Purification and characterization of the NADPH–cytochrome *P*-450 (cytochrome *c*) reductase from higher-plant microsomal fraction

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NADPH–cytochrome *P*-450 (cytochrome *c*) reductase (EC 1.6.2.4) was solubilized by detergent from microsomal fraction of wounded Jerusalem-artichoke (*Helianthus tuberosus* L.) tubers and purified to electrophoretic homogeneity. The purification was achieved by two anion-exchange columns and by affinity chromatography on 2',5'-bisphosphoadenosine–Sephrose 4B. An M_r value of 82000 was obtained by SDS/polyacrylamide-gel electrophoresis. The purified enzyme exhibited typical flavoprotein redox spectra and contained equimolar quantities of FAD and FMN. The purified enzyme followed Michaelis–Menten kinetics with K_m values of 20 μM for NADPH and 6.3 μM for cytochrome *c*. In contrast, with NADH as substrate this enzyme exhibited biphasic kinetics with K_m values ranging from 46 μM to 54 mM. Substrate saturation curves as a function of NADPH at fixed concentration of cytochrome *c* are compatible with a sequential type of substrate-addition mechanism. The enzyme was able to reconstitute cinnamate 4-hydroxylase activity when associated with partially purified tuber cytochrome *P*-450 and dilauroyl phosphatidylcholine in the presence of NADPH. Rabbit antibodies directed against plant NADPH–cytochrome *c* reductase affected only weakly NADH-sustained reduction of cytochrome *c*, but inhibited strongly NADPH–cytochrome *c* reductase and NADPH- or NADH-dependent cinnamate hydroxylase activities from Jerusalem-artichoke microsomal fraction.

INTRODUCTION

NADPH–cytochrome *P*-450 (cytochrome *c*) reductase plays a central role in the oxidoreduction processes taking place in animal microsomal fractions (referred to below simply as microsomes). Following two-electron reduction by NADPH, the FAD–FMN bis-flavoprotein (Iyanagi & Mason, 1973) sequentially transfers the 2 reducing equivalents to suitable acceptors, mainly complexed transition metals, e.g. haemoproteins, iron chelates etc. These may in turn react with molecular O_2 or H_2O_2 , resulting in cytochrome *P*-450-dependent oxidations, cytochrome b_5 -dependent fatty acid desaturation and lipid peroxidation.

The occurrence of cytochrome *P*-450 in higher-plant microsomes has been firmly established during the last decade. The haemoprotein is implicated in important biosynthetic pathways: the production of lignins and flavonoids (Benveniste & Durst, 1974; Potts *et al.*, 1974; Hagmann *et al.*, 1983; Grand, 1984), hydroxy fatty acids (Soliday & Kolattukudy, 1977; Salaun *et al.*, 1978), cutins and suberins (Kolattukudy, 1980), terpenic hormones (Hasson & West, 1976), alkaloids (Madyastha *et al.*, 1976) and phytoalexins (Fujita *et al.*, 1982). Cytochrome *P*-450 is also involved in the metabolism of lipophilic exogenous compounds by plant tissues (Frear *et al.*, 1969; Young & Beevers, 1976).

The presence in plant microsomes of a functional equivalent of the NADPH–cytochrome *P*-450 reductase from animals is clear from a number of considerations. The plant cytochrome *P*-450 enzymes are absolutely

dependent upon reducing equivalents, NADPH being preferred over NADH and ascorbate being ineffective (Soliday & Kolattukudy, 1976; Benveniste *et al.*, 1977). The inhibition of NADPH–cytochrome *c* reductase activity by selective inhibitors produces concomitant inhibition of microsomal cytochrome *P*-450-dependent plant mono-oxygenases (Benveniste *et al.*, 1977; Salaun *et al.*, 1978). Madyastha & Coscia (1979) purified from *Catharanthus roseus* a NADPH–cytochrome *c* reductase resembling the mammalian microsomal enzyme.

The present paper describes the purification and properties of a NADPH–cytochrome *c* reductase from wound-induced Jerusalem-artichoke tuber microsomes. The involvement of this enzyme in the reduction of cytochrome *P*-450 by NADPH (or NADH) is clearly established by immunological studies and by the ability of the reductase to reconstitute mono-oxygenase activities, in the presence of partially purified cytochrome *P*-450. Despite the functional analogies, the plant enzyme differs from the animal reductase in terms of M_r , chromatographic behaviour, lability of the flavin prosthetic groups and the effect of salt concentration on the catalytic turnover number.

MATERIALS AND METHODS

Plant material

Jerusalem-artichoke (*Helianthus tuberosus* L., var. blanc commun) tubers, grown locally, were stored in polyethylene bags at 4 °C in darkness.

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Tuber slices (1 mm thick) were rinsed first with tap water for 30 min to wash off polyphenols released by wounding, and then with distilled water before they were incubated for 46 h in the dark at room temperature (600–700 g of tissue in 6 litres of distilled water). A stream of filtered water-saturated air was bubbled through the incubation flask.

Preparation of microsomes

Microsomal pellets were prepared by homogenizing the aged slices at 4 °C, in a Moulinex mixer, in 100 mM-sodium phosphate buffer, pH 7.4, containing 25 mM-2-mercaptoethanol, 1 mM-EDTA and 250 mM-sucrose, in a ratio of 1.3:1 (v/w). Phenylmethanesulphonyl fluoride, a proteinase inhibitor, was added before the grinding to a final concentration of 1 mM. The homogenate was filtered through cheesecloth and centrifuged at 10000 *g* for 20 min. Microsomes were sedimented by centrifugation at 100000 *g* for 60 min, or aggregated with 25 mM-MgCl₂ for 30 min and sedimented at 18000 *g* for 1 h. The resulting pellets were washed with 100 mM-sodium pyrophosphate buffer, pH 7.4, containing 1 mM-EDTA, 10 mM-2-mercaptoethanol and 0.5 mM-phenylmethanesulphonyl fluoride and resedimented by a 100000 *g* centrifugation for 45 min. The washed microsomes were resuspended in 10 mM-sodium phosphate buffer, pH 7.8, containing 1.5 mM-2-mercaptoethanol and 20% (v/v) glycerol, homogenized in a Potter-Elvehjem homogenizer and stored at –20 °C. The final protein concentration was 4–7 mg/ml.

Solubilization and purification of NADPH-cytochrome *c* reductase

The frozen microsomes were thawed in presence of 0.5 mM-phenylmethanesulphonyl fluoride. A 20% (w/v) sodium cholate (or Emulgen 911) solution was rapidly added to the microsomal suspension to obtain a detergent/microsomal protein ratio of 2:1 and the suspension was gently stirred at 4 °C for 30 or 45 min. The solubilized microsomes were diluted 2-fold with 10 mM-sodium phosphate buffer, pH 7.8, containing 1.5 mM-2-mercaptoethanol and 20% (v/v) glycerol, before centrifugation at 100000 *g* for 90 min.

Chromatography on DEAE-Trisacryl M. The clear supernatant was loaded on a DEAE-Trisacryl column (2.6 cm × 20 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA, 1.5 mM-2-mercaptoethanol, 20% (v/v) glycerol and 0.2% (w/v) Emulgen 911 (buffer A). The column was washed with 2–3 vol. of buffer A. The reductase was eluted by a linear 50–300 mM-KCl gradient in buffer A (460 ml). The enzyme fractions were pooled and directly applied to the 2',5'-bisphosphoadenosine-Sepharose 4B column.

Chromatography on 2,5-bisphosphoadenosine-Sepharose 4B. The column (1.3 cm × 15 cm) was equilibrated with buffer A. After loading of the reductase, the column was washed with 175 ml of 200 mM-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA, 1.5 mM-2-mercaptoethanol, 20% (v/v) glycerol and 0.2% (w/v) Emulgen 911, followed by buffer A. The reductase was then eluted with buffer A containing 1 mM-2'-AMP.

Second chromatography on DEAE-Trisacryl M. This small column (3 cm × 3 cm), equilibrated with 10 mM-potassium phosphate buffer, pH 7.8, containing 0.1 mM-

EDTA, 1.5 mM-2-mercaptoethanol, 20% (v/v) glycerol and 0.6% (w/v) sodium cholate (buffer B), was used to replace Emulgen 911 by sodium cholate. The reductase fractions were loaded on the gel, which was washed with buffer B until the absorption at 280 nm (due to Emulgen) became lower than 0.03. The flavoprotein was then eluted with a linear 0–300 mM-KCl gradient (200 ml) in buffer B.

The concentration of sodium cholate of the active reductase was decreased to 0.05% by ultrafiltration through an Amicon YM30 membrane and dilution with 10 mM-potassium phosphate buffer, pH 7.8, containing 1.5 mM-2-mercaptoethanol and 20% (v/v) glycerol. The purified NADPH-cytochrome *c* reductase was frozen at –20 °C.

The rat liver NADPH-cytochrome *c* reductase used to compare the mammalian and plant enzymes was prepared by the same procedures, starting from hepatic microsomes from non-induced rats.

Solubilization and partial purification of cytochrome *P*-450

Microsomes from MnCl₂-induced Jerusalem-artichoke tubers (Reichhart *et al.*, 1980) were solubilized with CHAPS {3-[(3-cholamidopropyl)dimethylammonio] propanesulphonic acid} (2 mg of detergent/mg of microsomal protein). The solubilized material was applied to a DEAE-Trisacryl column equilibrated with 20 mM-Tris/HCl buffer, pH 7.8, containing 0.1 mM-EDTA, 1.5 mM-2-mercaptoethanol and 20% (v/v) glycerol (buffer C) and 3.25 mM-CHAPS. After development of the column with a linear 50–500 mM-KCl gradient in buffer C, the major peak of cytochrome *P*-450 was eluted with buffer C containing 100 mM-KCl and 0.2% (w/v) Emulgen 911. The peak fractions were pooled, dialysed against buffer C and applied to a DEAE-Trisacryl column equilibrated with buffer C containing 0.2% (w/v) Emulgen 911. Cytochrome *P*-450 was then eluted with a linear 50–150 mM-KCl gradient in the same buffer. The cytochrome-containing fractions were pooled, dialysed, concentrated on sucrose and stored at –20 °C.

Analytical methods

Polyacrylamide-slab-gel electrophoresis was performed in the presence of 0.1% SDS, with a 7.5–15% acrylamide gradient for the separating gel and 5% acrylamide for the stacking gel. The protein samples, in 100 mM-Tris/HCl buffer, pH 6.8, containing 1.25% (w/v) SDS, 1.25% (v/v) 2-mercaptoethanol, 15% (v/v) glycerol and 0.05% Bromophenol Blue, were boiled for 3 min before being loaded on the gel. Electrophoresis was carried out at room temperature. Proteins were detected by silver nitrate staining (Morrissey, 1981).

Specific FMN and FAD contents of the reductase were determined by the microfluorimetric method of Faeder & Siegel (1973). Fluorimetric analyses were conducted with a thermostatically controlled Jobin-Yvon spectrofluorimeter.

Protein was determined by the method of Lowry *et al.* (1951), as modified by Schacterle & Pollack (1973). When non-ionic detergent was present in the sample, protein was determined in the presence of SDS, as described by Dulley & Grieve (1975). Bovine serum albumin was used as a standard.

Kinetic analyses were performed by using the iterative non-linear least-square methods developed by Cleland

(1979). The programs were written in FORTRAN and run on a DEC PDP 11/03 computer.

'Reconstitution' of NADPH-cytochrome *c* reductase

The purified plant reductase was incubated for 2 h at 4 °C in the dark, with 10 μ M-FMN or -FAD. The mixture was then dialysed against 10 mM-sodium phosphate buffer, pH 7.8, containing 1.5 mM-2-mercaptoethanol, 20% (v/v) glycerol and 0.05% sodium cholate.

Preparation of antibodies

Rabbit antibodies to Jerusalem-artichoke NADPH-cytochrome *P*-450 reductase were prepared. Approx. 400 μ g of reductase was mixed with Freund's incomplete adjuvant and administered by intramuscular injection. After two injections, at a 1-month interval, blood samples were collected from the ear vein and serum was prepared. The presence of antibodies directed against the plant reductase was revealed by enzyme-linked immunosorbent assay tests.

The effect of rabbit anti-reductase immune serum was tested on the activity of purified reductase and on microsomal mono-oxygenation and electron-transfer reactions. Purified reductase or microsomes were incubated for 15 min at room temperature with an identical volume of rabbit anti-(plant reductase) serum. In the control, serum was replaced by an identical volume of 100 mM-sodium phosphate buffer, pH 7.4. Control experiments showed that serum from non-immunized rabbit did not inhibit to any extent the microsomal NADPH-cytochrome *c* reductase activity.

Enzyme assays

NADPH- or NADH-dependent reduction of cytochrome *c* was monitored by the increase of absorbance at 550 nm, at 25 °C, in the presence of 100 mM-sodium phosphate buffer, pH 7.4, and 0.05 mM-cytochrome *c*. The volume of the reaction mixture was 1 ml. A molar absorption coefficient of 21 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ for horse heart cytochrome *c* was used. One unit of enzyme activity is that which reduces 1 μ mol of cytochrome *c*/min.

NADPH- or NADH-dependent reduction of $\text{K}_3\text{Fe}(\text{CN})_6$ (1 mM) was measured at 25 °C, in the presence of 100 mM-sodium phosphate buffer, pH 7.4, by the decrease of absorbance at 420 nm. The molar

absorption coefficient of $\text{K}_3\text{Fe}(\text{CN})_6$ at 420 nm is 1.02 $\text{mm}^{-1}\cdot\text{cm}^{-1}$.

The concentration of solubilized cytochrome *b*₅ was measured from the difference spectra of samples reduced with $\text{Na}_2\text{S}_2\text{O}_4$ ($A_{424} - A_{490}$) by using the molar absorption coefficient 171 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ (Omura & Takesue, 1970).

Cytochrome *P*-450 concentration was calculated from the $A_{450} - A_{490}$ in a dithionite-reduced CO-cytochrome difference spectrum by using a molar absorption coefficient of 91 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ (Omura & Sato, 1964).

Cinnamate hydroxylase and laurate in-chain hydroxylase activities were measured radiochemically, as described by Benveniste *et al.* (1977) and Salaun *et al.* (1978) respectively.

RESULTS

Purification of NADPH-cytochrome *c* reductase

Table 1 summarizes a typical purification of the detergent-solubilized NADPH-cytochrome *c* reductase from Jerusalem-artichoke tuber microsomes. Sodium cholate (2 mg/mg of microsomal protein) released 90% of the reductase and 50% of the total protein from the membrane. A more effective solubilization was achieved by the non-ionic detergent Emulgen 911 (2 mg/mg of protein), since the activity of the solubilized reductase exceeded that of the membrane-bound enzyme (detailed results not shown).

Chromatography on a DEAE-Trisacryl M column of the supernatant of the solubilized microsomes achieved a 4.5-fold purification of the reductase. The solubilized NADPH-cytochrome *c* reductase was entirely retained on the ion-exchange gel. As shown in Fig. 1, a linear gradient of KCl achieved the separation of NADPH-cytochrome *c* reductase from cytochrome *P*-450 and NADH-ferricyanide reductase; however, cytochrome *b*₅ contaminated the NADPH-cytochrome *c* reductase significantly. The fractions enriched in NADPH-cytochrome *c* reductase activity were directly loaded on 2',5'-bisphosphoadenosine-Sepharose 4B affinity column. When the equilibration medium contained 0.2% Emulgen 911, NADPH-cytochrome *c* reductase was selectively retained on the gel, whereas cytochrome *b*₅ and remaining traces of NADH-ferricyanide reductase were

Table 1. Purification of NADPH-cytochrome *c* (cytochrome *P*-450) reductase from microsomes of wounded Jerusalem-artichoke tubers

For experimental details see the text. Cytochrome *c* reduction was measured at 25 °C in the presence of 100 mM-sodium phosphate buffer, pH 7.4, 2.5 μ M-FMN, 0.05 mM oxidized cytochrome *c* and 0.05 mM-NADPH. KCN was added for reductase activity determinations in microsomes.

Step	Total protein (mg)	Total activity (μ mol/min)	Specific activity (μ mol/min per mg)	Purification (fold)	Yield (%)
Microsomes	672	94	0.14	—	100
Solubilized microsomes	439	85	0.19	1.4	90
1st DEAE-Trisacryl column eluate	108	97	0.90	6.4	104
2',5'-Bisphosphoadenosine-Sepharose 4B eluate	6.9	68	9.77	70	72
2nd DEAE-Trisacryl column eluate	1.4	47	33.33	238	50

Fig. 1. Chromatography of cholate-solubilized Jerusalem-artichoke tuber microsomes on DEAE-Trisacryl M

The column was equilibrated with 10 mM-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA, 1.5 mM-2-mercaptoethanol, 20% glycerol and 0.2% Emulgen. ●—●, NADPH-cytochrome *c* reductase (μmol of cytochrome *c* reduced/min per 1 ml of eluate); ○—○, NADH-ferricyanide reductase (μmol of ferricyanide reduced/min per 1 ml of eluate); ▲···▲, cytochrome P-450 (nmol/1 ml of eluate); □—□, cytochrome *b*₅ (nmol/1 ml of eluate); —, KCl gradient.

excluded. After the column had been washed at high salt concentration (200 mM-phosphate buffer), the flavo-protein was specifically detached by 2'-AMP. This step brought a 10-fold increase of the reductase specific activity (Table 1).

In contrast, in the presence of 0.2% sodium cholate instead of Emulgen 911, the plant reductase was not tightly bound to the affinity gel and was eluted shortly after cytochrome *b*₅ during washing. Under identical conditions, NADPH-cytochrome *c* reductase isolated from rat liver microsomes was retained on the affinity column. The affinity of Jerusalem-artichoke NADPH-cytochrome *c* reductase for the ligand was probably not responsible for this chromatographic behaviour, since 2',5'-bisphosphoadenosine is a potent competitive inhibitor ($K_i = 3 \mu\text{M}$) for this enzyme.

A second DEAE-Trisacryl M column, equilibrated with 0.6% cholate, achieved a further 3-fold purification and permitted the replacement of Emulgen by sodium

cholate. In addition, the 'proteolytic' form of the reductase (peak I), which is eventually produced during the solubilization and the purification, was separated from the native enzyme (peak II), which was eluted at higher ionic strength (Fig. 2). Peak I was identified as the 'proteolytic' form of the reductase since the trypsin-solubilized enzyme produced exclusively peak I. When phenylmethanesulphonyl fluoride was present during the preparation of microsomes and the solubilization and purification of the enzyme, this peak was greatly decreased.

The purified reductase was concentrated and the sodium cholate concentration lowered to 0.05% by ultrafiltration on an Amicon YM30 membrane.

By using the procedure described here, plant NADPH-cytochrome *c* reductase was purified more than 200-fold with a 50% yield. After SDS/polyacrylamide-gel electrophoresis and silver staining, the enzyme appeared as a single band.

Fig. 2. Elution profile of NADPH-cytochrome *c* reductase from the second DEAE-Trisacryl M column

This DEAE-Trisacryl M column was equilibrated with 10 mM-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA, 1.5 mM-2-mercaptoethanol, 20% glycerol and 0.6% sodium cholate. ●—●, NADPH-cytochrome *c* reductase (μ mol of cytochrome *c* reduced/min per 1 ml of eluate); —, KCl gradient.

Reconstitution of cytochrome *P*-450-dependent mono-oxygenase activities

The purified detergent-solubilized NADPH-cytochrome *c* reductase was able to reduce cytochrome *P*-450 isolated from Jerusalem-artichoke tuber microsomes (Table 2). Cinnamate hydroxylase activity, probably the major cytochrome *P*-450-dependent mono-oxygenase in Mn^{2+} -induced Jerusalem-artichoke tuber, was reconstituted in the presence of cytochrome *P*-450, reductase and NADPH. Under the conditions used, the reconstituted activity was proportional to the quantity of reductase. Addition of dilauroyl phosphatidylcholine improved the

reconstitution. The low activity of cinnamate hydroxylase measured in the reconstituted system (0.055 pmol of *p*-coumarate formed/min per pmol of cytochrome *P*-450) as compared with that in microsomes (20 pmol/min per pmol of cytochrome *P*-450) may be due to excessive detergent concentration remaining in the cytochrome *P*-450 and reductase preparations.

Very low activities of the laurate in-chain hydroxylase, another mono-oxygenase present in our plant material, were also detected in the reconstituted system (detailed results not shown).

Immunochemical studies

The physiological role of the plant NADPH-cytochrome *c* reductase was confirmed by immunological studies. Rabbit antibodies raised to purified native NADPH-cytochrome *c* reductase from Jerusalem artichoke were tested on both NADPH- and NADH-cytochrome *c* reductase activities. When Jerusalem-artichoke microsomes were diluted 1:1 by the immune serum, NADPH-cytochrome *c* reductase was inhibited by 93%, whereas NADH-cytochrome *c* reductase activity was decreased by only 13%. Since the purified reductase has a low affinity for NADH, the fraction of NADH-supported cytochrome *c* reduction inhibited by the antibodies is probably that part mediated by NADPH-cytochrome *c* reductase.

Under identical conditions, the cytochrome *P*-450-dependent cinnamate hydroxylase was inhibited by 98.4% when NADPH was the electron donor, and by 92.5% in the presence of a high concentration (10 mM) of NADH.

These results confirm that the NADPH-cytochrome *c* reductase that we purified is part of the microsomal electron-transport chains and is responsible for the transfer of reducing equivalents between NADPH (and NADH) and cytochrome *P*-450.

M_r

The M_r of the Jerusalem-artichoke reductase was assessed by using SDS/polyacrylamide-gel electrophor-

Table 2. NADPH-dependent cinnamate hydroxylation in a reconstituted system

Reconstitution was achieved at 0 °C by addition to N_2 -dried DLPC (dilauroyl phosphatidylcholine), in order, of cytochrome *P*-450 in 10 mM-sodium phosphate buffer, pH 7.8, 1.5 mM-2-mercaptoethanol, 20% (v/v) glycerol and near 1% (w/v) Emulgen 911, and of NADPH-cytochrome *c* reductase in the previous buffer. (Just before reconstitution, FMN was added to the purified NADPH-cytochrome *c* reductase.) This mixture was vigorously stirred. Cinnamate hydroxylase activity was measured at 28 °C, for 20 min, in the presence of 100 mM-sodium phosphate buffer, pH 7.4, 0.1 mM-NADPH, 0.02 mM-[3- ^{14}C]cinnamic acid and the components of the reconstituted system; 26 pmol of cytochrome *P*-450 was present per assay. The final volume of incubation was 200 μ l.

Conditions	Cinnamate hydroxylase activity (pmol of <i>p</i> -coumarate formed/min)
Cytochrome <i>P</i> -450	0
Cytochrome <i>P</i> -450 + DLPC (100 μ g)	0
Reductase (46 munits)	0
Cytochrome <i>P</i> -450 + reductase (18.5 munits)	0.35
Cytochrome <i>P</i> -450 + reductase (18.5 munits) + DLPC(100 μ g)	0.63
Cytochrome <i>P</i> -450 + reductase (46 munits)	1.04
Cytochrome <i>P</i> -450 + reductase (46 munits) + DLPC(100 μ g)	1.44

Fig. 3. SDS-polyacrylamide-slab-gel electrophoresis of purified NADPH-cytochrome *c* (cytochrome *P*-450) reductase from Jerusalem-artichoke and rat liver microsomes

Effect of trypsin on detergent-solubilized plant enzyme: native enzyme (0.38 mg/ml) was incubated at 4 °C with trypsin (2 µg/mg of reductase). After 30 min, trypsin inhibitor was added (40 µg/mg of reductase). Samples were then diluted 1:1 with electrophoresis sample buffer containing 2.5% 2-mercaptoethanol and 2.5% SDS, and boiled for 3 min. A 7.5–15% acrylamide separating gel was used. Proteins were stained by the silver nitrate method. Lane a, Jerusalem-artichoke reductase (3.3 µg); lane b, Jerusalem-artichoke reductase (3.3 µg) plus soya-bean trypsin inhibitor; lane c, trypsin-treated Jerusalem-artichoke reductase (3.3 µg); lane d, markers (M_r values indicated at right-hand side of Figure) (2 µg); lane e, rat liver reductase (3.2 µg).

esis, with the following standards: phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 20100) and α -lactalbumin (M_r 14400). Fig. 3 compares the electrophoretic mobility of the purified Jerusalem-artichoke reductase (lanes a and b) and the purified rat liver microsomal reductase (lane e). The M_r of the plant reductase is clearly higher than that of the rat liver enzyme: 81000–82000 for the Jerusalem-artichoke reductase, 76000–77000 for the liver enzyme. These values correspond probably to the native forms of the reductases; the M_r measured for the liver reductase is in accordance with published values for the native form of the enzyme (Vermilion & Coon, 1978). For the plant reductase, the fact that a trypsin treatment (2 µg of trypsin/mg of reductase) converted the enzyme into a smaller protein, with M_r 76000–77000 (lane c), which had an electrophoretic mobility similar to that of purified trypsin-solubilized reductase, corroborate this assumption. The fragment cleaved by the tryptic treatment, which may correspond to the hydrophobic portion of the Jerusalem-artichoke reductase, would have an M_r of 5000–6000.

Flavoprotein nature

When FMN and FAD were omitted from the equilibration buffers of the chromatography columns in the procedure used to purify NADPH-cytochrome *c* reductase, a progressive loss of specific activity of the

Fig. 4. Absorbance spectra of purified NADPH-cytochrome *c* (cytochrome *P*-450) reductase from Jerusalem-artichoke tuber microsomes

The reductase concentration was 0.34 mg of protein/ml in 10 mM-sodium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA, 20% glycerol and 0.1% sodium cholate. The total volume in the cuvettes was 1 ml. The reference cuvette contained the same buffer, without reductase. The different additions to the sample cuvette were compensated by the same ones in the reference. An equal absorbance baseline (----) was established with buffer in the two cuvettes. Spectrum a, 'reconstituted reductase, without any addition (—); spectrum b, aerobically NADPH-reduced reductase (NADPH concn. 15 µM) (·····); spectrum c, aerobically NADPH-reduced reductase after reoxidation with $K_3Fe(CN)_6$ (concn. 85 µM) (— · — · —); spectrum d, fully reoxidized reductase (ferricyanide concn. 190 and 300 µM) (— · — · —).

enzyme was observed during the procedures. The addition of FAD to the purified enzyme had only a small effect, whereas FMN exerted increasing stimulatory effects during the course of purification (4-fold on the purified reductase). In contrast, ferricyanide reduction by the purified enzyme was not influenced by the addition of FMN, indicating that in plant NADPH-ferricyanide reductase, as in the animal enzyme (Vermilion *et al.*, 1981), FMN is not implicated.

On account of the lability of the FMN prosthetic group, the absorption spectra and the flavin content of the reductase were measured on 'reconstituted' enzyme, i.e. purified reductase incubated in the presence of FMN and then dialysed to eliminate the unbound flavin. Under these conditions, the visible-region spectrum (Fig. 4 spectrum a) showed a peak at 455 nm with a shoulder at 478 nm, and another shoulder at 385 nm. As isolated, the enzyme was probably not obtained under a fully oxidized state. After aerobic reduction with NADPH (Fig. 4, spectrum b), the peak at 455 nm decreased, a distinct peak with lower absorbance appeared at 385 nm and the absorbance increased at wavelengths above the 500 nm isosbestic point. Since reduction took place under aerobic conditions, this spectrum does probably not correspond to the totally reduced flavoprotein. Reoxidation of the

Fig. 5. Double-reciprocal plots of the initial rate of cytochrome *c* reduction by various amounts of NADPH at different cytochrome *c* concentrations

NADPH-cytochrome *c* reductase activity was measured at 25 °C in the presence of 100 mM-sodium phosphate buffer, pH 7.4, containing 2.5 μM-FMN, 2.3 pmol of purified Jerusalem-artichoke reductase and various concentrations of NADPH and cytochrome *c* in a final volume of 1 ml. The following concentrations of cytochrome *c* were utilized: ▽, 31.6 μM; ▼, 15.8 μM; ○, 8.5 μM; ●, 4.25 μM. Activity is expressed as ΔA_{550} /min per ml of enzyme.

NADPH-reduced enzyme by successive additions of $K_3Fe(CN)_6$ (85, 190 and 300 μM in the sample) produced typical flavoprotein spectra (Fig. 4, spectra c and d). Spectrum d corresponds to the fully oxidized flavoenzyme, since the increase of the ferricyanide concentration from 190 to 300 μM produced no further increase of the 455 nm absorbance. A concentration of 15 nmol of flavins/mg of reductase was calculated from spectrum d.

The flavin content of the Jerusalem-artichoke reductase was measured spectrofluorimetrically, by the procedure of Faeder & Siegel (1973). On the basis of an M_r of 82000, the plant enzyme contained 0.41 mol of FMN and 0.31 mol of FAD per mol of reductase, indicating equimolar quantities of FMN and FAD.

Kinetic properties

The reaction mechanism of the purified Jerusalem-artichoke reductase was investigated. This enzyme has two substrates. One substrate is the electron donor: the K_m obtained by steady-state kinetic analyses on the purified reductase showed a good apparent affinity for NADPH (K_m for NADPH = 20 μM).

When the rate of cytochrome *c* reduction was studied as a function of NADH concentration a biphasic kinetic was observed, i.e. low apparent affinity ($K_m = 54$ mM) and high velocity at high NADH concentration, and high apparent affinity ($K_m = 46$ μM) and low velocity for low NADH concentrations.

An electron acceptor constitutes the other substrate: data presented above indicate that cytochrome *P*-450 isolated from Jerusalem artichoke was reduced by the purified enzyme, as well as exogenous acceptors [cytochrome *c* ($K_m = 6.3$ μM) or $K_3Fe(CN)_6$].

The sequence of interaction of NADPH and oxidized cytochrome *c* with the purified reductase was investigated. The double-reciprocal plots of the initial rate of

Fig. 6. Comparison of the effects of ionic strength on NADPH-cytochrome *c* reductases purified from rat liver microsomes and from Jerusalem-artichoke tuber microsomes

Rat reductase: ○, effect of NaCl; ●, effect of KCl; Jerusalem-artichoke reductase: △, effect of NaCl; ▲, effect of KCl. Activities were measured at 25 °C in the presence of 50 mM-sodium phosphate buffer, pH 7.4, containing 2.5 μM-FMN and 4.44 pmol of purified rat liver reductase or 4.7 pmol of purified Jerusalem-artichoke reductase in a final volume of 1 ml. NADPH-cytochrome *c* reductase activities are expressed as percentages of the activity of each reductase measured without any salt addition (0.96 μmol of cytochrome *c* reduced/min per ml of rat reductase and 13.7 μmol/min per ml of Jerusalem-artichoke reductase).

cytochrome *c* reduction by various amounts of NADPH at different cytochrome *c* concentrations were convergent lines (Fig. 5). These results are in agreement with a ternary-complex mechanism for the interaction of cytochrome *c* and NADPH with the reductase. The apparent K_m values, determined on the basis of a sequential mechanism, were 30.8 μM for NADPH and 18.2 μM for cytochrome *c*. Kinetic studies of product inhibition (NADP⁺) in the presence of various cytochrome *c* concentrations and a fixed NADPH concentration indicated a non-competitive inhibition, which is consistent with a sequential rather than a ping-pong mechanism (Fromm, 1979).

Effect of ionic strength

Fig. 6 shows a comparison of the effects of the concentrations of NaCl and KCl on NADPH-cytochrome *c* reductases isolated from plant and rat liver microsomes. In contrast with the liver enzyme, the plant cytochrome *c* reductase was only slightly stimulated by increasing ionic strength (maximum at 1.02), and inhibition appeared above 1.05. This lack of effect could contribute to the lower specific activity of the purified plant cytochrome *c* reductase.

DISCUSSION

NADPH-cytochrome *c* (cytochrome *P*-450) reductase, isolated from wound-induced Jerusalem-artichoke tuber microsomes, was purified 240-fold to apparent homogeneity.

The flavoprotein nature of the enzyme was established by characteristic flavin spectra. However, the flavin content (15 nmol of flavins/mg of protein) was lower than that measured for the mammalian liver reductase (24–25 nmol/mg of protein) (Vermilion & Coon, 1978). This low apparent content of flavins, 0.41 mol of FMN and 0.31 mol of FAD per mol of 'reconstituted' artichoke reductase, contrasted with the nearly 1 mol of each flavin per mol of rat reductase (Iyanagi & Mason, 1973; Vermilion & Coon, 1974). Low contents of flavins were also measured for the *Catharanthus roseus* reductase, another plant enzyme, by Madyastha & Coscia (1979).

The low FAD content of the artichoke reductase was probably the result of an irreversible dissociation of the flavin from its apoenzyme. FMN was also highly labile, but this dissociation was reversible. In comparison, the loss of FMN by the rat liver reductase that we purified, using the same procedure, was limited, since the addition of FMN produced only a weak activation of NADPH-dependent cytochrome *c* reduction. It is likely that the low flavin contents observed here are due either to an overevaluation of protein content or to the presence of a contaminating protein, with the same electrophoretic mobility.

Cinnamate hydroxylase and laurate in-chain hydroxylase are microsomal cytochrome *P*-450-dependent mono-oxygenases, present in the Jerusalem-artichoke tuber after wounding (Benveniste *et al.*, 1977; Salaun *et al.*, 1978) and after treatment with different xenobiotics (Reichhart *et al.*, 1980). The ability of the purified reductase to reconstitute these mono-oxygenase activities in the presence of partially purified cytochrome *P*-450 and a phospholipid confirms its biological role as electron carrier between NADPH and cytochrome *P*-450. Madyastha & Coscia (1979) also observed a crucial role of purified NADPH-cytochrome *c* reductase in the transfer of electrons from NADPH to cytochrome *P*-450 during geraniol hydroxylation.

The functional role of NADPH-cytochrome *c* reductase in tuber microsomes was confirmed by immunological studies. Rabbit antibodies, directed against the plant NADPH-cytochrome *c* reductase, inhibited strongly not only the NADPH-supported but also the NADH-supported cinnamate hydroxylase activities from artichoke microsomes. Since these antibodies are highly specific for NADPH-cytochrome *c* reductase, our results indicate that the same flavoprotein mediates NADPH-dependent and, at least partly, NADH-dependent cinnamate hydroxylation in our microsomes. They confirm the results obtained in previous studies on microsomal electron transport in plant microsomes, with the use of specific inhibitors or partial solubilization of the microsomal flavoproteins (Benveniste *et al.*, 1982). It was concluded that during NADH-supported hydroxylation the first electron was transferred to cytochrome *P*-450 via NADPH-cytochrome *c* reductase.

Although NADPH-cytochrome *c* (cytochrome *P*-450) reductase plays a similar functional role in plant and in mammalian microsomes, the plant enzyme differs on several accounts from the enzyme of animal origin. A first

difference was noted during the purification procedure. Guinea-pig liver reductase binds to 2',5'-bisphosphoadenosine-Sepharose 4B at high cholate concentrations (Kobayashi & Rikans, 1984), and under our conditions the rat liver reductase was well bound in the presence of 0.2% cholate. In contrast, we observed repeatedly that binding of the plant reductase to the affinity gel was much weaker. One may assume that this behaviour is due to the active site of the plant enzyme being close to the negatively charged groups of sodium cholate. This would prevent the interaction with the negatively charged 2'-phosphate group of the ribose of 2',5'-bisphosphoadenosine, which seems to be crucial for binding and subsequent elution from the 2',5'-bisphosphoadenosine-Sepharose column (Yasukochi & Masters, 1976). When the non-ionic detergent Emulgen 911 was used in place of cholate, the plant reductase bound to the affinity gel.

Another distinct chromatographic behaviour of the artichoke reductase was observed at the stage of enzyme elution from the 2',5'-bisphosphoadenosine-Sepharose. Several authors used this step, not only to purify the enzyme, but also to exchange non-ionic detergent by an ionic one (Dignam & Strobel, 1977; Vermilion & Coon, 1978; Guengerich & Martin, 1980; Williams *et al.*, 1983; Shephard *et al.*, 1983). In contrast, the plant reductase could not be eluted from the gel by 2'-AMP, after replacement of Emulgen 911 by 0.2% or 0.6% cholate in the equilibration medium. Only return to Emulgen 911 in the affinity gel allowed elution of the reductase by 2'-AMP.

The purest reductase preparation obtained from Jerusalem-artichoke microsomes had a cytochrome *c* reduction specific activity of 33 $\mu\text{mol/min per mg}$. This value is higher than that described for *Catharanthus roseus* reductase (17 $\mu\text{mol/min per mg}$) by Madyastha & Coscia (1979), but lower than those reported for mammalian liver reductase (nearly 60 $\mu\text{mol/min per mg}$) (Yasukochi & Masters, 1976; Vermilion & Coon, 1978; Shephard *et al.*, 1983). The lack of stimulation of the artichoke reductase by ionic strength, associated with a greater lability of FAD, could explain this discrepancy. It should also be noted that the plant reductase was assayed at 25 °C instead of 37 °C for the animal enzymes. The low stimulatory effect produced by increasing salt concentrations is not characteristic of the artichoke reductase, since it was also observed for yeast (Kubota *et al.*, 1977), housefly (Wilson & Hodgson, 1971; Mayer & Prough, 1977; Mayer & Durrant, 1979), southern-armyworm midgut (Crankshaw *et al.*, 1979) and guinea-pig liver (Kobayashi & Rikans, 1984) reductases.

The detergent-solubilized artichoke reductase has an M_r , assessed by SDS/polyacrylamide-gel electrophoresis, of 81 000–82 000, whereas the trypsin-solubilized reductase and the protein resulting from digestion by trypsin of the purified detergent-solubilized enzyme show an M_r of 76 000–77 000. The detergent-solubilized *Catharanthus roseus* reductase and its proteolytic form exhibited M_r values of 78 000 and 63 000 respectively. The M_r of the detergent-solubilized mammalian liver reductases ranged from 76 000 to 79 500, and of the proteinase-solubilized enzymes from 68 000 to 71 000. An M_r of 83 000 was described for the reductase from housefly (Mayer & Durrant, 1979) and the yeast *Saccharomyces cerevisiae* (Aoyama *et al.*, 1978). Thus, in comparison with reductases from different sources, the artichoke NADPH-cytochrome *P*-450 reductase is characterized by a high M_r .

for the native enzyme and by a short trypsin-removable region. Fujita & Asahi (1985) reported M_r values of 81 000 for native NADPH-cytochrome *c* reductase from sweet potato and of 75 000 and 72 000 for probably two distinct proteolytic forms.

The reaction mechanism of the enzyme was investigated. Two types of kinetic mechanisms are generally reported for NADPH-cytochrome *c* reductases from different origins: (1) a Bi Bi Ping Pong mechanism, in which the binding of the electron acceptor is dependent on the previous binding and oxidation of NADPH (Masters *et al.*, 1965; Fan & Masters, 1974; Mayer & Prough, 1977; Hiwatachi & Ichikawa, 1979; Mayer & Durrant, 1979; Crankshaw *et al.*, 1979); (2) a Bi Bi sequential mechanism, where the two substrates bind on the enzyme, in a random or an ordered manner. The first report from Phillips & Langdon (1962) was in favour of a random Bi Bi sequential mechanism for the rat liver enzyme. Such a mechanism was also assigned by Dignam & Strobel (1977) with cytochrome *c* or cytochrome *P*-450 as electron acceptor and by Kobayashi & Rikans (1984) for the NADPH-dependent reduction of cytochrome *c*. Our experimental findings are in good agreement with this second mechanism, even if our results, at this stage, do not discriminate between an ordered or a random interaction of NADPH and cytochrome *c* with the enzyme.

Numerous studies show that in animal systems the NADPH-cytochrome *c* (cytochrome *P*-450) reductase plays a central role in microsomal electron transfer: the flavoprotein distributes reducing equivalents to cytochrome *P*-450 but also to cytochrome *b₅* and fatty acid desaturase, and to different iron chelates implicated in the production of oxygen radicals. In plants, apart from the participation to cytochrome *P*-450-dependent oxidations, clearly confirmed by the present report, the role of this enzyme is poorly understood. Purification of the reductase and production of antibodies against it will allow to further investigate its regulation and the role that it plays in the expression of plant cytochrome *P*-450 enzymes.

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REFERENCES

- Aoyama, Y., Yoshida, Y., Kubota, S., Kumaoka, H. & Furumichi, A. (1978) Arch. Biochem. Biophys. **185**, 362–369
- Benveniste, I. & Durst, F. (1974) C. R. Hebd. Séances Acad. Sci. Ser. D. **278**, 1487–1490
- Benveniste, I., Salaun, J. P. & Durst, F. (1977) Phytochemistry **16**, 69–73
- Benveniste, I., Gabriac, B., Fonne, R., Reichhart, D., Salaun, J. P., Simon, A. & Durst, F. (1982) in Cytochrome *P*450, Biochemistry, Biophysics and Environmental Implications (Hietanen, E., Laitinen, M. & Hanninen, O., eds.), pp. 201–208, Elsevier Biomedical press, Amsterdam
- Cleland, W. W. (1979) Methods Enzymol. **63**, 103–138
- Crankshaw, D. L., Hetnarski, K. & Wilkinson, C. F. (1979) Biochem. J. **181**, 593–605
- Dignam, J. D. & Strobel, H. W. (1977) Biochemistry **16**, 1116–1123
- Dulley, J. R. & Grieve, P. A. (1975) Anal. Biochem. **64**, 136–141
- Faeder, E. J. & Siegel, L. M. (1973) Anal. Biochem. **53**, 332–336
- Fan, L. L. & Masters, B. S. S. (1974) Arch. Biochem. Biophys. **165**, 665–671
- Frear, D. S., Swanson, H. R. & Tanaka, F. S. (1969) Phytochemistry **8**, 2157–2169
- Fromm, H. J. (1979) Methods Enzymol. **63**, 467–486
- Fujita, M. & Asahi, T. (1985) Plant Cell Physiol. **26**, 397–405
- Fujita, M., Oba, K. & Uritani, I. (1982) Plant Physiol. **70**, 573–578
- Grand, C. (1984) FEBS Lett. **167**, 7–11
- Guengerich, F. P. & Martin, M. V. (1980) Arch. Biochem. Biophys. **205**, 365–379
- Hagmann, M. L., Heller, W. & Grisebach, H. (1983) Eur. J. Biochem. **134**, 547–554
- Hasson, E. P. & West, C. A. (1976) Plant Physiol. **58**, 473–484
- Hiwatachi, A. & Ichikawa, Y. (1979) Biochim. Biophys. Acta **580**, 44–63
- Iyanagi, T. & Mason, H. S. (1973) Biochemistry **12**, 2297–2308
- Kobayashi, S. & Rikans, L. E. (1984) Comp. Biochem. Physiol. B **77**, 313–318
- Kolattukudy, P. E. (1980) Science **208**, 990–1000
- Kubota, S., Yoshida, Y., Kumaoka, H. & Furumichi, A. (1977) J. Biochem. (Tokyo) **81**, 197–205
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
- Madyastha, K. M. & Coscia, C. J. (1979) J. Biol. Chem. **254**, 2419–2427
- Madyastha, K. M., Meehan, T. D. & Coscia, C. J. (1976) Biochemistry **15**, 1097–1102
- Masters, B. S. S., Bilimoria, M. H., Kamin, H. & Gibson, Q. H. (1965) J. Biol. Chem. **240**, 4081–4088
- Mayer, R. T. & Durrant, J. L. (1979) J. Biol. Chem. **254**, 756–761
- Mayer, R. T. & Prough, R. A. (1977) Comp. Biochem. Physiol. B **57**, 81–87
- Morrissey, J. H. (1981) Anal. Biochem. **117**, 307–310
- Omura, T. & Sato, R. (1964) J. Biol. Chem. **239**, 2370–2378
- Omura, T. & Takesue, S. (1970) J. Biochem. (Tokyo) **67**, 249–257
- Phillips, A. H. & Langdon, R. G. (1962) J. Biol. Chem. **237**, 2652–2660
- Potts, J. R. M., Weklych, R. & Conn, E. E. (1974) J. Biol. Chem. **249**, 5019–5026
- Reichhart, D., Salaun, J. P., Benveniste, I. & Durst, F. (1980) Plant Physiol. **66**, 600–604
- Salaun, J. P., Benveniste, I., Reichhart, D. & Durst, F. (1978) Eur. J. Biochem. **90**, 155–159
- Schacterle, G. R. & Pollack, R. L. (1973) Anal. Biochem. **51**, 654–655
- Shephard, E. A., Pike, S. F., Rabin, B. R. & Phillips, J. R. (1983) Anal. Biochem. **129**, 430–433
- Soliday, C. L. & Kolattukudy, P. E. (1977) Plant Physiol. **59**, 1116–1121
- Vermilion, J. L. & Coon, M. J. (1974) Biochem. Biophys. Res. Commun. **60**, 1315–1322
- Vermilion, J. L. & Coon, M. J. (1978) J. Biol. Chem. **253**, 2694–2704
- Vermilion, J. L., Ballou, D. P., Massey, V. & Coon, M. J. (1981) J. Biol. Chem. **256**, 266–277
- Williams, D. E., Becker, R. R., Potter, D. W., Guengerich, F. P. & Buhler, D. R. (1983) Arch. Biochem. Biophys. **225**, 55–65
- Wilson, T. G. & Hodgson, E. (1971) Insect Biochem. **1**, 171–180
- Yasukochi, Y. & Masters, B. S. S. (1976) J. Biol. Chem. **251**, 5337–5344
- Young, O. & Beevers, H. (1976) Phytochemistry **15**, 379–385