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MULTIPLE FORMS OF NADPH-CYTOCHROME P450 REDUCTASE IN HIGHER PLANTS

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SUMMARY : We report on the presence of multiple forms of NADPH-cyt P450 reductase in microsomes from higher plants. This contrasts with the animal cyt P450 monooxygenases, where the numerous cyt P450 isoforms are reduced by a single form of reductase. Three NADPH-cyt c reductases have been resolved from Jerusalem artichoke tuber microsomes by chromatography on Reactive Red Agarose and Concanavalin A-Sepharose. Their molecular weights, determined by sodium dodecylsulfate-gel electrophoresis, are 80,000, 82,000 and 84,000. The three proteins share common epitopes and are dependent upon FMN for catalytic activity. They are highly selective for NADPH as electron donor, and allowed effective reconstitution of *trans*-cinnamic acid and 3,9-dihydroxypterocarpan 6a-hydroxylase activities with purified cyt P450 fractions from *Helianthus tuberosus* and *Glycine max*, respectively. As such, they appear as true isoenzyme forms of NADPH-cyt P-450 reductase.

In eukaryotes, the microsomal cyt P450-dependent monooxygenases are multienzyme complexes consisting essentially of the cyt P450, which serves as the terminal oxidase, and of an FAD and FMN containing flavoprotein, the NADPH-cyt P450 reductase, which transfers two electrons from NADPH to the hemoprotein. In animals, the existence of multiple cyt P450s is well established since many years (1). In plants, the multiplicity of the cyt P450 is also becoming obvious (2-4). In contrast, no isozymes of NADPH-cyt P450 reductase have been detected in a particular animal source (5). However, we have recently observed, in Western blots from wound-induced Jerusalem artichoke microsomes, the presence of three proteins (6), which cross-reacted with specific antibodies (7) raised against the purified artichoke reductase (8). To elucidate the question of the existence of isozymes of the NADPH-cyt P450 reductase in plant microsomes, we have attempted to isolate the three proteins.

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This communication describes, for the first time, the separation and characterization of three isoenzymes which are able to transfer reducing equivalents from NADPH to cyt P450s. The activity of the three enzymes is dependent upon FMN, as generally described for the NADPH-cyt P450 reductase. Finally, evidence for multiple reductase forms in other plants, based on Western blot analysis, is presented.

MATERIALS AND METHODS

Materials : Emulgen 911 was a generous gift from Kao Chemical Co.. CHAPS (3-([3-cholamidopropyl]-dimethylammonio)-1-propane sulfonate), Reactive Red 120-Agarose with diverse concentrations of dye, Concanavalin A-Sepharose and α-methylmannoside were from Sigma Chemical Co., Bio-beads SM-2 from Bio-Rad, DEAE Trisacryl from IBF and 2'5' ADP-Sepharose 4B and molecular weight marker proteins from Pharmacia. Nitrocellulose (45 µm pore size) was from Schleicher and Schüll.

Enzyme assays : NADPH- or NADH-dependent reduction of cytochrome *c* was measured spectrophotometrically as described elsewhere (7). For kinetic studies, 14 concentrations ranging from 2.2 µM to 565 µM for NADPH, and from 4 µM to 12 mM for NADH, were used. Reactivation of NADPH-cyt *c* reductase with FMN was achieved by including different concentrations of the cofactor directly into the spectrophotometric assay, without preincubation with the purified reductases.

SDS-polyacrylamide gel electrophoresis : The purity of the NADPH-cyt c reductase preparations was checked by SDS-polyacrylamide gel electrophoresis (7.5 or 12.5 % acrylamide, w/v), after Laemmli (9). Protein bands were stained by silver nitrate. Molecular weights were estimated using standard proteins (14-93 kilodaltons) electrophoresed under identical conditions.

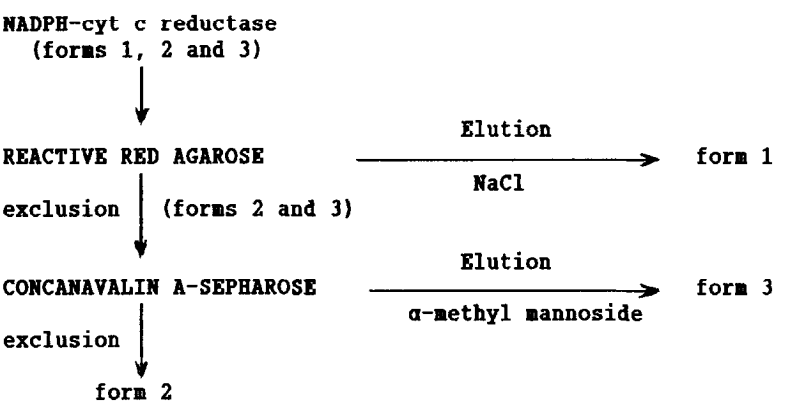
Western blot analysis : The microsomal proteins separated by SDS-polyacrylamide gel electrophoresis were electrotransferred onto nitrocellulose, after Towbin et al. (10). The remaining protein-binding sites on the nitrocellulose were blocked by 5% bovine serum albumin and 5% non-fat dry milk, in solution in phosphate-buffered saline containing 0.4% Tween 20. The rabbit anti-reductase serum was diluted 20,000 times. The specific antigen-antibody complexes were revealed by goat anti-rabbit immunoglobulins coupled with alkaline phosphatase. BCIP (5-Bromo-4-chloro-3-indolyl phosphate) and NBT (Nitroblue tetrazolium), the substrates of the phosphatase were incubated at pH 9.6 in 0.1 M diethanolamine.

Isolation of the multiple forms of NADPH-cyt c reductase : The NADPH-cyt c reductase was purified from 1-2 g microsomal protein from Jerusalem artichoke tuber by chromatography on DEAE-Trisacryl M and 2'5' ADP-Sepharose 4B columns, in the presence of Emulgen 911, as described previously (8). This preparation was then submitted to dye affinity chromatography, on a 20 ml Reactive Red 120 Agarose column (3.4 µmoles dye/ml gel), equilibrated with 10 mM KPi, pH 7.4 containing 20% glycerol, 0.2% Emulgen 911, 1.5 mM β-mercaptoethanol and 1mM EDTA. The fraction of reductase activity partially retained on the dye gel (form 1) was eluted by increasing salt conc.. The unbound reductase was applied onto a Concanavalin A-Sepharose column (15 ml) equilibrated with 10 mM Tris-HCl buffer, pH 7.2, containing 20% glycerol, 0.1% Emulgen 911, 1.5 mM β-mercaptoethanol and 0.4M NaCl. This step resolved the reductase into two forms : form 2 was excluded from the lectin column, form 3 was retained and eluted by 0.6M α-methylmannoside. In order to replace Emulgen 911 by the zwitterionic detergent CHAPS, the enzymes were applied onto a small DEAE-Trisacryl column (5 ml), and eluted by a gradient of KCl (0-0.4 M) in the presence of CHAPS, and finally concentrated on carboxy methylcellulose.

Reconstitution of monooxygenase activity : The cyt P450 isoform hydroxylating cinnamic acid was purified by chromatography on DEAE-Trisacryl and on Hydroxylapatite (4). This preparation was totally free from any NADPH-cyt c reductase contamination. For reconstitution of monooxygenase activity, 26 pmoles cyt P450, in solution in 10mM NaPi buffer, pH7.4, 10% glycerol and 0.9% Emulgen 911, were mixed with different quantities of each reductase form. No phospholipids were added. The mixtures were treated by 100 µl Biobeads, equilibrated with 0.1 M NaPi buffer, pH 7.4, to remove excess detergent. These reconstituted systems were then incubated at 26°C for 1 hour in the presence of 10 µM FAD and FMN, 500 µM NADPH and 22 µM [^{14}C] cinnamate, and cinnamate hydroxylation measured as described (11). Pterocarpan hydroxylase activity reconstitution was as described in (3).

RESULTS

Isolation of the isoforms of Jerusalem artichoke NADPH-cyt c reductase: Affinity chromatography on 2'5' ADP-Sepharose 4B is very efficient for the purification of NADPH-cyt P450 reductase : this step yields a single protein from mammalian liver microsomes (12, 13). The NADPH-cyt c reductase from Jerusalem artichoke tuber microsomes was also specifically isolated by this procedure (8), however high resolution SDS-PAGE showed that in fact three proteins were co-purified (Fig.1). Their apparent molecular weight, measured by comparison with standard proteins after SDS-polyacrylamide gel electrophoresis, is 80,000 (form 1), 82,000 (form 2) and 84,000 (form 3).



To separate these proteins, we have deviced the purification scheme summarized above. It is based on the differential affinities of the three isoforms for lectins and substrate-mimicking dyes.

Reactive Red-Agarose has been shown to resolve $NADP^+$ dependent enzymes (14). Form 1 was retained by a gel grafted with 3.4 µmoles dye per ml agarose, while form 2 was only retarded and form 3 was readily excluded. After abundant washing of the column with equilibration buffer to remove loosely bound form 2, form 1 was eluted with 150 mM NaCl. Two chromatographic cycles were used to eliminate any contaminating form 2. To avoid binding of form 2 to the gel, we have also tested gels with lower dye concentrations (0.2, 0.5 and 0.96

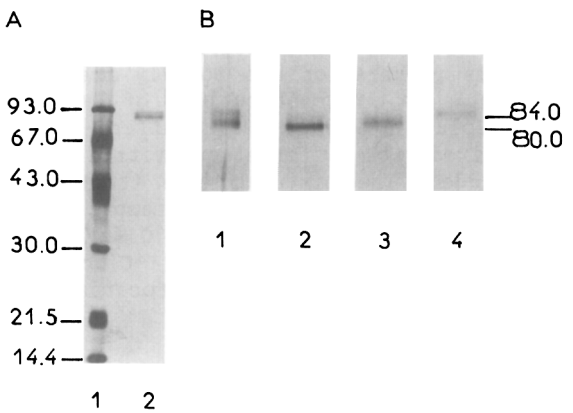


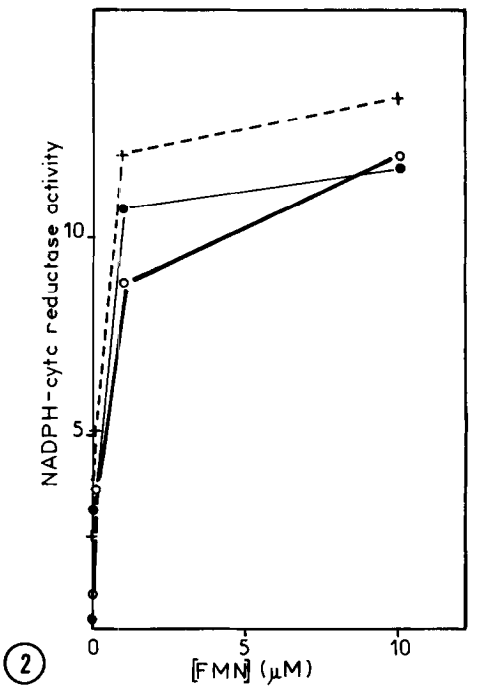
Figure 1 A. SDS-polyacrylamide gel (12.5 %) electrophoresis profile of Jerusalem artichoke NADPH-cyt P450 reductase after affinity chromatography on 2'5'ADP-Sepharose. **Lane 1** : molecular weight scaled in dalton x 10^{-3}. **Lane 2** : NADPH cyt c reductase.
Figure 1 B. SDS-polyacrylamide gel (7.5%) electrophoresis profile of the purified forms of NADPH-cyt P450 reductase. **Lane 1** : NADPH-cyt P450 reductase after purification on 2'5'ADP-Sepharose. **Lane 2** : form 1 of the NADPH-cyt c reductase. **Lane 3** : form 2. **Lane 4** : form 3 (glycoprotein). Proteins were silver-stained.

µmole dye per ml agarose). These gels, however, did not bind any of the three isoforms, form 1 being only retarded.

Form 2 and 3 were separated by Concanavalin A-Sepharose chromatography. Form 2 was not retained by the lectin column, while form 3 was bound and specifically eluted with α-methylmannoside, indicating a glycoprotein nature of this form of reductase. Fig.1B shows the SDS-polyacrylamide gel electrophoresis profile of the three purified NADPH-cyt c reductases.

Kinetic studies : We have measured the apparent affinity of the three forms of reductase for NADPH, in the presence of 50µM cyt c. The three enzymes exhibited typical Michaelis-Menten kinetics when the concentration of NADPH was varied from 2.2µM to 565µM. The apparent Km (=20 µM) for NADPH was identical for the three purified enzymes. Kinetics of cyt c reductase in the presence of NADH could not be measured, since NADH reduced non-enzymatically the cyt c (2.5 µM FMN was present in the assay) : the reactions were not saturable, with respect to the NADH concentrations used (up to 12 mM).

Reactivation by FMN of NADPH-cyt c reductase : Microsomal NADPH-cyt P450 reductase is characterized by the presence of both FAD and FMN (8, 15), whereas NADH-cyt b_5 reductase possesses only FAD (16). Loss of FMN, which occurs readily in reductase preparations (8), results in enzyme inactivation. Activity can be restored *in vitro* by addition of FMN to the incubation medium (17). Fig.2 shows the progressive restoration of the NADPH-cyt c reductase activity of the three forms, after addition of increasing amounts of FMN.



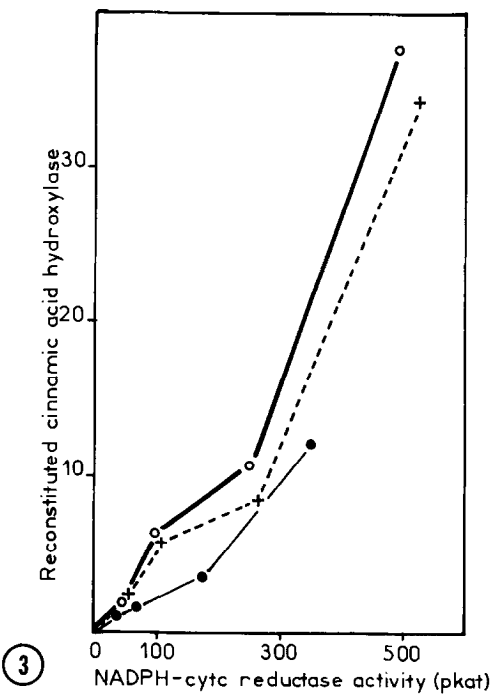


Figure 2. Reactivation of the NADPH-cyt c reductase activity of the three isoforms as a function of the concentration of FMN added. NADPH-cyt c reductase activity is expressed in nkat reduced cyt c per ml isoform . (●—●) form 1 ; (+---+) form 2 ; (o▬o) form 3.

Figure 3. Reconstitution of cinnamic acid 4-hydroxylase activity by the three NADPH-cyt P450 reductase isoforms. Reductases are expressed as enzyme units (pkat cyt c reduction activity). Cinnamic acid hydroxylase activity is expressed as pkat p-coumarate formed/nmole cyt P450. (●—●) form 1 ; (+---+) form 2 ; (o▬o) form 3.

Reconstitution of monooxygenase activity : A cyt P450 fraction deprived of reductase activity and highly enriched in the hemoprotein catalysing the hydroxylation of cinnamic acid was prepared. Monooxygenase activity was reconstituted by the addition of increasing concentrations of each of the three forms of reductase (Fig.3). All three isolated reductases from plant microsomes were able to transfer electrons to the cyt P450 catalysing cinnamate hydroxylation and may, therefore, be considered as isoforms of the NADPH-cyt P450 reductase.

These isoforms showed no selectivity for the physiological electron acceptor, since reconstitution of 3,9-dihydroxypterocarpan 6a-hydroxylase activity was achieved by addition of each isoenzyme to cyt P450 (3) isolated from elicitor-challenged soybean cell cultures (results not shown).

Evidence for reductase isoforms in other plant microsomes : Polyclonal antibodies raised against the purified artichoke NADPH-cyt P450 reductases recognized specifically these flavoproteins (7). They were used as tools to investigate the existence of immunorelated multiple proteins in other plant

species, by Western blot analysis on microsomal proteins. Specific proteins from other plant microsomes, with electrophoretic mobility similar to those of the artichoke reductases (molecular weights between 80 and 84 kDa), cross-reacted with the anti-artichoke reductase antiserum. Three proteins were detected in microsomes from maize embryos, *Catharanthus* cell suspension culture, *Vicia sativa* seedlings. Two immunoreactive proteins were revealed in wounded potato tuber, avocado pear, and in *Veronica* and wheat cell suspension cultures.

DISCUSSION

Three microsomal NADPH-cyt c reductases have been isolated from wound-induced Jerusalem artichoke tubers, in spite of similar apparent affinity for NADPH and very close molecular weights (80, 82 and 84 kD). Surprisingly, the three forms behaved differently on the dye affinity column. Reactive Red, like other triazine dyes, is believed to mimic the diphosphate link of pyridine nucleotides, thus binding $NAD(P)^+$-depending enzymes. The affinity of the three proteins for Reactive Red appeared weak, since only form 1 was bound onto the gel with the highest commercially available dye concentration per ml of gel. Lack of binding of forms 2 and 3 could result from folding of the polypeptide chains hindering the access of the active site to the immobilized dye. Alternatively, differences in the hydrophobic and ionic interactions with the gel matrix could also explain the observed chromatographic behaviour.

The interaction of form 3 with Concanavalin A-Sepharose and its selective elution by α-methylmannoside are consistent with the existence of an oligosaccharide moiety, rich in mannose and/or glucose. This is the first example of a glycosylated form of a microsomal NADPH-cyt *c* reductase, in animals and in plants. However, at this stage, the glycoproteic nature of forms 1 and 2 cannot be excluded, since the binding to other lectins, characteristic of other glycanes, has not yet been tested.

All three enzymes are NADPH-cyt *c* reductases with similar apparent Km for NADPH. Furthermore, they share common immunogenic domains since several monoclonal antibodies that we prepared cross-react with all forms. Therefore, one could suppose that the three isoforms would originate from proteolytic cleavage of a single native enzyme. However, all three forms were active and efficient NADPH-cyt P450 reductases. This excludes proteolytic degradation since it has been shown that the reductase lacking the hydrophobic domain, readily cleaved at very low protease concentration, becomes unable to transfer electrons to cyt P450 monooxygenases (18).

Cinnamic acid hydroxylase activity was reconstituted by mixing the purified cyt P450 fractions and each of the three reductases, without addition of phospholipids. Apparently, the detergent present in the cyt P450

preparation (1% Emulgen 911) replaced efficiently the phospholipids. After reconstitution of the monooxygenase complex, lowering the detergent concentration increased the activity, which reached about 10% of the corresponding microsomal activity. This should be improved by using higher reductase concentrations since activity increased linearly as a function of added flavoprotein and saturation was not reached under the conditions used.

The three artichoke reductase isoforms were also able to reconstitute 3,9-dihydroxypterocarpan 6a-hydroxylase with cyt P450 purified from elicitor-challenged soybean cell cultures (3). Catalytic interaction between NADPH-cyt P-450 reductase and cyt P450s from different origins is a general phenomenon. For example, trout reductase was as effective as rat reductase in a reconstituted system that contained rat cyt P448 (19). Likewise, p-chloro-N-methylaniline demethylase of purified avocado cyt P450s was reconstituted with rat liver reductase (20). Preliminary experiments in our laboratory also showed that reductase purified from hepatic rabbit microsomes would reconstitute cinnamate hydroxylase with artichoke cyt P450.

Two or three proteins, immunorelated with the artichoke NADPH-cyt P450 reductases, were detected in the microsomes from all higher plants that we tested. Although this evaluation may be under-estimated since low-represented immunoreactive proteins would not be detected by Western blotting of microsomes, multiple forms of reductase seem to be the rule in higher plants. In animal systems, it is admitted that a single microsomal NADPH-cyt P450 reductase is involved in cyt P450-dependent oxidations, in fatty acid desaturation and elongation, in heme oxygenation and in squalene epoxidation. The reason why plants have multiple NADPH-cyt P-450 reductase forms and this situation has not evolved or was not conserved in other organisms, remains open. More detailed studies, using several purified cyt P450s, will be needed to determine if all isoforms are equally competent for productive interaction with different hemoproteins. Their involvement in the other redox reactions cited above will also be studied. Another question raised by our findings concerns the origin of reductase multiplicity: are these isoforms encoded by one or several genes or/and could they result from a different post-translational maturation. Finally, the temporal and spatial distribution, in terms of subcellular or tissular localization, of these enzymes during plant development, will be investigated.

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REFERENCES

1. Guengerich, F.P., Dannan, G.A., Wright, S.T., Martin, M.V. and Kaminsky, L.S. (1982) Biochemistry 21, 6019-6030.

2. Benveniste, I., Salaün, J.P., Simon, A., Reichhart, D., and Durst, F. (1982) Plant Physiol. 70, 122-126.
3. Kochs, G. and Grisebach, H. (1989) Arch. Biochem. Biophys. 273, 543-553.
4. Gabriac, B., Werck-Reichhart, D., Teutsch, H., and Durst, F. (1991) Arch. Biochem. Biophys.,in press.
5. Porter, T.D., Beck, T.W. and Kasper, C.B. (1990) Biochemistry 29, 9814-9818.
6. Lesot, A., Benveniste, I., Hasenfratz, M.P. and Durst, F. (1990) Plant Cell Physiol. 31, 1177-1182.
7. Benveniste, I., Lesot, A., Hasenfratz, M.P. and Durst, F. (1989) Biochem. J. 259, 847-853.
8. Benveniste, I., Gabriac, B. and Durst, F. (1986) Biochem. J. 235, 365-373.
9. Laemmli, U.K. (1970) Nature 227, 680-685.
10. Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci USA 76, 4350-4354.
11. Benveniste, I., Salaün, J.P. and Durst, F. (1977) Phytochemistry 16, 69-73.
12. Yasukochi, Y. and Masters, B.S.S. (1976) J. Biol. Chem. 251, 5337-5344.
13. Shephard, E.A., Pike, S.F., Rabin, B.R. and Phillips, I.R. (1983) Anal. Biochem. 129, 430-433.
14. Watson, D.H., Harvey, M.J. and Dean, P.D.G. (1978) Biochem. J. 173, 591-596.
15. Iyanagi, T. and Mason, H. (1973) Biochemistry 12, 2297-2308.
16. Jollie, D.R., Sligar, S.G. and Schuler, M. (1987) Plant Physiol., 85, 457-462
17. Vermilion, J.L. and Coon, M.J. (1978) J. Biol. Chem. 253, 8812-8819.
18. Gunn, J.R. and Strobel, H.W. (1979) J. Biol. Chem. 254, 4177-4185.
19. Williams, D.E., Becker, R.R., Potter, D.W., Guengerich, F.P. and Buhler D.R. (1983) Arch. Biochem. Biophys. 225, 55-65.
20. O'Keefe, D.P. and Leto, K.J. (1989) Plant Physiol. 89, 1141-1149.