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# PURIFICATION OF NADPH-FERREDOXIN REDUCTASE FROM RAT LIVER MITOCHONDRIA

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## Summary

NADPH-ferredoxin reductase (NADPH:ferredoxin oxidoreductase, EC 1.6.7.1) has been identified in rat liver mitochondria and purified to homogeneity as judged by sodium dodecyl sulfate (SDS) gel electrophoresis.

The protein was detected by its ability to reconstitute NADPH-cytochrome *c* reductase in the presence of adrenal ferredoxin.

The purified protein had properties very similar to adrenal NADPH-ferredoxin reductase. The molecular weight was 52 000, as estimated by gel filtration. On SDS-polyacrylamide gels, mobility was identical to that of adrenal NADPH-ferredoxin reductase ($M_r$ = 52 000). The enzyme exhibited a typical oxidized flavoprotein absorbance spectrum with maxima at 269, 377 and 450 nm and gave an absorbance ratio $A_{450nm}/A_{269nm}$ of 0.138.

The fluorescence excitation spectrum was identical to that of FAD. In the presence of NADPH and a ferredoxin, the reductase was found to be active in a reconstituted cytochrome *P*-450-dependent steroid 26-hydroxylase, which was recently isolated from rat liver mitochondria (Pedersen, J.I. (1978) FEBS Lett. 85, 35—39).

## Introduction

A cytochrome *P*-450, functioning as a steroid hydroxylase, has recently been identified and solubilized from rat liver mitochondria [1]. The catalytic activity of the enzyme depends on a ferredoxin (an iron-sulphur protein) and NADPH-ferredoxin reductase (NADPH:ferredoxin oxidoreductase, EC 1.6.7.1) that mediate electrons from NADPH to the heme protein [1]. A ferredoxin has already been isolated and partly purified from bovine liver mitochondria, and

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; MOPS, morpholinopropane sulphonic acid; SDS, sodium dodecyl sulfate.

shown to be active in a reconstituted steroid hydroxylation system [2]. The present communication describes the isolation and purification of NADPH-ferredoxin reductase from rat liver mitochondria. It is shown that the enzyme (a flavoprotein) has properties very similar to NADPH-ferredoxin reductase from adrenal cortex mitochondria. This communication demonstrates that the reductase is active in a reconstituted cholesterol 26-hydroxylation system composed of soluble rat liver mitochondrial cytochrome *P*-450, ferredoxin and NADPH.

## Methods

*Materials.* DEAE-cellulose, type 40 was from Schleicher and Schuell, Inc. Keene, U.S.A. CNBr-activated Sepharose-4B and Sephadex G-100 were from Pharmacia Fine Chemicals, Uppsala, Sweden. [1$\alpha$,2$\alpha$,($n$)-$^3$H]Cholesterol (specific activity 43 Ci/mmol) from The Radiochemical Centre, Amersham, England, was purified before use by thin-layer chromatography [3].

Bovine adrenal ferredoxin (adrenodoxin) was prepared essentially as described by Orme-Johnson and Beinert [4] except that the last electrophoretic step was replaced by gel filtration on Sephadex G-100. The ratio of absorbance, $A_{414\,nm}/A_{280nm}$ of the preparation was 0.79 and it was homogeneous as judged by polyacrylamide gel electrophoresis both in the absence and presence of SDS. No NADPH-cytochrome *c* reductase activity was detected in the absence of adrenodoxin reductase, which demonstrates that the preparation was free of NADPH-ferredoxin reductase.

Bovine adrenal NADPH-ferredoxin reductase was partly purified as described previously [2]. This material was further purified by adrenodoxin-Sepharose 4B affinity chromatography [5]. The resulting preparation was homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 2). The ratio $A_{450nm}/A_{270nm}$ was 0.128 and the specific adrenodoxin-dependent NADPH-cytochrome *c* reductase activity was 12 500 nmol · mg protein$^{-1}$ · min$^{-1}$.

Cytochrome *P*-450 was solubilized from rat liver mitochondria as described previously [2]. The specific content of the preparation was 0.14 nmol · mg protein$^{-1}$. The preparation contained some NADPH-ferredoxin reductase activity corresponding to 1.3 nmol cytochrome *c* reduced · mg protein$^{-1}$ · min$^{-1}$ by NADPH in the presence of excess adrenodoxin.

Other chemicals and biochemicals were standard commercial high purity materials.

*Preparation of rat liver mitochondria.* Rat livers were rinsed, minced and homogenized in 4 vols. (w/v) ice-cold 0.25 M sucrose/15 mM HEPES buffer (pH 7.4)/0.5 mM EDTA. The homogenate was centrifuged at 770 $\times g$ (at $R_{max}$) for 10 min in the Sorvall RC2B centrifuge (HS 4 rotor, 4°C). The precipitate was resuspended once and the centrifugation repeated. The combined supernatants were centrifuged at 9400 $\times g$ for 30 min. The mitochondrial pellets were resuspended and the suspension was centrifuged at 650 $\times g$ for 10 min in the HB 4 rotor. This treatment removed most of the contaminating erythrocytes. The supernatant solutions were centrifuged at 16 000 $\times g$ for 10 min in the HB 4 rotor. The mitochondrial pellets were resuspended four times more and finally frozen and stored in liquid $N_2$ until further processed.

*Purification of NADPH-ferredoxin reductase from rat liver mitochondria.*

Mitochondrial pellets (corresponding to 250 g liver tissue) were thawed and suspended in an equal volume of ice-cold distilled water. The suspension was left on ice for 20 min and subsequently diluted with 2 vols. buffer to a final concentration of 10 mM MOPS (pH 7.4) and 50 mM KCl (62 mg/ml protein). The suspension was sonicated three times for 30 s in a Branson sonifier at maximum output.

The sonicated mitochondria were centrifuged at 100 000 $\times g$ for 1 h in a Beckman L2-65K ultracentrifuge (30 rotor). The pellet was resuspended in approx. 1/3 the original volume of 50 mM KCl/10 mM MOPS buffer, resonicated for 30 s and recentrifuged as above. To the combined supernatant solutions was added 1/10 vol. packed DEAE-cellulose equilibrated with 10 mM MOPS buffer (pH 7.4). The suspension was stirred on ice for 2 h and centrifuged at 27 000 $\times g$ for 20 min in the SS 34 rotor of the Sorvall centrifuge.

The supernatant containing the NADPH-ferredoxin reductase was subjected to ammonium sulphate fractionation using a saturated solution, pH adjusted to 7.4 with Tris base. The protein precipitating between 35 and 65% saturation was collected by centrifugation (27 000 $\times g$ for 15 min, SS 34 rotor). The pellet was dissolved in 0.3 M MOPS buffer (pH 7.4)/20% glycerol to a total volume of approx. 20 ml. This solution was applied to a Sephadex G-100 column, 2.5 cm $\times$ 100 cm, equilibrated in 0.3 M MOPS buffer (pH 7.4)/20% glycerol/0.5 mM dithiothreitol. The protein was eluted with the same buffer at a flow rate of 20 ml/h.

The fractions containing the highest specific adrenodoxin-dependent NADPH-cytochrome *c* reductase activity were collected. NaCl was added to a final concentration of 0.04 M and the preparation was further purified by affinity chromatography. 1 g CNBr-activated Sepharose-4B was coupled to adrenodoxin essentially as described by Sugiyama and Yamano [5]. The 2.6 ml prepared gel was equilibrated in 0.3 M MOPS buffer (pH 7.4)/20% glycerol/0.04 M NaCl.

The protein solution was applied to the gel and washed with MOPS/glycerol/NaCl buffer until protein could not be detected (by absorbance at 280 nm) in the eluate. The enzyme activity was subsequently eluted as a sharp peak with 0.5 M NaCl/0.3 M MOPS (pH 7.4)/20% glycerol. The peak fraction was used for the characterization of the enzyme without further treatment. The preparation was stored in liquid $N_2$.

*Analytical procedures.* Ferredoxin-dependent NADPH-cytochrome *c* reductase was measured following the reduction of cytochrome *c* at 550 nm on a Shimadzu MPS-50L recording spectrophotometer. The sample and reference cuvettes contained the following in 0.6 ml 0.1 M MOPS buffer (pH 7.4): 0.6 $\mu$mol glucose 6-phosphate, 0.1 unit glucose-6-phosphate dehydrogenase, 10 nmol NADP, 0.25 $\mu$mol KCN, 0.25 $\mu$mol cytochrome *c* and a suitable amount of enzyme. The reaction was started by the addition of 20 pmol adrenodoxin. An extinction coefficient of 20.5 $mM^{-1} \cdot cm^{-1}$ for the reduced minus the oxidized form of cytochrome *c* was used for calculation [6].

Optical spectra were recorded at room temperature on a Cary 118 recording spectrophotometer. The fluorescence spectra were recorded on an Aminco SPF 500 spectrofluorimeter.

Cholesterol 26-hydroxylase activity by reconstituted soluble enzyme compo-

nents was assayed as described previously [1] except that the buffer was 25 mM MOPS (pH 7.4) and the amount of NADP was 100 nmol. The amounts of enzymes are given in Table II. The incubations were extracted and chromatographed as described [1].

Protein was determined by the Lowry [7] or by the Coomassie Blue [8] method using bovine serum albumin as standard, When the Coomassie Blue method was tested with the homogeneous preparation of adrenal ferredoxin reductase, good agreement was found between estimated and determined amount of protein. An extinction coefficient of 11.3 $mM^{-1} \cdot cm^{-1}$ was used for the determination of the molar concentration of flavoprotein based on absorbance at 450 nm [9].

The molecular weight of the liver NADPH-ferredoxin reductase was estimated by gel filtration on Sephadex G-100 [10].

Electrophoresis was performed in 7.5% polyacrylamide gels according to Weber et al. [11]. Samples were incubated at 37°C for 2 h in 0.1% SDS/0.1% β-mercaptoethanol in 10 mM sodium phosphate (pH 7.2) and applied to 6-mm diameter gels (prerun for 2 h at 4 mA/gel). The electrode buffer was 0.1 M sodium phosphate (pH 7.2), 0.1% SDS. The buffer was recirculated between the two chambers during the electrophoresis (cold room, 8 mA/gel, approx. 4 h). Protein was stained with Coomassie Blue [12].

## Results

Detection of the NADPH-ferredoxin reductase during the purification procedure was based on the ability of the enzyme to reconstitute a NADPH-cytochrome *c* reductase activity in the presence of added adrenal ferredoxin. In the sonicated preparation of mitochondria, a NADPH-cytochrome *c* reductase activity could be detected in the absence of added ferredoxin. Since it could not be decided whether this was solely due to the presence of a ferredoxin in the sonicate or also to the presence of an additional, different species of NADPH-cytochrome *c* reductase no value for total enzyme activity is quoted for the starting material in Table I. After treatment of the mitochondrial

TABLE I

PURIFICATION OF NADPH-FERREDOXIN REDUCTASE FROM RAT LIVER MITOCHONDRIA

| | Protein (mg) | Total activity (units) * | Specific activity (units/mg) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| Sonicated mitochondria | 6127 | | | | |
| Mitochondrial extract | 3536 | | | | |
| DEAE-cellulose-treated extract | 2750 | 11 277 | 4.1 | 1 | 100 |
| Ammonium sulphate fractionation | 1735 | 9 369 | 5.4 | 1.3 | 88 |
| Sephadex G-100 | 242 | 5 829 | 24.1 | 5.9 | 52 |
| Adrenodoxin-Sepharose 4B affinity chromatography (peak fraction) | 0.11 | 1 018 | 9255 | 2257 | 9 |

* NADPH-cytochrome *c* reductase activity measured as described under Methods. One activity unit is defined as the amount of enzyme catalyzing the reduction of 1 nmol of cytochrome *c* per min under the given experimental conditions.

extract with DEAE-cellulose and removal of the ferredoxin, NADPH-cytochrome *c* reductase activity became totally dependent on added ferredoxin. The evaluation of the purification is therefore based on the total enzyme activity at this stage of the procedure.

The Sephadex G-100 step removed a considerable amount of protein although the purification was not extensive.

As is evident from Table I the important step of the purification is the affinity chromatography. In fact, most of the protein from the Sephadex G-100 chromatography passed unabsorbed through the adrenodoxin-Sepharose-4B column (Fig. 1). By increasing the ionic strength of the buffer with 0.5 M NaCl the NADPH-ferredoxin activity was eluted as a sharp peak. The specific activity in the peak fraction was 9255 nmol of cytochrome *c* reduced · mg protein$^{-1}$ · min$^{-1}$, and the total purification was more than 2000-fold with a yield of 9%.

The material in the peak fraction was highly pure and only one band was seen on SDS-polyacrylamide gel electrophoresis (Fig. 2). In several runs the mobility was found to be identical to that of adrenal NADPH-ferredoxin reductase which indicates that the two proteins had similar molecular weights. The molecular weight as determined by gel filtration on Sephadex G-100 was found to be of the order of 52 000.

In the oxidized form the NADPH-ferredoxin reductase displayed a characteristic flavoprotein absorption spectrum with peaks at 269, 377 and 450 nm, and shoulders at 420 and 475 nm (Fig. 3). The spectrum is identical to that reported for the adrenal ferredoxin reductase [13—15]. The ratio of the absorbance at 450 nm to that at 269 nm, an index of purity, was 0.138.

The fluorescence excitation spectrum (Fig. 4) was similar to that reported for the adrenal NADPH-ferredoxin reductase [15]. Qualitatively it was identical to that of a solution of pure FAD in water, but the intensity was on a molar basis slightly lower than that of FAD. This indicates that there cannot be more than 1 mol FAD per mol of the protein.

The enzyme was found to be active in the reconstitution of a cytochrome

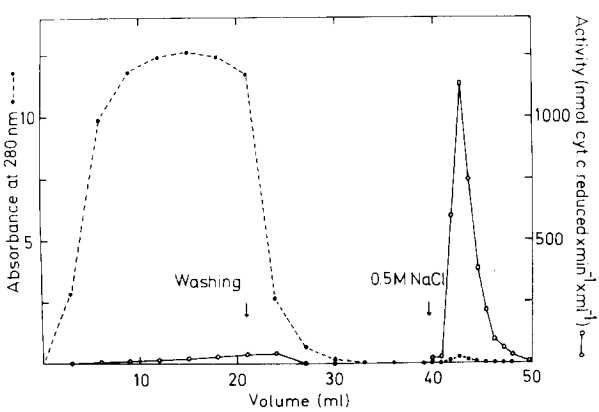


**Fig. 1. Affinity chromatography of NADPH-ferredoxin reductase from rat liver mitochondria on adrenodoxin-Sepharose-4B column. The experimental procedure is given under Methods. 3-ml fractions were collected up to the start of the elution after which 0.9-ml fractions were collected. The enzyme activity represents the ferredoxin-dependent NADPH-cytochrome *c* reductase.**

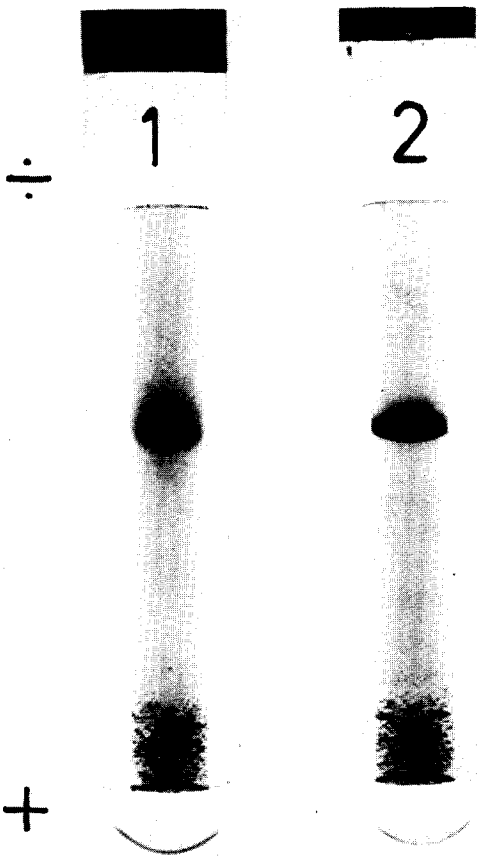


Fig. 2. SDS-polyacrylamide gel electrophoresis of NADPH-ferredoxin reductase from rat liver mitochondria (gel 1). The electrophoretic pattern under the same experimental conditions of bovine adrenal NADPH-ferredoxin reductase is shown for comparison (gel 2). 6 and 5.5 $\mu$g protein was applied to gel 1 and gel 2, respectively. The tracking dye is marked by wires. (The opacities at the bottom of the gels represent some unremoved SDS.)

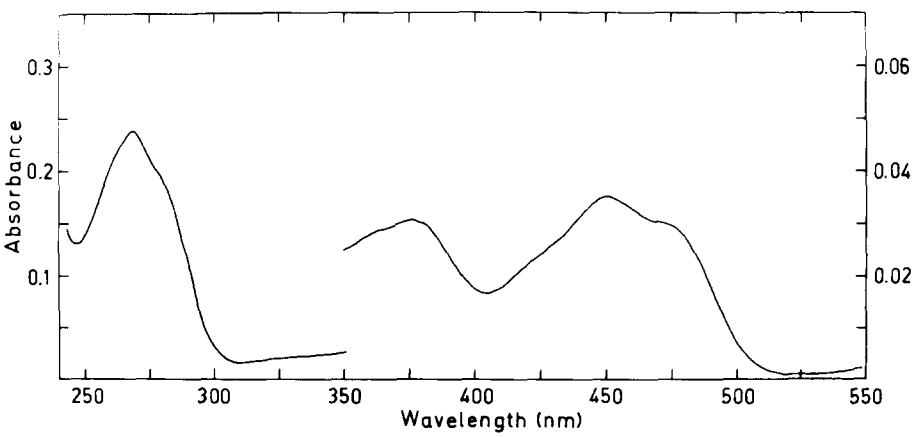


Fig. 3. Absorption spectrum of oxidized NADPH-ferredoxin reductase from rat liver mitochondria. The spectrum is that of the peak fraction from affinity chromatography. The visible part of the spectrum is expanded five times compared to the ultraviolet region.

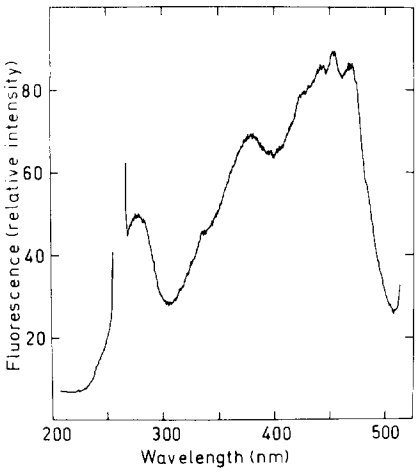


Fig. 4. Fluorescence excitation spectrum of NADPH-ferredoxin reductase from rat liver mitochondria. The spectrum was recorded at room temperature monitoring fluorescence emission at 520 nm (2 nm slit) with an excitation slit width of 2 nm.

*P*-450-catalyzed steroid 26-hydroxylation reaction. The system was composed of cytochrome *P*-450 solubilized from rat liver mitochondria, adrenodoxin and NADPH. Increasing amounts of NADPH-ferredoxin reductase added to this system increase the conversion of cholesterol to 26-hydroxycholesterol (Table II). That some conversion takes place even in the absence of added ferredoxin reductase is explained by the presence of some reductase in the cytochrome *P*-450 preparation (see Methods).

We have previously shown that liver mitochondrial ferredoxin reductase can be replaced by the corresponding adrenal reductase in this system [2], and also that the liver and adrenal ferredoxins are equally active [2].

TABLE II

RECONSTITUTION OF RAT LIVER MITOCHONDRIAL STEROID 26-HYDROXYLATION FROM SOLUBLE ENZYME COMPONENTS

For incubation conditions see Methods.

| Components present in the incubation medium | Incubation number | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Rat liver mitochondrial cytochrome *P*-450 (nmol) | 0.11 | 0.11 | 0.11 | 0.11 | 0.11 |
| Bovine adrenal ferredoxin (nmol) | — | 1.2 | 1.2 | 1.2 | 1.2 |
| Rat liver mitochondrial NADPH-ferredoxin reductase (pmol) | — | — | 1.5 | 15.5 | 77.5 |
| Product formation * (percent) | 0.5 | 7.0 | 8.4 | 9.9 | 11.7 |

* Identified to be mainly 26-hydroxycholesterol but with a small (<10%) amount of 25-hydroxycholesterol (to be published).

## Discussion

It is now well established that liver mitochondria contain a three component cytochrome *P*-450-dependent hydroxylation system similar to the one present in adrenal mitochondria [1,2,16]. The characteristic feature of this system is that the reduction of the cytochrome by NADPH requires the participation of a ferredoxin (an iron-sulphur protein) and a NADPH-ferredoxin reductase (a flavoprotein) [1,16]. One main physiological function of the liver mitochondrial cytochrome *P*-450 is certainly the hydroxylation of sterols in the formation of bile acids [17,18].

We have previously reported on the isolation and solubilization from rat liver mitochondria of a cytochrome *P*-450 [1] and on the isolation and purification of a ferredoxin from bovine liver mitochondria [2]. The present work conclusively demonstrates that liver mitochondria also contain a NADPH-ferredoxin reductase that takes part in the steroid hydroxylation reaction.

This enzyme, a flavoprotein, has been purified to homogeneity as based on SDS-polyacrylamide gel electrophoresis. The data presented show that it has properties very similar to those reported for the adrenal NADPH-ferredoxin reductase. Thus, the index of purity, $A_{450nm}/A_{270nm}$ of 0.138 is in the same range or even higher than that reported for homogeneous bovine adrenal NADPH-ferredoxin reductase (0.116—0.132) [5,13—15]. The specific ferredoxin-dependent NADPH-cytochrome *c* reductase activities are approximately the same when tested under the same experimental conditions (12 500 nmol cytochrome *c* reduced · mg protein$^{-1}$ · min$^{-1}$ for the adrenal, versus 9255 for the liver enzyme).

Also the molecular weight of the enzyme is very similar to that of the adrenal reductase. A value of 52 000 was obtained by gel filtration. A value of 54 000 has been reported for the adrenal ferredoxin reductase using the same method [14]. On SDS-polyacrylamide gel electrophoresis the two proteins showed identical migration distances. Values of 50 000—54 000 have been reported for the adrenal enzyme when determined by SDS gel electrophoresis [5,13—15] and 49 500 when determined by sedimentation equilibrium ultracentrifugation [15].

The fluorescence excitation spectrum strongly indicates the presence of FAD in the enzyme, as has been found for the adrenal enzyme [5,13—15]. The exact determination of the flavine content and a more detailed study of the structural and catalytic properties of the enzyme was precluded by the extremely small quantity of enzyme purified.

The function of this enzyme is, as demonstrated in the reconstitution experiments, to serve as an electron mediator between NADPH and ferredoxin in the reduction of cytochrome *P*-450. The electron transport chain of the liver mitochrondrial steroid hydroxylation system is thus similar to that of the steroid hydroxylation system in the adrenal cortex mitochondria [19,20]. In the adrenal cortex mitochondria the hydroxylation system is located to the inner membrane [21]. Most likely the same is true for the liver mitochondrial steroid hydroxylase.

Finally it should be mentioned that the presence of a ferredoxin-dependent NADPH cytochrome *c* reductase activity in liver mitochondria represents a

confirmation of the previous findings of NADPH-cytochrome *c* reductase in these mitochondria [21]. Whether an additional and different species of NADPH-cytochrome *c* reductase like the one that has been reported to be present in chick kidney mitochondria [22] is also present in liver mitochondria has yet to be ascertained.

After the submission of this paper the isolation of a similar enzyme from rabbit liver mitochondria has been published [23]. The specific ferredoxin-dependent NADPH-cytochrome *c* reductase activity of that preparation (637 nmol $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$) is considerably lower than that reported here, but the general conclusions are in agreement.

## Acknowledgements

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