mg of adrenodoxin, average ratio of 0.86, from 20 g of mitochondrial protein. It was stored at −20° in 0.5 *M* Tris (pH 8.0) (room temperature value), for months with no apparent loss in ratio or activity.

Results

The elution profiles from DEAE-cellulose and hydroxylapatite chromatography employed in the purification of adrenodoxin reductase are shown in Figures 1 and 2. The four parameters used to monitor the extent of purification have been included in these plots: A_{280}, A_{450}, the ratio A_{450}/A_{280}, and specific activity (relative to A_{450}) in the reduction of cytochrome *c* with NADPH as electron donor. A single region of activity was observed in each chromatography, including the intermediate Sephadex G-100 step. This activity paralleled the absorbance at 450 nm and the A_{450}/A_{280} ratio, except in the initial chromatography on DEAE-cellulose (Figure 1) where the broad NADPH-cyt *c* reduction peak was displaced from the others.

NADH-cytochrome *c* reduction, by contrast, was insignificant in all fractions eluted from DEAE-cellulose. This activity was present in mitochondrial extract prepared by centrifuging sonicated mitochondria at 150,000*g* for 1–2 hr but centrifugation for at least 12 hr reduced the NADH-cyt *c* reaction of the supernatant to background levels.

Fractions 68–77 from DEAE-cellulose chromatography were combined, concentrated with ammonium sulfate, and applied to the Sephadex G-100 column. Following elution of a smaller peak of protein, a much larger protein peak was eluted coincident with a single symmetrical yellow band. The peak fractions containing the greatest A_{450}/A_{280} ratio (0.12) and the greatest NADPH-cyt *c* reductase activity were pooled and applied to the hydroxylapatite column without further treatment.

The terminal chromatography, on hydroxylapatite (Figure 2), further increased the A_{450}/A_{280} ratio of the applied material and resulted in a virtually constant specific activity over the entire measurable region, suggesting that high purity was achieved with this step. Although the NADPH-cyt *c* specific activity relative to absorbance at 450 nm increased very little with chromatography on Sephadex and hydroxylapatite (indicating that contamination by other flavoproteins was slight), Table I demonstrates that purification was substantial on the basis of total protein. Net purification from mitochondrial extract was 19-fold; the final yield of adrenodoxin reductase was 13 mg/kg of adrenal

Table I: Purification of Adrenodoxin Reductase.

Purification Step	Total Protein (mg)	Total Activity (units[a] × 10^{-5})	Yield (%)	Specific Activity (units/mg)	Purification
Mitochondrial extract[b]	2600	23.4	100	901	1.0
35–65% saturation ammonium sulfate	1340	21.4	91	1600	1.8
DEAE-cellulose	51.4	3.70	16	7220	8.0
Sephadex G-100	23.4	2.71	12	11,500	13
Hydroxylapatite	9.6	1.64	7	17,000	19

[a] One activity unit is defined as the amount of enzyme catalyzing the reduction of 1 nmol of cytochrome *c*/min. [b] The starting material was 21 g of mitochondrial protein from 720 g of adrenal cortex.

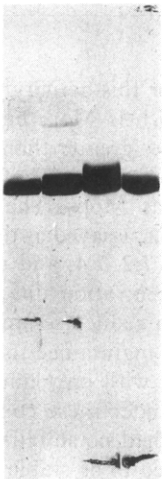

FIGURE 3: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of adrenodoxin reductase. Experimental conditions are described in the text. Gels 1 and 2 are 5 mm in diameter; 3 and 4, 6 mm (numbering is from left to right). The Bromophenol blue dye front is marked by India ink in each case. For gels 1 and 4, 2 and 3 μg of protein from the same reductase preparation were applied, respectively. Gels 2 and 3 (10 and 6 μg of protein, respectively) are from two other reductase preparations. The A_{450}/A_{270} ratio for the samples was 0.124–0.128.

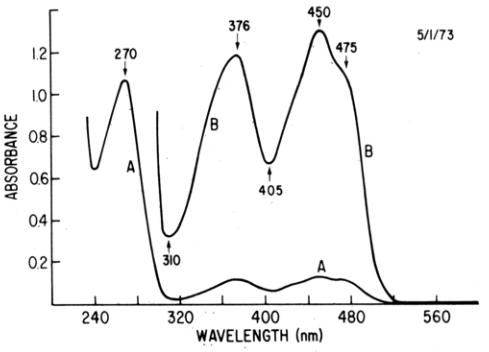

FIGURE 4: Absolute absorbance spectrum of adrenodoxin reductase. Spectra were recorded in cuvets with an optical path of 0.445 cm, utilizing both a standard 0–2 absorbance slidewire (A) and a 10× expanded scale (0–0.2) absorbance slidewire (B). The adrenodoxin reductase sample, in 0.2 *M* phosphate buffer (pH 7.4) exhibited an A_{450}/A_{270} ratio of 0.123.

cortex scrapings. The A_{450}/A_{280} ratio of the purified material of this preparation was 0.14 (A_{450}/A_{270} 0.126).

The purity of the combined peak fractions from hydroxylapatite chromatography was assessed by SDS polyacrylamide gel ectrophoresis and analytical ultracentrifugation. Figure 3 displays the gel patterns obtained with three different reductase preparations (different amounts of protein were loaded). In general, a single, intense band was obtained. Gel 2 exhibits the position and relative intensity of a contaminant which was sometimes present when lower quality fractions were carried along in the purification sequence.

Sedimentation of purified reductase in the analytical ultracentrifuge equipped with schlieren optics resulted in a single, symmetrical peak. The sedimentation coefficient $s_{20,w}$ was determined to be 3.85 S, independent of protein concentration (1–3 mg/ml). This is in agreement with the value of 3.93 S reported by Suhara et al. (1972b). Adrenodoxin reductase prepared according to the methods of this paper, possessing an A_{450}/A_{280} ratio of 0.14–0.15 (A_{450}/A_{270} = 0.124 or greater), therefore appears to be homogeneous according to these two criteria.

The molecular weight of homogeneous adrenodoxin reductase was estimated by the methods of SDS polyacrylamide gel electrophoresis and sedimentation equilibrium ultracentrifugation. The average value from 13 electrophoresis experiments was 51,500.

Using standard sedimentation equilibrium techniques and assuming a partial specific volume of 0.730, the molecular weight of the reductase by ultracentrifugation was computed to be 49,500 (average of 10 values). These values are similar to those obtained by Chu and Kimura (1973a) who reported a molecular weight of 54,000 using SDS polyacrylamide gel electrophoresis and gel filtration with Sephadex G-100 and to those obtained by Suhara et al. (1972b), who reported a value of 50,000 by SDS polyacrylamide gel electrophoresis and 54,000 with Sephadex G-100.

Adrenodoxin reductase in the oxidized form (Figure 4) displays a characteristic flavoprotein absorption spectrum, with maxima at 270, 376, and 450 nm and shoulders near 280, 425, and 475 nm. Absorption minima were present at 310 and 405 nm. This spectrum of the highly purified reductase is similar to those reported by Chu and Kumara (1973a) and Suhara et al. (1972b); the major difference is the depth of the trough at 310–315 nm, which is here one-half the absorbance (relative to A_{450}) shown in their spectra. The A_{450}/A_{270} ratio, an index of purity, varied from 0.122 to 0.128 in these preparations. The reciprocal values, 7.8–8.2, are significantly less than that reported by Chu and Kimura (A_{270}/A_{450} = 8.6) or Suhara et al. (A_{272}/A_{450} = 8.4).

The fluorescence excitation spectrum of adrenodoxin reductase is shown in Figure 5. This spectrum is similar to that of free flavine under the same experimental conditions, differing qualitatively in having two distinct maxima below

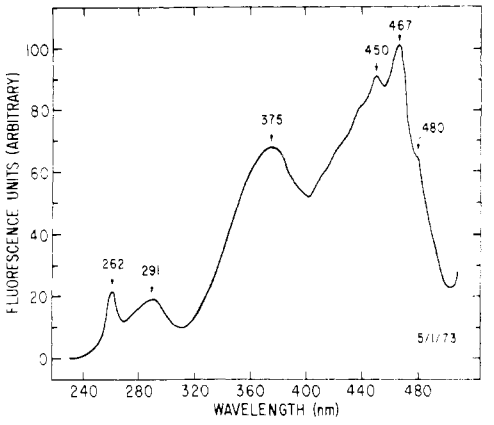

FIGURE 5: Fluorescence excitation spectrum of adrenodoxin reductase. Spectra were recorded monitoring fluorescence emission at 520 nm (8-nm slit) with an excitation slit width of 6 nm. Temperature, 25°; optical path, 1 cm. The sample of adrenodoxin reductase used exhibited an absorbance at 450 nm of 0.172 and an A_{450}/A_{270} ratio of 0.125. The buffer was 0.2 *M* phosphate (pH 7.4).

300 nm. As reported by Chu and Kimura (1973a), the fluorescence intensity of the FAD moiety bound to this flavoprotein was comparable to that of an equal concentration of free FAD.

The flavine released from adrenodoxin reductase by immersion in boiling water had the same mobility as standard FAD by thin-layer chromatography on both plastic sheets and glass plates, using either 0.7 *M* phosphate or 0.2 *M* acetate as the developing system. The mobility relative to authentic FAD varied from 0.95 to 1.00 in several determinations; chromatography of the flavine obtained from the reductase did not reveal any fluorescent components other than that corresponding to FAD. The reductase analyzed had an A_{450}/A_{270} ratio of 0.122 (A_{450} = 0.428).

Five of the six analyses by fluorimetry gave no detectable amount of FMN and the FMN detected in the sixth case amounted to 2.8% of the total flavine. The fluorescence of the flavine separated from boiled adrenodoxin reductase was in all cases typical of standard FAD. Six determinations gave an average value of 3.73×10^{-7} *M* (range of $3.56\text{–}3.86 \times 10^{-7}$ *M*) whereas the A_{450} value (assuming ϵ = 11.3 mM^{-1} cm^{-1} at 450 nm; Whitby, 1953) permits calculation of 3.78×10^{-7} *M*. Within experimental error, this result indicates that the flavoprotein absorbs light at 450 nm to the same extent as does free FAD.

Similarly, knowing the quantity of protein used in the analyses (19–78 μg) one can calculate that the protein mass associated with 1 mol of FAD is 52,000 g (range 50,200 to 54,000 g). Based on FAD content, Suhara et al. (1972b) determined the minimum molecular weight to be 55,400, whereas a value of 52,000 may be computed from the data reported by Chu and Kimura (1973a). A minimum molecular weight of 52,000 as estimated here by FAD content compares well with the absolute values obtained by ultracentrifugation (49,500) and by gel electrophoresis (51,500).

In agreement with Chu and Kimura (1973a), highly purified adrenodoxin reductase was found to contain 1.7% carbohydrate by weight, determined here as glucose; corrections were included for light absorption in the colorimetric assay due to sample protein, FAD content, and buffer.

When purified to homogeneity, adrenodoxin reductase was found to reduce cytochrome *c* with NADPH at a rate of 17,000 nmol min^{-1} mg of protein^{-}, corresponding to a turnover number of 850 min^{-1} (Table II). Adrenodoxin was

Table II: Electron Transfer Activity Exhibited by Adrenodoxin Reductase.

Acceptor	Specific Activity[a] Donor NADH	NADPH
DPIP	5,000	1,400
FeCN	27,000	87,000
Cyt *c*	630	17,000
Cyt P450	5	1,800

[a] nmoles acceptor reduced per minute per mg of adrenodoxin reductase. In the case of cyt P450 reduction, the cuvet contained mitochondrial fragments depleted of reductase prepared as described under Materials and Methods, equivalent to 2.1 nmol of cyt P450/ml. The A_{450}/A_{270} ratio of the reductase used here was 0.126.

absolutely essential for this activity. The reaction was routinely assayed using 0.1 *M* Mops buffer (pH 7.4) in which rates were several times greater than with 0.1 *M* phosphate (pH 7.4). Tris, Tricine, or Hepes buffer gave the same electron transport rates as Mops. The pH optimum for the NADPH–cyt *c* reaction assayed as described under Materials and Methods was 7.2–7.4, and using one-half or twice the usual Mops concentration did not affect the rate of reaction. Adrenodoxin reductase stored at −20° in 0.1 *M* Mops buffer (pH 7.4) maintained its original specific activity even after a year, with only minor loss of the holoenzyme. When stored under these conditions, 1 m*M* EDTA and mercaptoethanol had no stabilizing effect on AR. The reductase also appeared to be stable when stored in Tricine or Hepes buffer. Measurable loss of flavoprotein (resulting in precipitation of apoprotein) and specific activity was observed after storage for several days to weeks in 0.1 *M* phosphate or Tris buffer (pH 7.4) or in lower concentrations of Mops.

Other electrons transfer activities catalyzed by adrenodoxin reductase are also presented in Table II. It is interesting that although this reductase is physiologically a NADPH-specific protein, diaphorase activity with DPIP (but not FeCN) is several times greater with NADH than with NADPH.

The requirement for adrenodoxin reductase in cytochrome P450 reduction is illustrated in Figure 6, using adrenal cortex mitochondrial fragments depleted of reductase and adrenodoxin. The depleted fragment used here exhibited a very low background rate of cyt P450 reduction, which was enhanced several hundred-fold upon addition of reductase. The rate of electron transport from NADH to cytochrome P450 in the presence of adrenodoxin (saturating amounts) and reductase is barely measurable (trace A and Table II) and several hundred times less than obtained when NADPH is added (traces A and B). Trace B indicates that the reductase is virtually absent from the cyt P450 preparation used and both traces demonstrate the requirement for NADPH and for the reductase in order to obtain cyt P450 reduction.

The ability of adrenodoxin reductase (in the presence of NADPH and adrenodoxin) to reconstitute the cytochrome P450 catalyzed 11β-hydroxylation of deoxycorticosterone, and side-chain cleavage of 20α-hydroxycholesterol and cholesterol were also examined. No conversion of either of these substrates was obtained with the reductase-depleted mitochondrial fragments unless supplemented with this en-

zyme. When sufficient amounts of adrenodoxin and adrenodoxin reductase were used, substantial rates of mixed function oxidation were observed. Several studies gave rates (nmol substrate converted per min per nmol of cyt P450) of: 3-6 for 11β-hydroxylation of deoxycorticosterone, 4-9 for side-chain cleavage of 20α-hydroxycholesterol, and 9-23 for side-chain cleavage of cholesterol.

Discussion

The purification sequence reported here, including chromatography on DEAE-cellulose, Sephadex, and hydroxylapatite, is a simple and direct method for both adrenodoxin and its reductase in homogeneous form and in reasonable yield. From 1 kg of adrenal cortex, 13 mg of homogeneous reductase (A_{450}/A_{270} ratio = 0.126) and 50 mg of adrenodoxin at an average A_{415}/A_{280} ratio of 0.86 were obtained.

The chromatography sequence used, DEAE-cellulose followed by Sephadex (rather than the reverse order used by other workers), is partly responsible for the success of our method. This sequence was chosen because it is more convenient when the purification of adrenodoxin as well as the reductase is desired and because better yields and purity of the reductase are achieved. When the mitochondrial extract (after ammonium sulfate fractionation) was applied to Sephadex G-100 first, separate regions containing adrenodoxin, adrenodoxin reductase, and adrenodoxin plus its reductase could be eluted in 10 m*M* phosphate buffer. The extent of this separation apparently depends on the size of the sample applied relative to the dimensions of the gel bed and formation of a complex of adrenodoxin and reductase (Chu and Kimura, 1973b) and subsequent purification can become complicated. Our DEAE-cellulose elution pattern cannot be directly compared to those of Omura et al. (1966) or Chu and Kimura (1973a), because these authors employed an initial Sephadex chromatography, and monitored DPIP reduction rather than cyt *c* reduction.

The terminal chromatography, on hydroxylapatite, is a second and major improvement on the other methods for the purification of adrenodoxin reductase and has allowed us to prepare milligram quantities of homogeneous flavoprotein from only several hundred grams of adrenal cortex. Furthermore, the reductase is stable in 0.1 *M* Mops buffer (pH 7.4) (but not phosphate), so that large quantities may now be prepared and stored for at least 1 year with no appreciable loss in activity.

The properties of homogeneous adrenodoxin reductase obtained here are essentially in agreement with those reported by other workers with preparations appearing to be less pure. These include molecular weight, sedimentation coefficient, absorption and fluorescence spectra, flavine content, carbohydrate content, and pyridine nucleotide specificity. However, we obtain a millimolar extinction coefficient similar to that of free FAD (11.3), somewhat greater than the value of 10.9 found by Chu and Kimura (1973a), and a slightly lower molecular weight (49,500-52,000).

As reported by Chu and Kumura (1973a), we find NADP to be a rather potent inhibitor of NADPH-cytochrome *c* reduction catalyzed by adrenodoxin reductase (and adrenodoxin). Use of the NADPH-generating system resulted in linear early rates, permitting ready estimation of reaction velocities. We have not yet assessed the effect of NADP on reconstituted cyt P450 reductase or steroid hydroxylating activities. The rate of cytochrome *c* reduction

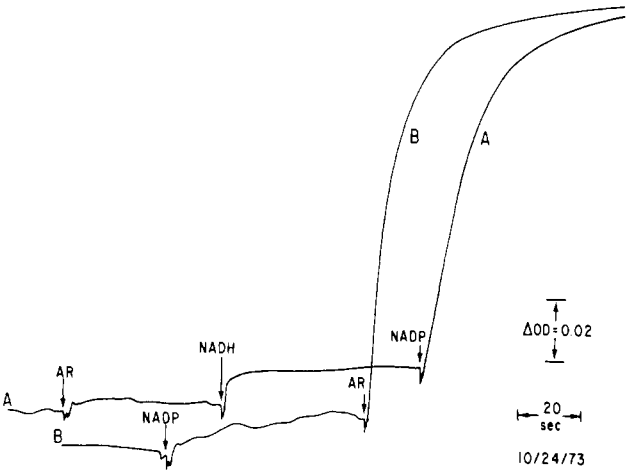

FIGURE 6: Requirement for adrenodoxin reductase and NADPH in cytochrome P450 reduction. Conditions were as described under Materials and Methods. Sonicated mitochondria equivalent to 6.2 nmol of cyt P450 and purified adrenodoxin, 1 nmol, were present in the 3.0-ml cuvet. When indicated, 0.13 μM adrenodoxin reductase and 20 μM NADP or NADH (trace A) were added to complete the reaction mixture. The reductase used in this experiment exhibited an A_{450}/A_{270} ratio of 0.126.

catalyzed by adrenodoxin reductase and adrenodoxin (Table II) is several fold greater than that reported by Suhara et al. (1972b) and is at least as rapid as may be calculated from the data of Chu and Kimura (1973a).

None of the reports describing the purification of adrenodoxin reductase (Suhara et al., 1972b; Chu and Kimura, 1973a,b; Katagiri and Takemori, 1973) have documented the reconstitution of cyt P450 reduction or steroid hydroxylation with the purified reductase. The requirement demonstrated here for adrenodoxin reductase in these activities confirms the earliest reports initially identifying this flavoprotein in adrenal cortex mitochondria (Nakamura et al., 1966; Omura et al., 1965, 1966; Kimura and Suzuki, 1965). The rate obtained here for 11β-hydroxylation in the reconstituted system is somewhat less than that reported using intact mitochondria (Harding et al., 1968; Cammer et al., 1968); the reason for this is unclear although it may be related to the use of suboptimal concentrations of adrenodoxin (Cooper et al., 1968; Huang and Kimura, 1971). The rates obtained for side-chain cleavage of 20α-hydroxycholesterol are similar to those in the literature (Wilson and Harding, 1973). In the case of cholesterol, the rates observed here are surprisingly high (Wilson and Harding, 1970; Shikita and Hall, 1973) since no effort was made to decrease the level of endogenous sterol.

Because homogeneous adrenodoxin reductase catalyzes both steroid 11β-hydroxylase activity and side-chain cleavage activity (using either cholesterol or 20α-hydroxycholesterol) at comparable rates, it is unlikely that this flavoprotein has any specific regulatory role in either of these cytochrome P450 dependent hydroxylation reactions.

Acknowledgments

We thank Mrs. Martie Smith for excellent technical assistance.

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A Pyrimidine Nucleoside Monophosphate Kinase from Rat Liver†

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ABSTRACT: A pyrimidine nucleoside monophosphate kinase has been purified 2100-fold from rat liver. With ATP and dATP as phosphate donors the kinase uses CMP, dCMP, and UMP as phosphate acceptors. Ara-CMP is also phosphorylated by the kinase. In contrast to dCMP and UMP, CMP can be phosphorylated by dCTP. CTP and ara-CTP cannot substitute for dCTP. The stringent specificity of the phosphate donor site for ATP and dATP is lost when CMP serves as acceptor. All nucleoside triphosphates act as donors to a significant extent. No evidence has been found to suggest more than one enzyme. All activities, to different degrees, are strictly dependent upon preincubation at 37° with a sulfhydryl reducing agent. Various reagents (85 m*M*) are ranked in order of increasing effectiveness of reactivation as follows: dithiothreitol > glutathione ≥ 2-mercaptoethanol > L-cysteine > DL-α-lipoic acid. A $NADP^+$-dependent thioredoxin (17 μ*M*)-thioredoxin reductase system from Novikoff ascites rat tumor was found to be the most powerful reducing agent tested. CTP, dCTP, UTP, and dTTP (1 m*M*) do not affect the kinase activity regardless of the phosphate acceptor.

Maley and Ochoa (1958) partially purified a nucleoside monophosphate kinase from *Azotobacter vinelandii* which phosphorylated CMP and dCMP. UMP was not tested as substrate. In *Escherichia coli* UMP kinase was found to be separable from CMP kinase. The latter enzyme could also phosphorylate dCMP (Hiraga and Sugino, 1966). However, in preparations from calf liver close association of CMP with UMP kinase activities occurred. dCMP was not tested (Strominger et al., 1959). Later, Sugino and coworkers reported a preparation purified from calf thymus which catalyzed phosphorylation of UMP, CMP, and dCMP (Sugino et al., 1966). In spite of the fact that dCMP was

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‡ Work done in partial fulfillment of the requirements for the Ph.D. degree at The University of Texas Graduate School of Biomedical Sciences at Houston and supported by a Rosalie B. Hite Predoctoral Fellowship.

§ Work supported by U.S. Public Health Service Grant CA-10407 and Research Grant G-460 from The Robert A. Welch Foundation.