Purification and Characterization of the Ferredoxin Component of 25-Hydroxycholecalciferol 1 α -Hydroxylase

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The chick kidney mitochondrial iron-sulphur protein (ferredoxin), a component of the NADPH-cytochrome *P*-450 reductase functional in the 1 α -hydroxylation of 25-hydroxycholecalciferol, was purified to homogeneity by chromatography on DEAE-cellulose, gel filtration on Sephadex G-100 and preparative electrophoresis on polyacrylamide gel. A novel NADPH-cytochrome *c* reductase assay utilizing crude renal NADPH-ferredoxin reductase was used for the detection of the ferredoxin. A mol.wt. of 53 000 was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and by Sephadex G-100 gel filtration of the ¹²⁵I-labelled ferredoxin. The ferredoxin has a sedimentation constant (*s*_{20,w}) of 2.66S, an *A*₄₁₁/*A*₂₈₀ of 0.4, and a molar absorptivity of 7300 cm⁻¹ · M⁻¹. The electron-paramagnetic-resonance spectrum after reduction with Methyl Viologen and dithionite was characteristic of ferredoxins with signals at *g*=1.956 and 2.025. Two iron and two labile sulphur atoms per molecule of ferredoxin were released by acid. Ouchterlony immunodiffusion tests by using goat anti-(bovine adrenal ferredoxin) antiserum showed precipitin reactions with the bovine adrenal ferredoxin and the chick renal ferredoxin as antigens, suggesting that the renal ferredoxin shares antigenic determinant(s) with the natural adrenal antigen. Amino acid analysis showed that of the total number of residues per molecule of ferredoxin, glutamic acid and aspartic acid are the most abundant residues, comprising 17 and 15% respectively.

The sequential hydroxylations of cholecalciferol first by liver microsomal fraction (Bhattacharyya & DeLuca, 1974) and then by kidney mitochondria (Fraser & Kodicek, 1970; Gray *et al.*, 1972) produce the physiologically active dihydroxylated metabolite of cholecalciferol identified as 1,25(OH)₂D₃. This metabolite is regarded as the hormonal form of the vitamin responsible for calcium and phosphate homeostasis (Haussler & McCain, 1977).

In a series of studies *in vitro* utilizing chick kidney mitochondria, the requirement of NADPH and the involvement of cytochrome *P*-450 as a terminal oxidase in the production of 1,25(OH)₂D₃ were established (Ghazarian *et al.*, 1973, 1974; Ghazarian & DeLuca, 1974; Henry & Norman, 1974). The reduction of the cytochrome by NADPH was postulated to involve two intermediate oxidation-reduction components, namely an iron-sulphur protein (ferredoxin) and a flavoprotein (Ghazarian *et al.*, 1974). In such a system, the renal ferredoxin, in a manner characteristic of the hydroxylases of a number of steroidogenic tissues, mediates the transfer of electrons from an NADPH-specific ferredoxin reduc-

tase to cytochrome *P*-450. The electron-transfer sequence ultimately culminates with the oxidation of the substrate 25(OH)D₃ to 1,25(OH)₂D₃. An enzymically functional ferredoxin was described in partially purified mitochondrial extracts of chick kidney (Pedersen *et al.*, 1976). Although the preparation was functional in the production of 1,25(OH)₂D₃ *in vitro*, it was heterogeneous, and on gel electrophoresis two major and two minor components could be detected (Pedersen *et al.*, 1976).

The present paper reports the purification of the chick kidney ferredoxin to homogeneity, and describes the molecular, optical and magnetic properties of the purified protein.

Experimental

Materials

The following materials were obtained from the sources indicated: DEAE-cellulose 40 no. 72 from Schleicher and Schuell, Keene, NH, U.S.A.; Sephadex G-100 from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.; Tris, crystalline horse heart cytochrome *c* (type III), rotenone, Mops (4-morpholine propanesulphonic acid), standard iron solution in 0.8M-HNO₃ (50 mg/100 ml), *NN'*-dimethyl-*p*-phenyl-

Abbreviations used: 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; 25(OH)D₃, 25-hydroxycholecalciferol.

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enediamine and 2,6-dichlorophenol-indophenol from Sigma Chemical Co., St. Louis, MO, U.S.A.; NADPH from P-L Biochemicals, Milwaukee, WI, U.S.A.; fluorescamine {4-phenylspiro[furan-2(3*H*),-1'-phthalan]-3,3'-dione} from Fisher Scientific Co., Itasca, IL, U.S.A.; Methyl Viologen hydrate (1,1'-dimethyl-4,4'-bipyridinium dichloride) from Aldrich, Milwaukee, WI, U.S.A.; ICI from Matheson, Coleman and Bell, Cincinnati, OH, U.S.A.; and Na¹²⁵I (sp. radioactivity 17Ci/mg) from New England Nuclear Corp., Boston, MA, U.S.A. Bovine adrenocortical ferredoxin (adrenodoxin) was purified up to and including the batch DEAE-cellulose step as previously reported (Orme-Johnson & Beinert, 1969). The preparation was dialysed for 16h against 0.05M-Tris/HCl, pH7.5, and stored at liquid-N₂ temperatures. The crude flavoprotein NADPH-ferredoxin reductase was obtained as the peak-I fraction (Pedersen *et al.*, 1976) of the DEAE-cellulose step during the purification of the renal ferredoxin. The NADPH-2,6-dichlorophenol-indophenol reductase activity of the flavoprotein was 1018nmol of 2,6-dichlorophenol-indophenol reduced/min per ml. The reductase activity was measured in 10mM-potassium phosphate, pH7.4, containing 10μM-2,6-dichlorophenol-indophenol (Savage, 1957). A millimolar absorptivity of 19cm⁻¹·mm⁻¹ at 600nm was used for the calculations.

Goat anti-(bovine adrenal ferredoxin) antisera

Both the non-immune and the immune goat antibodies were purified immunoglobulin fractions suspended in 0.1M-Tris/HCl, pH8.3, containing 50mM-NaCl and 0.02% (w/v) NaN₃ (Baron, 1976). The fractions were generous gifts from Dr. Jeffrey Baron.

Animals and preparation of mitochondria

White Leghorn cockerel chicks 1 day old were obtained from Northern Hatcheries, Beaver Dam, WI, U.S.A., and maintained for 5 weeks on a vitamin D-deficient rachitogenic test diet (U.S. Biochemicals, Cleveland, OH, U.S.A.) with free access to water until killed by cervical dislocation. Kidney mitochondria were prepared as previously described (Ghazarian & DeLuca, 1974).

NADPH-cytochrome c (cytochrome P-450) reductase assay

A novel approach was used in this study for the spectrophotometric measurement of NADPH-cytochrome *c* reductase activities. The measurements were made at room temperature (22°C) by following the reduction of cytochrome *c* at 550nm. A millimolar absorptivity of 19.6cm⁻¹·mm⁻¹ was used for reduced minus oxidized cytochrome (Jansson & Schenkman, 1977). The crude NADPH-ferredoxin reductase, NADPH and all the cofactors described

below were first mixed, then divided equally between reference and sample cuvettes. The reaction was initiated by adding to the sample cuvette a portion (50–100μl) of a fraction from column effluents. The reaction mixtures, in a final volume of 1 ml, contained 10mM-potassium phosphate, pH7.4, 6μM-cytochrome *c*, 50μM-NADPH, 25μl of the crude flavoprotein preparation, 5μM-rotenone from a 1mM ethanolic solution, and freshly prepared 0.6mM-NaCN. In this system all interfering cytochrome *c* reductase activities attributable either to impurities in the crude flavoprotein preparation or to fragments of mitochondrial respiratory electron-transport chain are eliminated. As a result, only two previously described NADPH-cytochrome *c* reductase activities are measured simultaneously. One is the peak-II activity (Pedersen *et al.*, 1976), which does not require either a flavoprotein or a ferredoxin-type protein for its activity, and the other is the renal ferredoxin-dependent activity, which also requires the flavoprotein. The significance of this assay system is that (a) it eliminates the need for the purification of the labile flavoprotein to remove contaminating NADPH-cytochrome *c* reductase activities, and (b) it eliminates the need to assay repetitively the same sequence of collected column-effluent fractions to distinguish between the fractions containing the two separate NADPH-cytochrome reductase activities, as was used earlier (Pedersen *et al.*, 1976).

Radioiodination

The renal ferredoxin was labelled by using the ICI method described by Kunimoto *et al.* (1972).

Iron-sulphur content

Non-haem iron was determined as the ferrous *o*-phenanthroline complex (Kimura & Suzuki, 1967). The colour formed was read at 510nm against reagent control. A millimolar absorptivity of 11.1cm⁻¹·mm⁻¹ was used. Labile sulphide was measured by the method of Fogo & Popowsky (1949) as modified by Kimura & Suzuki (1967).

Analytical electrophoresis

Polyacrylamide-gel electrophoresis in the absence of sodium dodecyl sulphate was carried out as described by Davis (1962). Electrophoresis in the presence of 1% sodium dodecyl sulphate and 100mM-dithiothreitol was carried out as described by Fairbanks *et al.* (1971). This method was also used to calibrate gels with reference proteins for the determination of the molecular weight of the renal ferredoxin. Gels were stained for protein with 0.025% Coomassie Brilliant Blue R.

Other methods

Ouchterlony double-immunodiffusion experiments, preparative polyacrylamide-gel electrophor-

esis, amino acid analysis and analytical-ultracentrifugation experiments were all carried out as previously described in detail (Ghazarian *et al.*, 1978a). E.p.r. measurements were performed on a Varian E-9 spectrometer as previously reported (Ghazarian *et al.*, 1978b). Protein determinations were made by the fluorescamine method (Böhlen *et al.*, 1973), except for the preparative-electrophoresis experiments, where the method of Lowry *et al.* (1951) was used. An Aminco model SPF-125 fluorimeter, adjusted for maximum slit widths and sensitivity, was used. All spectrophotometric measurements were carried out at room temperature with an Aminco DW-2 spectrophotometer. Gamma radiation was monitored in a Beckman GM-100 counter equipped with a data computer.

Purification of the ferredoxin

In the initial stages of the purification, the procedures followed were essentially those described in detail by Pedersen *et al.* (1976). Freshly prepared mitochondria representing a total of 14 g of mitochondrial protein were used. Briefly, the procedure consisted of (a) twice freezing and thawing the mitochondrial pellet, (b) osmotic shocking in 1.5 vol. of ice-cold water, (c) sonication at 0°C, (d) centrifugation at 105000g for 60 min and application of the supernatant to a DEAE-cellulose column (2.5 cm × 28 cm) equilibrated with 50 mM-KCl/10 mM-Mops, pH 7.4, (e) washing the column with 200 ml of 100 mM-KCl/10 mM-Mops, pH 7.4, and (f) linear gradient elution with 1000 ml of 10 mM-Mops, pH 7.4, from 100 to 500 mM-KCl. The fractions (10 ml each) containing the ferredoxin-dependent NADPH-cytochrome *c* reductase activities were concentrated in an Amicon cell fitted with a UM-2 membrane. The concentrated solution was applied to a Sephadex G-100 column (2.5 cm × 60 cm) equilibrated with 100 mM-KCl/10 mM-Mops, pH 7.4. The fractions (5 ml each) containing the ferredoxin-dependent reductase activities were concentrated as above. At this point, new procedures were added for the purification of the ferredoxin to homogeneity. The repetition of the Sephadex G-100 chromatography step used by Pedersen *et al.* (1976) was omitted in the

present study. Instead, the concentrated material was diluted with approx. 3 vol. of ice-cold water (to lower the KCl concentration) and again concentrated as above to 1.3 ml. A few milligrams of powdered sucrose were dissolved in the preparation and then the mixture was subjected to preparative polyacrylamide-gel electrophoresis in a Canalco apparatus by using a current of 8 mA (200–400 V). The stacking gel was 1 cm thick, and the separator gel was 5% in acrylamide and 4.5 cm thick. The elution buffer was 10 mM-Mops, pH 7.4; 3 ml fractions were collected at a flow rate of 36 ml/h. The fractions containing the ferredoxin (80–100; see Fig. 1) were combined, concentrated and used in all subsequent experiments.

Results

Table 1 summarizes the purification of the chick kidney ferredoxin. The total yield was 6.4 mg and represents approximately a 286-fold purification relative to the mitochondrial extract.

The purified ferredoxin showed a single homogeneous protein band when examined by analytical polyacrylamide-gel electrophoresis either in non-denaturing conditions (Fig. 2a) or in the presence of dithiothreitol and sodium dodecyl sulphate (Fig. 2b). Further evidence of homogeneity of the isolated ferredoxin was obtained from sedimentation-velocity studies. The ferredoxin, at a concentration of 3.3 mg/ml in 10 mM-Mops, pH 7.4, was centrifuged for a total of 150 min after the rotor had reached the maximum speed of 60000 rev./min at 5°C. From the plot of the rate of sedimentation as a function of time, a sedimentation constant ($s_{20,w}$) of 2.66S was calculated.

The molecular weight of the ferredoxin was estimated to be 53000 by its relative mobilities in two independent systems. The mobility of the ferredoxin in sodium dodecyl sulphate/polyacrylamide gel relative to reference proteins, a linear function of the log of molecular weight (Steck, 1974) was 0.51, compared with 0.9 for ribonuclease (mol.wt. 13700), 0.63 for glyceraldehyde 3-phosphate dehydrogenase (mol.wt. 35000), and 0.56 for ovalbumin (mol.wt. 45000). A similar molecular weight for the ferredoxin

Table 1. Summary of purification of chick renal ferredoxin

One unit of ferredoxin is the amount of material that catalyses the reduction of 1 nmol of cytochrome *c*/min as described under 'NADPH-cytochrome *c* (cytochrome P-450) reductase assay'.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Sonicated mitochondria	14919	36625	2.4	100	
Mitochondrial extract	14322	34794	2.4	95	1
DEAE-cellulose	257	18313	71.3	50	30
Sephadex G-100	32.7	12819	392	35	163
Electrophoresis	6.4	4395	687	12	286

Fig. 1. Preparative polyacrylamide-gel-electrophoresis profile obtained by subjecting to electrophoresis the renal ferredoxin band eluted from Sephadex G-100 chromatography (Pedersen *et al.*, 1976)

Approx. 33 mg of protein was applied to a 5% acrylamide column with a cross-sectional area of 320 mm². Electrophoresis was performed at 4°C at a current of 8 mA. Symbols: ○, protein; ●, cytochrome *c* reduction. Elution begins at the anode. The bar below the peak on the right indicates the fractions (80–100) that were collected and concentrated as described in the text. Each fraction contained 3 ml.

Fig. 2. Analytical gel electrophoresis of the purified renal ferredoxin under (a) non-denaturing conditions and (b) in the presence of 1% sodium dodecyl sulphate and 100 mM-dithiothreitol

The amount of protein applied was 26 µg for gel (a) and 13 µg for gel (b).

Fig. 3. Ouchterlony immunodiffusion patterns

The centre well of pattern (b) contained 1 mg of immunoglobulin G protein obtained from fractionated goat anti-(bovine adrenodoxin) antisera. The outer wells contained 8.7 µg of renal ferredoxin (upper left), 17.4 µg of renal ferredoxin (upper right), 3.6 µg of adrenodoxin (lower left), and 1.5 µg of adrenodoxin (lower right). The centre well of pattern (a) contained 988 µg of immunoglobulin G protein from non-immune goat sera. The outer wells contained the amount of antigens in the same order as used for pattern (b).

was also estimated by Sephadex G-100 gel filtration of the ¹²⁵I-labelled ferredoxin. A value of 0.21 for the elution constant (K_{av}), a linear function of the log of molecular weight (Andrews, 1964), was calculated for the radioactively labelled ferredoxin. The corresponding K_{av} values for the reference proteins were 0.38 and 0.24 for cytochrome *c* (mol.wt. 12000) and ovalbumin respectively.

The absolute spectrum of the purified oxidized ferredoxin is similar to that reported previously (Pedersen *et al.*, 1976), with a peak at 411 nm and a broad shoulder around 460 nm. The ratio A_{411}/A_{280} is 0.4, a 4-fold improvement over the earlier value of 0.11. An apparent molar absorptivity of $7.3 \times 10^3 \text{ cm}^{-1} \cdot \text{M}^{-1}$ at 411 nm was determined, based on a mol.wt. of 53000.

To characterize the isolated ferredoxin further, double-immunodiffusion experiments were carried out by using goat anti-(bovine adrenodoxin) antisera. As shown in Fig. 3 a definite cross-reactivity with the renal ferredoxin is clearly apparent, suggesting immunochemical similarity between bovine adrenal and chick renal ferredoxins. These results confirm the previous cross-reactivities demonstrated with the impure renal ferredoxin by using immunized rabbit antisera.

When the non-haem iron content of the ferredoxin was determined with *o*-phenanthroline, the ratio of iron to protein [measured by the Lowry *et al.* (1951) method] was 34 ng-atoms of iron/mg of protein.

Fig. 4. E.p.r. spectrum of the purified renal ferredoxin at a concentration of $32\mu\text{M}$ in 10mM -Mops, pH 7.4

The sample in a total volume of 0.5ml was reduced with Methyl Viologen and sodium dithionite at a final concentration of 6 and $4\mu\text{M}$ respectively. The conditions of spectroscopy were: microwave frequency, 9GHz ; microwave power, 5mW ; modulation frequency, 100kHz ; amplitude, 0.1mT ; temperature, 77K ; time constant, 0.25s ; scanning rate, 10mT/min . The numbers on the spectrum are the measured positions of the absorption maxima on the g -value scale calculated on the basis of an internal field marker with $g=2.003$.

indicate the apparent presence of equimolar amounts of iron and sulphide in the renal ferredoxin.

The e.p.r. spectrum of the Methyl Viologen-dithionite-reduced ferredoxin (Fig. 4) was characteristic of iron-sulphur proteins of the ferredoxin type. The g values of 1.956 and 2.025 were calculated on the basis of an internal Varian field marker with $g=2.003$.

The results of amino acid analysis of the renal ferredoxin are shown in Table 2. Because of the limited availability of the ferredoxin, the analysis represents uncorrected values based on a single hydrolysate. Glutamic acid and aspartic acid appear to be the most abundant residues in the protein. The ratio of the acidic to the basic residues, $(\text{Glu} + \text{Asp})/(\text{Lys} + \text{Arg})$, is 2.8 , a value in general agreement with those reported for a variety of iron-sulphur proteins (Kimura, 1969). The high content of the acidic residues in the renal ferredoxin is consistent with its high rate of anodic mobility on electrophoresis (see Fig. 2), and with the requirement of high salt concentrations for the elution in ion-exchange chromatography. In general, these properties are analogous to those described for the bovine adrenodoxin.

Table 2. Amino acid composition of chick renal ferredoxin

Residue	Estimated no. of residues/molecule
Trp	4
Lys	35
His	7
Arg	14
Asp	68
Thr	26
Ser	30
Glu	70
Pro	31
Gly	76
Ala	41
$\frac{1}{2}\text{Cys}$	3
Val	24
Met	8
Ile	18
Leu	25
Tyr	9
Phe	13
Total	502

Discussion

There is now ample evidence to support the concept that the kidney mitochondrial $25(\text{OH})\text{D}_3$ 1α -hydroxylase is a mixed-function oxidase composed of three electron-transfer proteins. The first line of evidence, found in 1974, indicated the involvement of the haem protein cytochrome $P-450$ as the terminal oxygen-activating component of the oxidase system (Ghazarian *et al.*, 1974; Henry & Norman, 1974). This was followed in 1976 by the isolation of a partially purified mitochondrial iron-sulphur protein (ferredoxin) and the demonstration that it was a functional component of the 1α -hydroxylase system (Pedersen *et al.*, 1976). The third component, a primary flavin dehydrogenase, was also shown to be essential for $1,25(\text{OH})_2\text{D}_3$ production *in vitro* (Ghazarian *et al.*, 1974). Hence the pathway of electron transport associated with the conversion of $25(\text{OH})\text{D}_3$ into $1,25(\text{OH})_2\text{D}_3$, in a manner analogous to the hydroxylases of steroidogenic tissues, may be depicted as follows:

The properties of the partially purified renal ferredoxin reported previously indicated certain similarities to those of the bovine adrenodoxin (Pedersen *et al.*, 1976). The similarities, which included electron paramagnetism and immunochemical cross-reactivity, have been confirmed in the present

From this result a minimum mol.wt. of 29400 could be calculated. Therefore, assuming a mol.wt. of 53000 , there are two iron atoms per molecule of the ferredoxin. The ferredoxin also contains sulphide, which is released on acidification. The ratio of sulphide to protein was estimated to be 40ng-atoms/mg of protein. The calculated minimum molecular weight based on sulphide is 25000 . These results

study. Moreover, our finding that the renal ferredoxin contains two iron and two labile sulphur atoms per molecule provides added support to the functional similarities between the two proteins.

Calculations based on the relative yield of the purified renal ferredoxin (Table 1) revealed that this ferredoxin represents approx. 0.35% of the protein of chick kidney mitochondria, compared with 2% for adrenodoxin in adrenal-gland mitochondria. This value approximates to 0.07 nmol of renal ferredoxin/mg of mitochondrial protein. In isolated chick kidney mitochondria, the cytochrome *P*-450 is present at a concentration of about 0.1 nmol/mg of protein. Hence the two components of the 1 α -hydroxylase appear to exist in a stoichiometric ratio of 1:1. This estimated molar equivalence of cytochrome *P*-450 and renal ferredoxin suggests a close intramitochondrial association of these electron-transfer components. The need for detergents and organic solvents for the solubilization of the cytochrome from mitochondria have been taken as evidence for its localization in the mitochondrial inner membrane. This, therefore, would lead to the likely conclusion that the renal ferredoxin is not uniformly distributed within the mitochondrial matrix, but must exist in a spatial proximity to the inner-membrane-bound cytochrome *P*-450.

The calculated A_{411}/A_{280} ratio of 0.4 for the homogeneous renal ferredoxin is distinctly different from that of 0.8 for the bovine adrenodoxin. This may reflect marked differences in their contents of aromatic amino acid residues. Although the tyrosine and phenylalanine contents of the renal ferredoxin are comparatively low relative to the other residues, tryptophan is present at 4 mol/mol of the ferredoxin. This is in distinct contrast with adrenodoxin, with no reported tryptophan residues (Kimura, 1969; Tanaka *et al.*, 1973).

In conclusion, we note several similarities and differences between chick renal ferredoxin and bovine adrenodoxin: (a) their biochemical and catalytic functions are analogous and are partly interchangeable (Ghazarian *et al.*, 1974); (b) although they demonstrate immunochemical cross-reactivity, the two antigens are structurally dissimilar; (c) their aromatic amino acid contents are different, whereas their ratios of acidic to basic residues are comparable.

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