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Purification and Partial Characterization of NADPH-Cytochrome c Reductase from *Petunia hybrida* Flowers[1]

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NADPH-cytochrome c reductase was solubilized from the microsomal fraction of *Petunia hybrida* flowers by 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate detergent and purified by adenosine 2′,5′-bisphosphate-Sepharose chromatography, followed by high-performance anion-exchange chromatography. Two proteins with molecular sizes of 75 and 81 kD were detected in the purified preparation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis showed that both purified proteins cross-reacted with two different monoclonal antibodies raised against *P. hybrida* NADPH-cytochrome c reductase and rabbit anti-Jerusalem artichoke NADPH-cytochrome P450 reductase antibodies. Only one 84-kD protein was detected by western blot analysis of fresh microsomal extracts. Amino acid sequence analysis of tryptic peptides revealed significant similarity to the NADPH binding region of plant and animal NADPH-cytochrome P450 reductases and *Bacillus megaterium* cytochrome P450:NADPH-cytochrome P450 reductase. The pH optimum for reduction of ferricytochrome c was 7.4 and the K_m values for the binding of NADPH and ferricytochrome c were 9.2 and 2.8 μM, respectively. We believe that the purified enzyme is a *P. hybrida* NADPH-cytochrome P450 reductase (EC 1.6.2.4).

The enzymic components of animal and plant microsomal monooxygenases are a NADPH-Cyt P450 (Cyt *c*) reductase and a Cyt P450 (Riviere and Cabanne, 1987). In higher plant membrane fractions, Cyts P450 have been implicated in the biosynthesis of phytoalexins (Kochs and Grisebach, 1989), lignins and tannins (Gabriac et al., 1985, 1991; Bolwell and Dixon, 1986), fatty acids (Soliday and Kolattukudy, 1978; Salaun et al., 1989), terpenes (Madyastha et al., 1976, 1977; Fujita and Asahi, 1985), alkaloids (Stadler and Zenk, 1993), GA (Hasson and West, 1976; Rademacher et al., 1987), and steroids (Rahier and Taton, 1986), and may have a role in the detoxification of xenobiotics in a manner analogous to nammalian liver xenobiotic detoxification (Hendry, 1986; Riviere and Cabanne, 1987; Mougin et al., 1990; Sandermann, 1992). The enzymes responsible for 3′ and 5′ hydroxyl substitution of anthocyanidin precursors in flowers (Forkmann et al., 1980; Forkmann and Stotz, 1981; Stotz and Forkmann, 1982; Stotz et al., 1985) and maize aleurone (Larson and Bussard, 1986) are also believed to be P450 dependent. Cyt P450-dependent flavonoid hydroxylation is conducted by microsomal flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase (Stafford, 1990). *Petunia hybrida* flower 3′ and 5′ hydroxyl modification is carried out by a microsomal Cyt P450 enzyme, dependent on NADPH, molecular oxygen, and NADPH-Cyt P450 reductase (Menting et al., 1994). None of the flavonoid 3′- or 3′,5′-hydroxylases have been purified.

NADPH-Cyt P450 reductase has been purified from plant and mammalian sources (Donaldson and Luster, 1991). Madyastha and Coscia (1979) first purified a plant NADPH-Cyt P450 reductase from *Catharanthus roseus* that was capable of reconstituting geraniol hydroxylase activity when combined with a Cyt P450. Benveniste et al. (1986) purified NADPH-Cyt P450 reductase from Jerusalem artichoke that was capable of reconstituting cinnamic acid 4-hydroxylase activity. Kochs and Grisebach (1989) were also able to reconstitute a monooxygenase from purified NADPH-Cyt P450 reductase and a Cyt P450.

In this paper, the results of the purification and partial characterization of a *P. hybrida* flower microsomal NADPH-Cyt *c* reductase are presented. The subcellular location of the enzyme, cross-reactivity with antibodies, and partial primary structure support the suggestion that the purified enzyme is a *P. hybrida* flower NADPH-Cyt P450 reductase.

MATERIALS AND METHODS

Preparation of Microsomal Membrane Fraction

Flowers were obtained from *Petunia hybrida* cv Old Glory Blue plants (Ball Seeds, Chicago, IL). During summer months plants were grown outdoors in vegetable beds under natural light, and in winter they were grown under glasshouse conditions with supplementary lighting to give 14 h of continuous lighting each day. Only the corolla limb of stages 5 (flower buds) through 8 (open flowers) were used (morphological stages were based on the series defined by Stotz et al., 1985). The microsomal fraction was isolated from either freshly harvested tissue or tissue stored for up to 3 d at 4°C.

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Abbreviations: 2′,5′-ADP-Sepharose, adenosine 2′,5′-bisphosphate-Sepharose 4B; 2′AMP, adenosine 2′-monophosphoric acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

All steps were carried out on ice or at 4°C except where stated otherwise. Flower bud limbs were ground in a mortar together with acid-washed sand and 1 mL of buffer A (100 mM potassium phosphate, 0.1% BSA, 10^{-7} M pepstatin A, 10^{-7} M leupeptin, 0.1 mg/mL PMSF, 0.25 M Suc, 0.25 M mannitol, 20 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM EGTA, 5 μM FMN, 1 μM FAD, pH 7.5) per g of tissue (wet weight), containing 10 mg/mL Polyclar AT (BDH, Poole, UK). The homogenate was filtered through Miracloth and centrifuged for 25 min at 12,000*g* (10°C). The pellet was discarded and the supernatant was centrifuged for 1 h at 105,000*g* (10°C). Pellets were suspended in buffer B (100 mM Mops-NaOH, 1 mM EDTA, 20% glycerol, 10^{-7} M leupeptin, 10^{-7} M pepstatin A, 0.1% BSA, pH 7.0) to a protein concentration of 10 mg/mL. The microsomal fraction was also isolated from the homogenate by using a second method, a modification of the method of Diesperger et al. (1974). The homogenate was centrifuged (12,000*g*, 20 min) and the supernatant was removed and filtered through Miracloth (Calbiochem, La Jolla, CA). The filtrate was adjusted to 40 mM $MgCl_2$ and incubated for 10 min on ice. Microsomal membranes were sedimented by centrifugation at 20,000*g* for 20 to 30 min. Membranes were suspended as described above.

NADPH-Cyt *c* Reductase Assay

Enzyme activity was assayed essentially as described by Benveniste et al. (1989). The reduction of ferricytochrome *c* (Sigma) to ferrocytochrome *c* was calculated from the rate of change of A_{550} in buffer C (100 mM potassium phosphate, 0.1 mM EDTA, 50 μM ferricytochrome *c*, 1 mM potassium cyanide, pH 7.5), assuming an extinction coefficient of 21.0 mM^{-1} cm^{-1} for horse-heart ferricytochrome *c* (Fan and Masters, 1974). One unit of activity corresponds to the reduction of 2 μmol of ferricytochrome *c* per min. Reactions were commenced by addition of 50 μM NADPH. With unpurified samples, baseline values in the absence of NADPH were subtracted from the NADPH-dependent determinations. Reactions were conducted at 25°C.

Determination of K_m for Cyt *c* and NADPH

Spectrophotometric assays were carried out using a Hewlett-Packard HP8452A diode-array spectrophotometer. For the determination of K_m for NADPH, ferricytochrome *c* concentration was 50 μM and each NADPH concentration was kept constant by means of an NADPH-generating system consisting of 100 mM sodium phosphate, 10 units/mL Glc-6-P dehydrogenase, 1 mM Glc-6-P, pH 7.4. Spectrophotometric determinations were carried out as rapidly as possible and data were collected over 60 s, during which the rates were zero order. For the determination of K_m of Cyt *c*, the initial rates at various Cyt *c* concentrations were calculated from data collected over no more than 10 to 15 s, during which the rates of reduction were constant. The buffer contained 100 mM sodium phosphate, 100 μM NADPH, 1 mM Glc-6-P, and 10 units/mL Glc-6-P dehydrogenase, pH 7.4. NADPH stock solution concentrations were determined spectrophotometrically, assuming an ϵ_{340} of 6.22 mM^{-1} cm^{-1} at 21°C. Zero-order reaction rates for ferricytochrome *c* reduction were determined at 550 nm. Ferricytochrome *c* concentration was calculated from the 550-nm absorption difference of $Na_2S_2O_4$ reduced minus oxidized samples and extinction coefficient of 21.0 mM^{-1} cm^{-1} (Fan and Masters, 1974).

Determination of Protein Concentrations

Protein concentration was determined by the Sedmak and Grossberg (1977) modification of the Bradford method with BSA as the standard protein. For the determination of particulate microsomal membrane protein concentration, an aliquot of microsomal suspension was mixed with an equal volume of 1% SDS. The mixture was thoroughly vortexed, diluted 1:10 with 0.9% saline and assayed. When used in this way the SDS did not affect the determinations.

Purification of NADPH-Cyt *c* Reductase

Microsomal NADPH-Cyt *c* reductase was solubilized by addition of solid CHAPS (Boehringer, Mannheim, Germany), final concentration 0.75%, 0.75 mg CHAPS/mg microsomal protein. The mixture was incubated for 45 min on ice with stirring. After centrifugation at 105,000*g* for 1 h at 10°C, the supernatant was rapidly desalted at room temperature on Sephadex G-25 (fine) equilibrated with buffer D (10 mM potassium phosphate, 15% glycerol, 0.1% CHAPS, 0.2 mM EDTA, 10^{-7} M leupeptin, 10^{-7} M pepstatin A, 5 μM FMN, 1 μM FAD, pH 7.2). Protein-containing fractions were green and these green fractions were pooled and loaded at 2 to 3 mL/min (30–50 cm/h) on a 20-mL 2′,5′-ADP Sepharose (Pharmacia) affinity column equilibrated with buffer E (10 mM potassium phosphate, 15% glycerol, 0.1% CHAPS, 0.2 mM EDTA, pH 7.2). The column was washed with 15 mL of buffer E, then with 15 mL of buffer F (0.2 M potassium phosphate, 15% glycerol, 0.1% CHAPS, 0.2 mM EDTA, pH 7.2), and finally with 15 mL of buffer E. The enzyme was eluted with 30 mL of buffer E containing 5 mM 2′-AMP. Fractions containing NADPH-Cyt *c* reductase activity were pooled, concentrated in an Amicon ultrafiltration cell, and stored at −75°C. Anion-exchange chromatography of the partly purified NADPH-Cyt *c* reductase was carried out using a fast protein liquid chromatography system and an 8-mL Q-Sepharose High Performance (Pharmacia) adsorbent at a flow rate of 1 mL/min (80 cm/h). The Q-Sepharose column was equilibrated with buffer G (10 mM Mops-NaOH, 0.3% CHAPS, pH 7.2), reductase was loaded, and unadsorbed protein was removed with 15 mL of buffer G. Adsorbed protein was eluted with three consecutive gradients of NaCl in buffer G: (1) 10 mL of 0 to 0.25 M NaCl; (2) 60 mL of 0.25 to 0.5 M NaCl; (3) 20 mL of 0.5 to 1 M NaCl. Protein and NADPH-Cyt *c* reductase elution were monitored at 280 nm and by measurement of enzyme activity, respectively. Fractions containing NADPH-Cyt *c* reductase activity were pooled, concentrated by ultrafiltration, and stored at −75°C in buffer G containing 20% glycerol.

HPLC Separation of Tryptic Peptides and Amino Acid Sequence Determination

Purified reductase (25 μg) was diluted 1:1 with 100 mM Tris-HCl, 0.1% SDS, 5 mM DTT (pH 8), boiled for 15 min,

cooled, and adjusted to 20% dimethylformamide (according to Houen and Sandø, 1991). Trypsin digestion by modified trypsin (Promega, Madison, WI) was carried out according to the Promega Technical Bulletin No. 2012. Digestion was checked by SDS-PAGE and was complete within 2 h.

Peptides were resolved by reversed-phase HPLC using a Vydac protein C_4 (250 mm × 4.6 mm) reversed-phase resolving column, and a C_4 Selectosil (5-μm C_4, 50 mm × 4.6 mm) guard column. Peptides were eluted with a 200-mL gradient of 0 to 70% acetonitrile and 0.1 to 0.085% TFA. HPLC-purified peptides were concentrated in vacuo to 20 to 30 μL. To maximize recovery, 0.02% Tween 20 was added to fractions. These fractions were sequenced on a gas-phase sequencer (470A Applied Biosystems Protein Sequencer) by the Edman degradation method.

PAGE

SDS-PAGE was carried out on 6% stacking and 10% separating gels prepared according to Laemmli (1970). Proteins were detected by staining with Coomassie brilliant blue R-250. Native-PAGE was a modification of the Laemmli (1970) SDS-PAGE procedure, in which 0.1% SDS was replaced by 0.1% Nonidet P40 (BDH) and tank buffer lacked SDS. NADPH-Cyt P450 activity staining (Fan and Masters, 1974) was carried out by placing native gels in a buffer consisting of 10 mM potassium phosphate, 15% glycerol, 0.1% CHAPS, 1 mg/mL neotetrazolium chloride, 0.2 mM NADPH, 5 μM FMN, 1 μM FAD, pH 7.2. Gels were stained for approximately 30 min. The molecular masses of activity-stained native-PAGE protein bands were determined by re-electrophoresis on SDS-PAGE.

Immunization of Mice and Production of Monoclonal Antibodies

Two 10-week-old female BALB/c mice were immunized by intraperitoneal injection of partially purified *P. hybrida* NADPH-Cyt *c* reductase and fusions of splenic plasma cells with P3-X38 Ag8 myeloma cells (Horibata and Harris, 1970) by the procedure of Galfré et al. (1977). Antigen binding by antibodies produced by hybridomal cultures was tested by probing western blots of NADPH-Cyt *c* reductase with hybridomal culture supernatants. Hybridomas that produced antibodies recognizing NADPH-Cyt *c* reductase were cloned by limiting dilution (Oi and Herzenberg, 1980). In this way, two polyclonal hybridomas yielded 10 and 8 monoclonal cultures. One representative of each group of monoclonal hybridomas was selected for experimental use.

Western Blotting, Dot Blotting, and Immunodetection

Dot blots were used to screen mouse sera for antibodies to NADPH-Cyt *c* reductase and were prepared by application of antigen to dry nitrocellulose, blocking (10 mM Tris-HCl, 1% gelatin, 2.5% BSA, 0.9% NaCl, pH 8), and then probing with 1:100 or 1:1000 diluted immunized mouse sera or 1:100 nonimmunized mouse sera in blocking solution for 2 h at room temperature. Filters were washed once (10 min) with buffer H (10 mM Tris-HCl, 0.9% NaCl, pH 8), twice with buffer H containing 0.05% Nonidet P40 (10 min each), and once again with buffer H (10 min). Filters were probed for 2 h with conjugated goat anti-mouse alkaline phosphatase in blocking buffer and washed as before. Alkaline phosphatase-labeled antigen was detected by washing filters twice with 0.1 M Tris-HCl, pH 9.2, then incubating at room temperature with 0.1 M Tris-HCl, pH 9.5, 0.33% (v/v) dimethylformamide, 0.1 M NaCl, 5 mM $MgCl_2$, 0.33 mg/mL nitroblue tetrazolium chloride, 0.17 mg/mL 5-bromo-4-chloro-3-indoyl phosphate.

Electrophoretic transfer of proteins from PAGE gels to nitrocellulose was carried out with a Bio-Rad Transblot apparatus. Transfers were carried out overnight at 4°C, 30 V, 100 to 300 mA, in a buffer containing 20 mM Tris, 192 mM Gly, 20% methanol. Transferred protein was detected on filters by staining with 0.3% Ponceau S in 3% TCA. Binding of rabbit or mouse antibodies to filters was detected by incubation of filters with conjugated goat anti-rabbit or anti-mouse alkaline phosphatase (respectively) and alkaline phosphatase was detected as above.

RESULTS

Reductase Solubilization and Extraction from *P. hybrida* Flowers

NADPH-Cyt *c* reductase activity was associated with the microsomal membrane fraction of flower buds irrespective of whether the membrane fraction was isolated by ultracentrifugation of the homogenate or by differential sedimentation with 40 mM $MgCl_2$ (see "Materials and Methods"). Isolation of the microsomal fraction by ultracentrifugation yielded more protein and enzyme (4- and 3-fold, respectively), although specific activity was slightly lower. On average, Old Glory Blue flower buds yielded 17 ± 5 units of reductase per kg flower buds at a specific activity of 10 to 60 milliunits/mg protein (determined from desalted CHAPS extracts of microsomal membranes). Nascent, open flowers were also a useful source of microsomal reductase, yielding 55% of the activity compared to flower buds (per g wet weight) and with similar specific activity. CHAPS, Nonidet P40, Tween 80 (Sigma), or octylglucoside (Boehringer-Mannheim) effectively solubilized NADPH-Cyt *c* reductase activity, but higher concentrations of Zwittergent 3–08 (Calbiochem) and hexylglucoside (Calbiochem) were required to extract the same amount of enzyme activity. Yields of microsomal reductase by CHAPS extraction were similar in the range 0.5 to 3% CHAPS (w/v). A NADH-Cyt *c* reductase activity was also solubilized from the microsomal fraction of flower buds by CHAPS. The NADH-Cyt *c* reductase was assayed in the same way as the NADPH-Cyt *c* reductase, apart from the replacement of NADPH by NADH. In one extract, the specific activity of the NADH-Cyt *c* reductase was measured at 66 milliunits/mg, compared to 26 milliunits/mg for NADPH-Cyt *c* reductase.

The supernatant of ultracentrifuged CHAPS-extracted microsomal membranes was separated into macromolecular and low molecular mass fractions by chromatography on Sephadex G-25. The low molecular mass fraction contained an apparent Cyt *c* reductase activity that in one instance gave a 25-fold higher rate of Cyt *c* reduction than the NADPH-Cyt

c reductase measured in the high molecular mass fraction. The Cyt *c* reductase activity of the low molecular mass fraction was independent of NADPH, it passed through a 10-kD cut-off ultrafiltration membrane, it was sensitive to brief boiling, and it was not a property of endogenous anthocyanin pigment. This nonenzymic Cyt *c* reduction was characterized by a rapid decrease in the rate of Cyt *c* reduction with time, whereas enzymic reductions of Cyt *c* were constant with time.

Chromatographic Purification of NADPH-Cyt c Reductase

The only effective purification procedure was to separate NADPH-Cyt *c* reductase in CHAPS-extracted microsomal protein first on 2′,5′-ADP-Sepharose followed by high-performance anion exchange on a Pharmacia fast protein liquid chromatography system. Affinity chromatography with FMN-agarose, Cyt *c*-agarose, and $NADP^+$-agarose failed to purify the reductase. The affinity adsorbent 2′,5′-ADP-Sepharose was effective in the purification of NADPH-Cyt *c* reductase, although recovery of activity was only 25% on average. $NADP^+$ and NADPH could be used to elute the reductase; however, the best and most reliable results were obtained with 2′-AMP used at a concentration of 5 mM. The enzyme was stable for months stored frozen at −75°C in the presence of this eluent. On average, 39% of the CHAPS-extracted enzyme activity failed to adsorb to 2′,5′-ADP-Sepharose. When an anti-NADPH-Cyt *c* reductase monoclonal antibody was used to probe western-blotted proteins from the unadsorbed fraction, no cross-reacting antigen was detected, indicating that the nonadsorbing activity was not due to the same NADPH-Cyt *c* reductase. Figure 1 shows proteins eluted from 2′,5′-ADP-Sepharose by 2′-AMP and separated on SDS-PAGE.

To further resolve the affinity-purified NADPH-Cyt *c* reductase, a high-resolution anion-exchange adsorbent was used. This adsorbent, Q-Sepharose High Resolution, resolved the reductase to a single activity-containing peak (Fig. 2). Results of a typical purification experiment are presented in

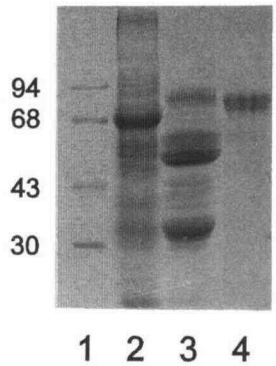

Figure 1. Coomassie-stained SDS-PAGE of NADPH-Cyt *c* reductase at various stages of purification. Lane 1, Molecular mass markers (kD); lane 2, 105,000*g* supernatant of CHAPS-extracted microsomal protein; lane 3, proteins eluted by 2′-AMP from 2′,5′-ADP-Sepharose; lane 4, pooled fractions from Q-Sepharose High Performance containing NADPH-Cyt *c* reductase activity.

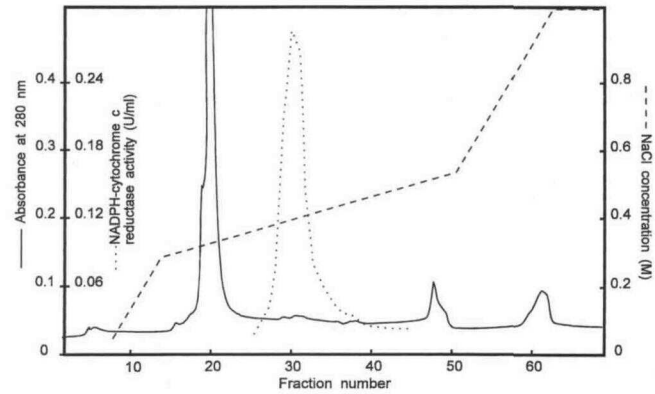

Figure 2. A typical elution profile of NADPH-Cyt *c* reductase activity and protein eluted from a Q-Sepharose High Performance anion-exchange column. The sample applied to the Q-Sepharose contained reductase activity that was partially purified by affinity chromatography on 2′,5′-ADP-Sepharose. NADPH-Cyt *c* reductase activity was determined by measurement of the rate of Cyt *c* reduction in the presence NADPH and protein was determined by measurement of A_{280}.

Table I. Recovery of NADPH-Cyt *c* reductase activity from Q-Sepharose anion exchanger was approximately 30% on average. The combination of affinity and anion-exchange chromatography was sufficient to purify the NADPH-Cyt *c* reductase to two major bands on SDS-PAGE (Fig. 1), although some samples displayed an additional band. Additional proteins appeared to result from an in vitro process, probably proteolysis, since stored samples initially containing two protein bands were later found to contain other, smaller proteins on SDS-PAGE. This occurred even though protease inhibitors were routinely added to extraction and chromatography buffers, although inhibitors were not included in anion-exchange and storage buffers. Concentrated, purified NADPH-Cyt *c* reductase was yellow, indicative of the presence of flavin nucleotide prosthetic groups.

Reductase Properties

NADPH-Cyt *c* reductase activity was stable at pH 6 to 8. When the pH of unpurified microsomal extract was lowered below 6, the reductase activity declined rapidly. Storage of reductase at −75°C for 4 months resulted in a slight activation of the enzyme activity, possibly due to the formation of a smaller but more active enzyme by proteolysis. Subsequently, a slow decline in activity was observed. The enzyme was stable in the presence of all detergents tried: CHAPS, Emulgen 911, Nonidet P40, and sodium cholate.

NADPH-Cyt *c* reductase was completely dependent on NADPH for activity; in the presence of 50 μM $NADP^+$, NADH or NAD^+ activity was not detected. Reductase activity was higher when FMN and FAD were added to the standard assay. When activities were determined in the presence of 5 μM FMN and 1 μM FAD, activity increased by 30 to 60% compared with the activities measured in their absence. The changes in activity were consistent irrespective of the degree of purification. It was sometimes necessary to add FMN/FAD

Table I. *Purification of NADPH-Cyt c (Cyt P450) reductase from P. hybrida flower buds*

Microsomal fraction isolated from 3.5-kg flower bud (limb) was solubilized with 0.75% CHAPS and centrifuged at 105,000g, and the supernatant was desalted. NADPH-Cyt c reductase in this preparation was purified by affinity chromatography followed by fast protein liquid chromatography/anion-exchange chromatography. The final amount of purified protein was determined by comparison of Coomassie-stained samples run on SDS-PAGE with a range of standard proteins. Therefore, the amount is subject to a large error and is expressed as a range of values.

Purification Step	Total Protein	Enzyme Activity	Specific Activity	Purification	Yield
	mg	*units*	*units/mg*	*-fold*	%
Microsomes	5000				
Desalted, CHAPS-extracted	2700	31.6	0.012	1	100
Affinity chromatography (2′,5′-ADP-Sepharose 4B)	1.2	8.2	6.8	610	26
Anion-exchange (Q-Sepharose High Performance)	0.05–0.1	2.8	30–60	2500–5000	9

to reconstitute reductase activity when eluted with NaCl from anion exchangers.

Amino Acid Sequence Analysis of Reductase

Peptides resulting from the digestion of NADPH-Cyt *c* reductase by trypsin were efficiently resolved by reversed-phase HPLC using a 200-mL gradient of 0 to 70% acetonitrile. About 70 peptides were detected at 214 nm and well-resolved peaks were collected and sequenced. Two peptides had overlapping sequences, and a comparison of this sequence with a sequence data base revealed that there was significant sequence similarity with the FAD-NADPH binding domains of several plant NADPH-Cyt P450 reductases (Table II). There was lesser sequence similarity to *Bacillus megaterium* Cyt P450:NADPH-Cyt P450 reductase precursor and to yeast and animal NADPH-Cyt P450 reductases.

Kinetic Characterization

The K_m for NADPH was determined to be 9.2 ± 1.0 μM (reduced, χ^2 was 3.4×10^{-2}) and the K_m for Cyt *c* was 2.8 ± 1.0 μM (reduced, χ^2 was 0.17). Eadie-Hofstee plots were linear, indicating simple Michaelis-Menten kinetics. The pH optimum for purified NADPH-Cyt *c* reductase activity was 7.3 ± 0.1. Enzyme activity was influenced by the ionic strength of the buffer. Activity measured in low-ionic-strength buffer (0.02 M) was one-fifth of the activity measured at optimum ionic strength (0.25 M). Activity in the presence of potassium or sodium phosphate was slightly higher than with sodium chloride or sodium sulfate. Enzyme activity in a low-ionic-strength buffer containing 15% (0.64 M) glycerol was similar to the activity measured in the absence of glycerol.

Immunological Characterization of NADPH-Cyt *c* Reductase

Two monoclonal antibodies were selected for experimental use by probing western blots of purified antigen with hybridomal supernatant. The antibody isotypes differed, one was IgG_1 and the other IgG_{2b}, and both had κ light chains. These monoclonal antibodies detected multiple minor bands on western blots of purified antigen as well as the major bands detected by protein staining. Purified *P. hybrida* NADPH-Cyt *c* reductase contained two and sometimes three major protein bands, indicating the presence of proteases. Anti-(Jerusalem artichoke) NADPH-Cyt P450 reductase polyclonal antibody cross-reacted with purified *P. hybrida* NADPH-Cyt *c* reductase. No protein A-Sepharose-purified rabbit preimmune serum was available as control antibody, but monoclonal antibodies raised against rat mitochondrial membrane proteins failed to cross-react with any of the reductase preparations, and mouse preimmune sera also failed to cross-react (data not shown). The patterns of protein bands detected by the monoclonal and polyclonal antibodies were similar (Fig. 3), although the smaller of the three major bands was poorly detected by the monoclonal antibody. The full size of the NADPH-Cyt *c* reductase was estimated by probing western blots of fresh CHAPS-extracted microsomal protein with monoclonal and polyclonal antibodies. In this way, a single 84-kD protein band was identified by the monoclonal and polyclonal antibodies, indicating that proteolysis gave rise to the other, smaller proteins (data not presented).

The monoclonal antibodies did not appear to recognize native NADPH-Cyt *c* reductase. The enzyme activity was not decreased by these antibodies and the antibodies did not immunoprecipitate the activity. However, the polyclonal antibody partly inhibited NADPH-Cyt *c* reductase activity but not a CHAPS-extracted microsomal NADH-Cyt *c* reductase activity (Table III). An activity-staining method utilizing the chromogenic redox compound neotetrazolium chloride labeled a protein with a molecular mass similar to that of the purified reductase, which was detected by an anti-NADPH-Cyt *c* reductase monoclonal antibody on a western blot (data not shown).

DISCUSSION

To date, only two protein components of plant monooxygenases, a Cyt P450 and a flavoprotein (NADPH-Cyt P450 [Cyt *c*] reductase), have been described (for reviews, see West, 1980; Donaldson and Luster, 1991). Both types of enzyme are intrinsic proteins present in the microsomal membrane fraction. In most eukaryotic tissues, apart from Jerusalem artichoke tubers (Benveniste et al., 1991), one type of NADPH-Cyt P450 reductase mediates the transfer of electrons from NADPH to multiple Cyts P450 (Porter et al.,

Table II. *Comparison of primary sequence of two tryptic peptides with computer data base sequences*

Purified NADPH-Cyt c reductase was digested with trypsin and peptides produced were separated by reversed-phase HPLC. The sequences of two tryptic peptides were compared with DNA sequences in ANGIS data bases (Australian National Genomic Information Service) and sequences to which greatest homology was found are presented. The identities of the residues in brackets are not certain. Acc. No., GenBank accession number.

Acc. No.	Sequence	Species
	- - R M D F I Y E E E L Q S F V D Q G V I A - -	*P. hybrida*
X66016	^{584}N R Q M D F I Y E D E L N N F V D Q G V I S E L	*A. thaliana*[a]
X66017	^{604}N R R M D F I Y E E E L Q R F V E S G A L A E L	*A. thaliana*[a]
L07843	^{582}N R Q M D F I Y E D E L K S F V E Q G S L S E L	*V. radiata*[b]
Z26252	^{584}N R Q M D F I Y E D E L N N F V Q Q G A I S E L	*V. sativa*[c]
Z26250	^{598}N R K V D F I Y E N E L N N F V E N G A L S E L	*H. tuberosus*[d]
X69791	^{606}N R K M D Y I Y E D E L N H F L E I G A L S E L	*C. roseus*[e]

[a] C. Mignote-Vieux, M. Kazmaier, F. Lacroute, D.M. Pompon Centre de Genetique Mol du Centre National de la Recherche Scientifique, France, unpublished sequences. [b] Shet et al. (1993). [c] I. Benveniste, C. Begue-Kirn, A. Lesot, M. Hasenfratz, F. Durst, Centre National de la Recherche Scientifique-Institut de Biologie Moleculaire des Plantes, Cellular and Molecular Enzymology, France, unpublished sequence. [d] M. Hasenfratz, J. Jeltsch, I. Benveniste, A. Lesot, F. Durst, Centre National de la Recherche Scientifique-Institut de Biologie Moleculaire des Plantes, Cellular and Molecular Enzymology, France, unpublished sequence. [e] Meijer et al. (1993).

1990). Although higher plant NADPH-Cyt P450 reductases exhibit immunological homology, higher plant and mammalian enzymes are immunologically distinct. A survey of NADPH-Cyt P450 reductases in higher plants demonstrated that all were 82 kD in size (Benveniste et al., 1989; Shet et al., 1993). The purified *P. hybrida* reductase was subjected to limited degradation during purification and storage, as indicated by antibody-probed western blots and SDS-PAGE. This degradation probably resulted from the co-purification of a low-abundance protease. The full, undegraded molecular mass was estimated to be 84 kD. The size of the *P. hybrida* NADPH-Cyt P450 reductase is therefore similar to the sizes of these enzymes, although the purified *P. hybrida* reductase preparation also contained a degraded protein of smaller size. The proteins to which the monoclonal antibodies have affinity also have the ability to reduce neotetrazolium chloride in a manner analogous to other NADPH-Cyt P450 (Cyt *c*) reductases (Fan and Masters, 1974; Fujita and Asahi, 1985).

We believe that our data indicate that the *P. hybrida* NADPH-Cyt *c* reductase is a component of the flavonoid 3′,5′-hydroxylase activity, which we have studied separately (Menting et al., 1994). It is interesting that the two essential monooxygenase components are expressed differently in developing flowers. The specific activity of the reductase is similar in flower buds and in open flowers, but the flavonoid

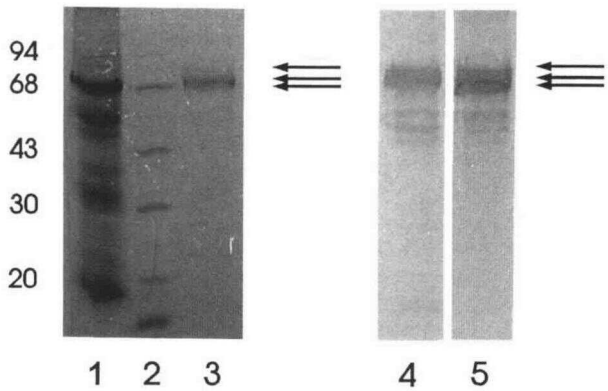

Figure 3. Detection of western-blotted NADPH-Cyt c reductase with anti-NADPH-Cyt P450 (Cyt c) reductase antibodies. Lanes 1 to 3, Coomassie-stained SDS-PAGE. Lane 1, 105,000g supernatant of CHAPS-extracted microsomes; lane 2, molecular mass markers (sizes in kD at left); lane 3, NADPH-Cyt c reductase purified by 2′,5′-ADP-Sepharose and Q-Sepharose chromatography. Replicates of lane 3 were electrophoretically transferred to nitrocellulose and probed with anti-NADPH-Cyt c reductase monoclonal antibody (lane 4) and a NADPH-Cyt P450 (Cyt c) reductase polyclonal antibody (lane 5). Arrows indicate three major proteins detected by Coomassie staining and the corresponding bands detected by the antibodies. In all cases control antibodies failed to cross-react with preparations containing NADPH-Cyt c reductase.

Table III. *Determination of the effect of a rabbit anti-Jerusalem artichoke NADPH-Cyt P450 (Cyt c) reductase polyclonal antibody on NADPH-dependent and NADH-dependent Cyt c reductase activities*

Unpurified, desalted CHAPS-solubilized microsomal NADPH- and NADH-Cyt c reductases were assayed following a brief incubation with anti-NADPH-Cyt P450 (Cyt c) reductase antibody. Results are averages of two determinations. Antibody was absent from the control assays.

Antibody Dilution	NADPH-Cyt c Reductase		NADH-Cyt c Reductase	
	units/mL	*% control*	*units/mL*	*% control*
Control	0.057	100	0.166	100
1:2500	0.053	93	0.158	95
1:25	0.014	25	0.162	98

3′,5′-hydroxylase is not active in open flowers (Menting et al., 1994). This may indicate that the same reductase is responsible for supplying reducing equivalents to several Cyt P450 isoenzymes. If so, then it must be expressed constitutively during the developing and mature stages of the corolla.

Reductase was purified from the microsomal fraction of *P. hybrida* flowers. This membrane fraction was isolated after a 20,000*g* centrifugation to remove mitochondria and other cellular components. Therefore, the NADPH-Cyt *c* reductase is likely to be derived from the ER or perhaps from another membrane such as the plasmalemma (West, 1980; Møller and Lin, 1986), which had NADPH-Cyt *c* reductase activity. It was necessary to remove low molecular mass material to accurately quantitate NADPH-Cyt *c* reductase activity. Reports of a similar phenomenon in other organisms have not been found.

The combination of 2′,5′-ADP-Sepharose and high-performance anion-exchange chromatography were effective in the purification of the reductase, and these were the only chromatographic media found to be effective. Most reports of NADPH-Cyt P450 (Cyt *c*) reductase purification procedures since about 1975 include the use of 2′,5′-ADP-Sepharose with 2′-AMP as the eluent. Here the method differs slightly in that the affinity adsorbent was used as the first chromatographic step. Once purified, the enzyme was relatively stable, but during chromatography losses of activity were high.

P. hybrida NADPH-Cyt P450 reductase K_m values for Cyt *c* (2.8 μM) and NADPH (9.2 μM) fall in the range of published values (Cyt *c*, 2–8 μM; NADPH, 6–20 μM) for NADPH-Cyt P450 reductase from other plants (Madyastha and Coscia, 1979; Fujita and Asahi, 1985; Benveniste et al., 1986; Petersen and Seitz, 1988; Shet et al., 1993). Ionic strength greatly affected the activity of the enzyme. This property of the *P. hybrida* NADPH-Cyt P450 reductase is similar to that of rat NADPH-Cyt P450 reductase, but the Jerusalem artichoke enzyme was only slightly influenced by ionic strength. This observation is unexpected, since the evidence points to greater similarities between the higher plant enzymes than between plant and mammalian enzymes.

The polyclonal antibody cross-reacted with *P. hybrida* NADPH-Cyt *c* reductase, so the enzyme has epitopic homology to Jerusalem artichoke NADPH-Cyt P450 (Cyt *c*) reductase (Benveniste et al., 1989) and sequence homology to mammalian, yeast, and bacterial NADPH-Cyt P450 (Cyt *c*) reductase enzymes. The *P. hybrida* reductase sequence obtained here shares conserved residues with the FAD-NADPH binding region of NADPH-Cyt P450 reductases (Vogel and Lumper, 1986; Porter et al., 1990). This region is also common to GSH reductase, Fd-$NADP^+$ reductase, and NADH-Cyt b_5 reductase genes (Porter and Kasper, 1986). Taken together, the data indicate that the enzyme purified here is a NADPH-Cyt P450 (Cyt *c*) reductase with similar properties to all other plant NADPH-Cyt P450 (Cyt *c*) reductases described.

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