Kidney and Adrenal Mitochondria Contain Two Forms of NADPH-adrenodoxin Reductase-dependent Iron-Sulfur Proteins

ISOLATION OF THE TWO PORCINE RENAL FERREDOXINS*

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Two NADPH-adrenodoxin reductase-dependent iron-sulfur proteins were detected in both porcine kidney and bovine adrenal mitochondria by using high resolution polyacrylamide electrophoresis. Adrenodoxin (M_r = 12,000) constituted the major ferredoxin activity in adrenal mitochondria and a similarly sized protein (M_r = 11,500) was isolated as the major renal ferredoxin activity. A second, higher molecular weight ferredoxin was observed in both adrenal (M_r = 13,300) and kidney (M_r = 13,000) mitochondria. The two renal ferredoxins were isolated by the use of ion exchange, gel exclusion, and preparative electrophoretic techniques. An absorption spectrum typical of [2Fe-2S] ferredoxins was obtained for each protein; however, the larger renal molecule had an unusually high 276 nm absorbance. Immunologic studies revealed a significant degree of antigenic commonality between the two renal proteins as well as specific cross-reactivity with two renal proteins. A possible precursor-product relationship between the paired renal and adrenal ferredoxins is discussed.

Iron-sulfur proteins are found ubiquitously in nature and typically function as electron-transferring molecules. Adrenodoxin is one of the most thoroughly characterized iron-sulfur proteins in vertebrate systems. It functions at the inner mitochondrial membrane to transfer electrons from NADPH-adrenodoxin reductase to cytochromes P-450 using adrenodoxins in pig kidney mitochondria. The major species had a molecular weight of 12,000 Da and its [2Fe-2S] chromophore imparts a reddish brown color to the protein (5-8). It has a unique optical spectrum with a low 276 nm absorbance and visible maxima at 414 nm and 454 nm (7). The class of proteins to which adrenodoxin belongs, i.e. the ferredoxins, contains molecules which have general similarities in structure and function, yet each appears to be species- and tissue-specific and functions most efficiently in vitro when reconstituted with its homologous NADPH-ferredoxin reductase and cytochrome P-450 (9, 10).

Due to the high homology between porcine and human proteins (11) and our interest in human diseases of vitamin D metabolism, we are using pig kidney to study the molecular regulation of cytochrome P-450s which hydroxylate 25-hydroxyvitamin D (9, 10, 12-14). Realizing the biological uniqueness of ferredoxins and the importance of using homologous components in our investigation of the pig renal cytochrome P-450s, we elected to isolate the iron-sulfur protein from pig kidney mitochondria.

This endeavor resulted in the discovery of two ferredoxins in pig kidney mitochondria. The major species had a molecular mass (11,500 Da) and spectral properties which were very similar to adrenodoxin, whereas the second ferredoxin molecule had a higher molecular mass (12,900 Da) and distinct spectral properties. This communication describes the isolation and initial characterization of these two proteins.

MATERIALS AND METHODS

Reagents and Biochemicals—Myoglobin (horse heart, type III), cytochrome c (horse heart, type III), goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate, p-nitrophenyl phosphate (as Sigma 104 phosphatase substrate tablets), and 2', 5'-ADP-agarose were obtained from Sigma. GelBond PAG film and AcrylAide Cross-Linker were purchased from FMC Corp., Marine Colloids Division. Other PAGE materials were electrophoresis purity obtained from Bio-Rad Laboratories. DEAE-Sephasel and Sephadex G-75 were obtained from Pharmacia Fine Chemicals. NUNC-Immuno Plate I microtiter plates were purchased from Southland Cryogenics. All chemicals were reagent grade or higher. Ultrafiltration was performed through Amicon Diaflo type YM10 membranes. Adrenodoxin and adrenodoxin reductase were prepared in this laboratory from bovine adrenal mitochondria. Adrenodoxin was purified through the G-75 step as described below. Adrenodoxin reductase was purified by the simplified method of Lambeth and Kamin (15) without the final Sephadex G-100 chromatography. Antiserum was raised in New Zealand white rabbits using Freund’s complete adjuvant. The primary inoculation (1 mg of protein) was made into the rear footpads. Four weeks later, a second injection (0.3 mg of protein) was made subcutaneously at several sites on the back. Serum was then collected 1 month after the booster.

Assay Procedures—Ferredoxin activity was measured by monitoring the reduction of cytochrome c at 550 nm under conditions described by Foster and Wilson (16). The transfer of electrons from NADPH to the iron-sulfur protein for the reduction of cytochrome c was absolutely dependent on the presence of NADPH-adrenodoxin reductase. ELISAs were performed as described by Spellman et al. (17). Protein concentrations were estimated by the method of Lowry et al. (18) using bovine serum albumin as the standard.

Analytical Electrophoresis—SDS-PAGE was performed as de-
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acrylamide ratio of 0.835. The SDS concentration in the tank buffer was reduced to 0.1%. Samples were boiled in application diluent for 5 min and applied without further manipulation. Gels were silver-stained by the method of Morrissey (20) with the following modifications. Fixation with 50% methanol, 10% acetic acid was followed directly by thorough rinsing in continuously flowing deionized water (1 h) prior to reduction with dithiothreitol (30 min). Reaction time in 0.1% silver nitrate was extended to 1 h.

Purification of Renal Ferredoxins—The iron-sulfur proteins were isolated as outlined below using the procedure of Kimura and Suzuki (21). All manipulations were performed at 4°C unless otherwise specified. The rich brown 100,000 x g supernatant obtained from sonicated porcine renal cortex mitochondria was applied to a column (2.6 X 10 cm) of DEAE-Sepharose. The column was washed extensively with buffer A (170 mM KCl, 10 mM KPi, pH 7.4, 1.5 mM mercaptoethanol) and the activity was eluted by a linear KCl gradient (buffer A to 350 mM KCl). Active fractions were concentrated by ultrafiltration and fractionated by molecular weight on a column (2.6 X 7.8 cm) of Sephadex G-75 equilibrated and run with buffer B (15 mM Tris/acetate, pH 8.0, 100 mM KCl, 1.5 mM mercaptoethanol). The concentrate of pooled active fractions was made 10% in sucrose (w/v) and spiked with bromphenol blue tracking dye. Cylindrical gels (20 ml of 18.7% separating gel and 2 ml of 4.4% stacking gel containing 10% sucrose) and running buffer were prepared as described under "Analytical Electrophoresis" except that SDS was omitted and catalyst concentrations were reduced by one-half. Following electrophoresis of the sample at 5 mA for 20 h, the cylinder in which the gel had been cast was removed from the electrophoresis apparatus and fractionated by molecular weight on a column (2.6 X 78 cm) of Sephadex G-75 equilibrated and run with buffer B (15 mM Tris/acetate, pH 8.0, 100 mM KCl, 1.5 mM mercaptoethanol). The concentrate of pooled active fractions was made 10% in sucrose (w/v) and spiked with bromphenol blue tracking dye. Cylindrical gels (20 ml of 18.7% separating gel and 2 ml of 4.4% stacking gel containing 10% sucrose) and running buffer were prepared as described under "Analytical Electrophoresis" except that SDS was omitted and catalyst concentrations were reduced by one-half. Following electrophoresis of the sample at 5 mA for 20 h, the cylinder in which the gel had been cast was removed from the electrophoresis apparatus and mounted (at room temperature) over a light box to enhance visualization of the two reddish brown bands. By carefully extruding short lengths of gel from the supporting tube, selected sections of the gel (dye front, visible bands, interband spaces) were cut cleanly with a razor blade. Individual slices were minced and eluted overnight at 4°C with constant agitation in 5-10 ml of buffer C (100 mM Tris/acetate, pH 8.0, 1.5 mM mercaptoethanol). The protein from each section, except the dye front, was concentrated by applying the gel eluate to a DEAE-Sepharose column (1 X 2.5 cm) equilibrated and run at room temperature with buffer C. The proteins were stripped from the column with buffer C 400 mM KCl and then stabilized in 500 mM Tris/acetate, pH 8.0. The gel elute from the dye front was diluted with buffer C, to lower the dye concentration, and reconstituted by ultrafiltration.

RESULTS

Pig kidney mitochondrial ferredoxin, obtained after DEAE-Sepharose and Sephadex G-75 chromatography, gave a single diffuse band when analyzed by 10–15% SDS-PAGE using Coomassie Blue staining (data not shown). However, this putatively homogeneous preparation gave an atypically high absorption at 276 nm, which prompted us to re-evaluate its purity. Realizing the ferredoxin's low molecular weight and poor affinity for Coomassie Blue staining, we selected a 20% SDS-PAGE system and the more sensitive silver stain to test the preparation's purity. When evaluated in this manner, three distinct bands (proteinase K-sensitive) were detected (Fig. 1, Rdx). Using the molecular weight order observed in the SDS-PAGE system, i.e. 13,000, 11,500, and 9,000, these proteins were respectively numbered 1, 2, and 3 (Fig. 1). Bovine adrenodoxin, prepared by the same method, gave a characteristic ferredoxin absorbance spectrum (data not shown). However, analogous to the kidney ferredoxin preparation, three distinct bands were also present in the adrenal material (Fig. 1, Adx). The adrenal preparation contained mainly adrenodoxin (M, 12,100) and two minor proteins with estimated molecular weights of 13,300 and 8,600 (Fig. 1). The minor protein bands were also detected in the renal ferredoxin material (Fig. 1, Rdx). The enrichment of these minor bands in the adenodoxin preparation was consistent with the adrenal content of the material. However, analogous to the kidney ferredoxin preparation, three distinct bands were also present in the adrenal material (Fig. 1, Adx). The adrenal preparation contained mainly adrenodoxin (M, 12,100) and two minor proteins with estimated molecular weights of 13,300 and 8,600 (Fig. 1).

Two reddish brown proteins which had ferredoxin activity could be detected for only protein-1 (Rdx-1) and protein-2 (Rdx-2). Protein-3 was totally inactive (Fig. 2B). Rdx-1 and Rdx-2 were excised from the preparative gel by taking narrow cuts, resulting in the incorporation of peripheral activity into the interband gel sections (Fig. 2B). Nearly 7 times more ferredoxin activity was contained in the smaller molecular weight Rdx-2 band than in the Rdx-1 band. However, it should be noted that although Rdx-1 constituted the major ferredoxin activity, it stained much more strongly in the silver-stained gel than in the Rdx-1 band. How-

FIG. 1. 20% SDS-polyacrylamide electrophoretic analysis of ferredoxin purification. The silver-stained gel presents bovine adrenodoxin (Adx) and pig renal ferredoxin (Rdx) activity peaks following DEAE-Sepharose and Sephadex G-75 chromatography. Isolated pig renal proteins 1, 2, and 3 were obtained by preparative electrophoresis as described in the text. Molecular weight markers were cytochrome c (12,400) and myoglobin (17,600).

FIG. 2. Preparative tube gel and corresponding areas of pig renal ferredoxin activity. A, schematic representation of nondenaturing PAGE of pig renal ferredoxin activity peak from the G-75 column. Two mg of protein was applied and electrophoresed for 20 h at 5 mA. Arrows denote migration pattern of the adrenal proteins. B, histogram depicting the gel sections and associated activity of pig renal ferredoxins in A. The numbering refers to protein bands described in Fig. 1.

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(Adx, Fig. 1) following analytical scale nondenaturing PAGE (see arrow notations in Fig. 2A). The ferredoxin activity was distributed between adrenodoxin (Adx-2), the major protein, and a minor higher molecular weight protein (Adx-1) which had a greater electrophoretic mobility.

The spectral properties of the two active kidney proteins were consistent with [2Fe-2S] iron-sulfur proteins, i.e., characteristic absorbance peaks at 414 nm and 454 nm (Fig. 3). A blunted 276 nm absorbance, typical of ferredoxin molecules, was obtained for the more abundant Rdx-2. In contrast, an abnormally high absorbance at 276 nm was recorded for the less abundant Rdx-1.

Antiserum raised against partially purified porcine renal ferredoxins (Rdx, Fig. 1) was positive by the ELISA technique for each of the three isolated renal proteins and also demonstrated cross-reactivity against the three-component adrenal preparation (Adx, Fig. 1) (data not shown). Using the heterologous antiserum in a competition ELISA (Fig. 4), it was possible to test the three isolated renal proteins for common antigenic determinants. With a constant amount of Rdx-2 adsorbed to the microtiter plate, liquid phase Rdx-1 and Rdx-2 reacted in an equivalent manner with the antiserum thereby inhibiting the binding of antibody to the solid phase antigen. Protein-3 and bovine serum albumin did not compete with adsorbed Rdx-2 for specific antibodies. Therefore, it is evident that Rdx-1 and Rdx-2 share common antigenic determinants.

DISCUSSION

The major pig kidney ferredoxin was very similar to formerly isolated bovine and avian kidney ferredoxins (9, 10). However, the previously undetected second ferredoxin protein was 1500 Da larger and had a greater number of acidic and aromatic amino acids. Yet, it was shown from a competition ELISA that the two ferredoxins have shared antigenic determinants. With a constant amount of Rdx-2 adsorbed to the microtiter plate, liquid phase Rdx-1 and Rdx-2 reacted in an equivalent manner with the antiserum thereby inhibiting the binding of antibody to the solid phase antigen. Protein-3 and bovine serum albumin did not compete with adsorbed Rdx-2 for specific antibodies. Therefore, it is evident that Rdx-1 and Rdx-2 share common antigenic determinants.

Using analytical scale techniques, we also detected two ferredoxin activities in bovine adrenal material prepared either by ourselves or kindly provided by Dr. David Lambeth (Emory University). Adrenodoxin constituted the major component, whereas the second activity was associated with a larger protein (M = 13,300). Minor contaminating higher molecular weight proteins have been reported in ferredoxin preparations from bovine adrenal and corpus luteum; however, they were not evaluated for ferredoxin activity (22). Our results clearly indicate that higher molecular weight ferredoxins exist in both kidney and adrenal which have gone undetected due, in part, to their low concentrations.

Although the larger and smaller molecular weight ferredoxins could arise as unique gene products or from differential RNA splicing (22), it is compelling to speculate that the larger molecular weight ferredoxins are unprocessed precursor forms of the smaller molecules. In the case of the renal proteins, the significant immunologic cross-reactivity supports this notion. We are currently conducting amino acid and gene sequencing studies to corroborate this hypothesis.

On the other hand, it is well documented that mitochondrial adrenodoxin, which is encoded by nuclear DNA, is synthesized as a larger precursor molecule (24, 25). Until recently, it had been assumed that the adrenodoxin precursor contained only a 7,000-Da N-terminal "signal" extension which was cleaved during mitochondrial incorporation to yield the mature 12,000- Da form. However, it was shown from a cDNA cloning study that adrenodoxin contains not only a 58-amino-acid N-terminal "signal" peptide but also a 14-amino-acid C-terminal extension (26). Although no one has documented the presence of a precursor adrenodoxin in adrenal mitochondria, the possibility that adrenodoxin could exist as a precursor molecule containing the small C-terminal extension is consistent with the documentation in this study of a higher molecular weight ferredoxin protein. The molecular weight difference between this larger protein and adrenodoxin is estimated by SDS-PAGE to be 1,300, which is in good agreement with the calculated molecular weight of 1,573 for adrenodoxin's C-terminal peptide (26). In addition, the calculated net charge of the C-terminal peptide (at pH 7) is approximately 6. Consequently, adrenodoxin with an intact C-terminal peptide would migrate faster in a nondenaturing PAGE system. Such a pattern was observed in our study of the larger adrenal

![Fig. 3. Absorption spectra of pig renal ferredoxins. Absorption spectra of Rdx-1 (---) and Rdx-2 (-- --) were recorded at room temperature in 1-cm quartz cuvettes on a Beckman DU-6 spectrophotometer. The inset shows the Rdx-1 spectrum at a 10-fold greater sensitivity.](image-url)
ferredoxin (Fig. 2A). Therefore, the results of this study are compatible with the hypothesis that the higher molecular weight ferredoxin in adrenal preparations could be a precursor form of adrenodoxin containing the C-terminal extension.

If the larger ferredoxins in kidney and adrenal are indeed precursors of the mature forms, then it becomes evident from the activity and spectral data that the iron atom is incorporated into these molecules after their passage through the outer mitochondrial membrane, but prior to C-terminal peptide cleavage. In fact, the apparent quantitative discrepancy between the two renal ferredoxins, observed when comparing the silver-stained protein with the recoverable ferredoxin activity (Figs. 1 and 2A, respectively) could be explained if there was a pool of unprocessed peptide which also lacked the iron component required for its characteristic chromophore and catalytic activity.

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REFERENCES