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Evidence for cytochrome b_5 as an electron donor in ricinoleic acid biosynthesis in microsomal preparations from developing castor bean (*Ricinus communis* L.)

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The major *b*-type cytochrome in microsomal membrane preparations from developing endosperm of castor bean (*Ricinus communis*) was cytochrome b_5. Cytochrome *P*-450 was also present. The microsomal membranes had Δ^{12}-hydroxylase activity and catalysed the NAD(P)H-dependent hydroxylation of oleate to yield ricinoleic acid. CO had no effect on the hydroxylase activity. Rabbit polyclonal antibodies were raised against the hydrophilic cytochrome b_5 fragment purified from cauliflower (*Brassica oleracea*) floret microsomes. The anti-(cytochrome b_5) IgG inhibited Δ^{12}-hydroxylase, Δ^{12}-desaturase and cytochrome *c* reductase activity in the microsomes. The results indicate that electrons from NAD(P)H were transferred to the site of hydroxylation via cytochrome b_5 and that cytochrome *P*-450 was not involved.

INTRODUCTION

The uncommon C_{18} hydroxy fatty acid ricinoleic acid (Δ^{12}-hydroxyoleic acid) is particularly abundant in the seed triacylglycerols of castor bean (*Ricinus communis*) (Achaya *et al.*, 1964). The substrate for the Δ^{12}-hydroxylase is microsomal oleoyl phosphatidylcholine (Moreau & Stumpf, 1981; Bafor *et al.*, 1991) and the esterified ricinoleate is released, for triacylglycerol assembly, from the complex lipid by the action of a phospholipase A_2 specific for oxygenated fatty acids (Bafor *et al.*, 1991). Many of the hydroxylation reactions in animals and plants which require molecular oxygen and the reductant NAD(P)H involve cytochrome *P*-450 (White & Coon, 1980; Donaldson & Luster, 1991). Previous studies on the Δ^{12}-hydroxylation reaction in castor bean showed that it had the properties of a mixed-function oxygenase (Galliard & Stumpf, 1966) and that the mechanism was by hydroxy substitution at the 12-position of oleate with retention of configuration at that position (Morris, 1967). Hydroxylase activity, however, was reported to be insensitive to CO, but inhibited by CN^- (Galliard & Stumpf, 1966). Moreau & Stumpf (1981), on the other hand, found that the hydroxylation was unaffected by CN^-. There is uncertainty, therefore, about its electron-transport components (Harwood, 1988). Spectrophotometric evidence has implicated cytochrome b_5 as an electron donor to the oleoyl-phosphatidylcholine Δ^{12}-desaturase in microsomal preparations from maturing safflower (*Carthamus tinctorius*) seed (Smith *et al.*, 1990*a*,*b*), and this was later confirmed with antibodies to cytochrome b_5, which inhibited desaturase activity (Kearns *et al.*, 1991; M. A. Smith, unpublished work). Microsomal preparations from castor-bean endosperm also have Δ^{12}-desaturase activity, and this makes it difficult to obtain unequivocal spectrophotometric evidence for particular electron-transport components of the hydroxylase. Here we identify putative redox carriers in the microsomal membrane preparations from developing castor bean and investigate the effect of rabbit antibodies, raised against purified plant cytochrome b_5, on Δ^{12}-hydroxylase activity.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Oleoyl-CoA (sp. radioactivity 52.9 μCi/μmol) was obtained from Amersham International, Amersham, Bucks., U.K. BSA (fraction V, fatty-acid-free), catalase, CoASH and reduced nicotinamide nucleotides were purchased from the Sigma Chemical Co. Goat anti-rabbit IgG–horseradish peroxidase conjugate was from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Plant material and microsomal preparation

Castor-bean (*Ricinus communis*, var. Rogusus) plants were grown from seed in a 16 h photoperiod at 25 °C and an 8 h night at 20 °C. Developing seeds were harvested 30–40 days after flowering. Endosperm tissue was removed, together with the small embryo, and placed in cold 0.1 M-phosphate buffer, pH 7.2. Microsomal preparations were as previously described (Bafor *et al.*, 1991) and either used immediately or stored at −80 °C in 0.1 M-phosphate buffer, pH 7.2, containing BSA (0.1%) and catalase (1000 units/ml) and used within 1 week. Cauliflower (*Brassica oleracea*) heads were purchased locally.

Cytochrome assays

Microsomal cytochrome content was determined by recording the steady-state reduced-minus-oxidized difference spectrum, between 400 nm and 600 nm, using a rapid-scanning split-beam spectrophotometer controlled by an ITT 2020 microcomputer. Reduction was achieved by the addition of excess NADH, ascorbate or sodium dithionite to microsomes suspended in 0.1 M-potassium phosphate buffer, pH 7.2. CO binding spectra were obtained after bubbling CO gas through a solution of dithionite-reduced microsomes. The *b*-type cytochrome content was estimated by using a difference absorption coefficient ($\Delta\epsilon_{558-575}$) of 21 mM^{-1}·cm^{-1} (Estabrook & Werringloer, 1978). Cytochrome *P*-450 was estimated from the (reduced+CO)-minus-reduced difference spectra, using a difference absorption coefficient ($\Delta\epsilon_{450-490}$) of 91 mM^{-1}·cm^{-1} (Omura & Sato, 1964).

Low-temperature (77K) difference spectroscopy was carried out on a scanning split-beam spectrophotometer designed to hold a Dewar flask containing liquid N_2 in which samples in cuvettes could be frozen and maintained at 77 K. Difference spectra of sample pairs, appropriately oxidized and reduced, were measured directly.

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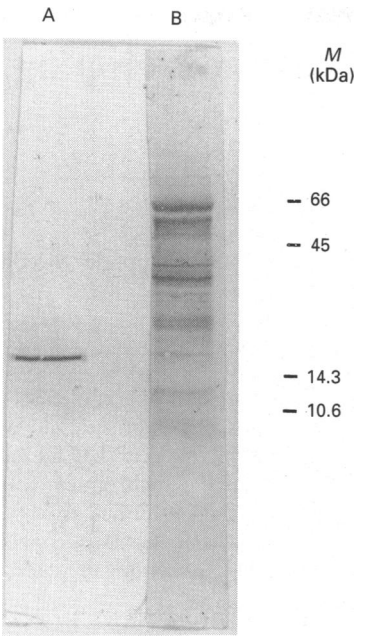

Fig. 1. Western blot of castor-bean microsomal cytochrome b_5

Microsomal membrane proteins were separated by SDS/PAGE and transfered to a Problott membrane by electrophoretic transfer. The membrane was cut longitudinally; one track (A) was used for Western blotting and the other (B) was stained with Coomassie Blue R250. Molecular masses (*M*) of marker proteins are shown on the right.

Purification of cytochrome b_5

The hydrophilic haem-containing fragment of plant cytochrome b_5 was purified to apparent homogeneity, as evidenced by SDS/PAGE electrophoresis, from a trypsin digest of microsomal membranes prepared from cauliflower florets, essentially as described by Reid & Mauk (1982). Polyclonal antibodies were raised in a male New Zealand White rabbit by subcutaneous injection of the purified cytochrome b_5 every 2 weeks for 6 weeks. The initial injection was in Freund's complete adjuvant with subsequent injections using Freund's incomplete adjuvant. IgG was purified from the immune serum by anion-exchange chromatography (Harlow & Lane, 1988). By Western blotting the antibody preparation was shown to react with cytochrome b_5 and to be monospecific for the microsomal cytochrome b_5 protein (Fig. 1).

SDS/PAGE and Western blotting

Electrophoresis in SDS/polyacrylamide gels was performed using the discontinuous procedure of Schägger & von Jagow (1987) with 16% and 10% separating gel and spacer gel respectively.

Proteins were transferred to Problott poly(vinylidene difluoride) membrane (Bio-Rad). Western blots (Towbin *et al.*, 1979) were developed using 1:3000 dilutions of purified IgG, with non-fat dried milk as blocking agent, and goat anti-rabbit IgG–horseradish peroxidase conjugate as the second antibody.

Enzyme assays

Cytochrome *c* reductase activity was measured as described by Moore & Proudlove (1983). The cytochrome *c* in assay mixtures was determined spectrophotometrically at 550 nm by using an absorption coefficient of 21 $mM^{-1} \cdot cm^{-1}$ (Moore & Proudlove, 1983).

Δ^{12}-Hydroxylase activity was measured in microsomal membranes labelled *in situ* with *sn*-2-[1-^{14}C]oleoyl phosphatidylcholine. Microsomes (equivalent to 270 nmol of phosphatidylcholine or 1.2 mg of protein) were incubated with 25 nmol of [1-^{14}C]oleoyl-CoA (2.9×10^6 d.p.m.), BSA (10 mg), CoASH (2 μmol) and catalase (2000 units) in 1 ml of potassium phosphate buffer (0.1 M, pH 7.2) at 25 °C. After 10 min the incubation mixture was diluted with 29 ml of ice-cold phosphate buffer, and the microsomal membranes recovered by centrifugation at 125000 *g* for 30 min. The pellets were resuspended in 0.5 ml of phosphate buffer containing catalase (2000 units/ml). Prelabelled microsomes [equivalent to 13.5 nmol of phosphatidylcholine (55 μg of protein) and containing 50000 d.p.m. of [^{14}C]oleate (0.42 nmol), of which 71% was in position 2 of *sn*-phosphatidylcholine] were incubated with the anti-(cytochrome b_5) IgG for 10 min, 25 °C, in phosphate buffer containing catalase (1000 units/ml) in a total volume of 0.35 ml. NADH (1 μmol) was then added and the reaction was allowed to proceed for 90 min with shaking. The incubations were stopped by the addition of acetic acid, and the lipids were extracted by a modification (Stobart & Stymne, 1990) of the method described by Bligh & Dyer (1959). The total lipid was methylated *in situ* with methanolic HCl (3.5%, w/w) (Kates, 1964) and the fatty acid methyl esters analysed by radio-g.l.c.

Protein was determined by the bicinchoninic acid (BCA) method (Pierce) after treatment of the microsomal preparation with 0.1% SDS; BSA was used as the standard.

RESULTS AND DISCUSSION

Room-temperature dithionite-reduced-minus-air-oxidized difference spectra of microsomes from developing castor beans indicated the presence of *b*-type cytochrome with characteristic α-, β- and Soret bands at 558.5, 528 and 425 nm respectively (Fig. 2). Total dithionite-reducible *b*-type cytochrome amounted to 140 pmol/mg of microsomal protein, of which some 74% and 67% was reduced by NADH and NADPH respectively. The *b*-type cytochrome consisted of at least two components. The major component was reduced by NADH but not by ascorbate, even in the presence of redox mediators, indicating that it had a redox mid-point potential of less than 58 mV [the mid-point potential of ascorbate (Wilson, 1978)]. An ascorbate-reducible component was also present which had a single α-band absorbance maximum at 560 nm in difference spectra at room temperature and 77 K (results not shown). This component accounted for only 10–15% of the total *b*-type cytochrome. Low-temperature (77 K) difference spectra of the NADH-reducible component (NADH-reduced-minus-ascorbate-reduced) resolved the α-band into two distinct peaks at 553 and 559 nm (Fig. 2), a feature characteristic of cytochrome b_5 (Kajihara & Hagihara, 1968). Reduction of the major cytochrome *b* component by NADH indicated an active cytochrome reductase in the microsomal membranes.

CO-binding spectra of dithionite-reduced microsomes showed the presence of cytochromes *P*-450 and *P*-420 (Fig. 3) at concentrations of some 42 and 25 pmol/mg of microsomal protein respectively.

Microsomes from developing castor bean, therefore, contain cytochromes *P*-450 and *P*-420 and at least two *b*-type cytochromes, the major one of which has spectral properties similar to those of the cytochrome b_5 reported for other oilseed species and animal preparations (Smith *et al.*, 1990*a*,*b*; Lemberg & Barrett, 1973). The cytochrome *P*-450 content of the microsomes from castor bean was similar to that found in other oilseeds, whereas the cytochrome b_5 level was some 50% lower than in most other species investigated (Smith *et al.*, 1990*b*).

The castor-bean microsomes had good Δ^{12}-hydroxylase activity, with rates of 16–20 pmol ricinoleate $\cdot min^{-1} \cdot$ mg of

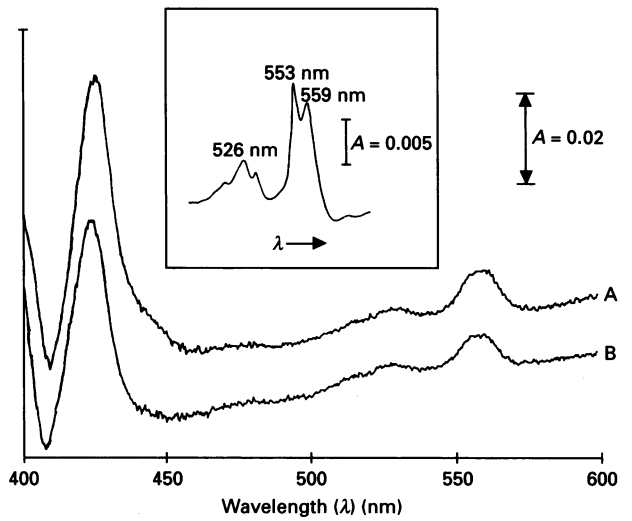

Fig. 2. Reduced-minus-oxidized difference spectra of microsomal membranes from castor bean

Microsomes (equivalent to 1.92 mg of protein) were suspended in 0.6 ml of 100 mM-potassium phosphate buffer, pH 7.2, and the reduced-minus-oxidized absorbance spectra recorded. For experimental details, see the Materials and methods section. Trace A, dithionite-reduced; trace B, NADH-reduced; trace C (inset), low-temperature (77 K) difference spectrum of the α- and β-bands of the NADH-reducible component.

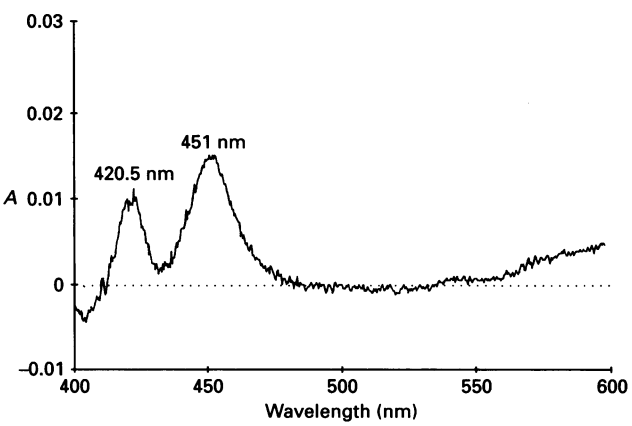

Fig. 3. CO-binding difference spectrum of dithionite-reduced castor-bean microsomal membranes

Microsomes (equivalent to 1.92 mg of protein) were suspended in 0.6 ml of 100 mM-potassium phosphate buffer, pH 7.2, and excess dithionite was added. Absorbance spectra were recorded before and after bubbling CO gas through the suspension.

microsomal protein^{-1} formed from [1-^{14}C]oleate, and this was unaffected by CO (results not shown), an observation in agreement with previous reports (Galliard & Stumpf, 1966; Moreau & Stumpf, 1981). CN^- was also found to inhibit Δ^{12}-hydroxylation (results not shown). The direct effect of CN^- on a hydroxylase component, however, was difficult to assess, since reaction mixtures contained, for high hydroxylase activity, catalase (Bafor *et al.*, 1991), and this was also inhibited by CN^- (results not shown). The observations, however, indicate that cytochrome *P*-450 was probably not concerned in the hydroxylation of oleate, but still raised the question of cytochrome b_5 involvement, which occurs in the Δ^{12}-desaturase of safflower (Smith *et al.*, 1990*b*). Spectrophotometric investigation of the participation of

Table 1. Effect of anti-(cytochrome b_5) IgG on the activity of Δ^{12}-hydroxylase, Δ^{12}-desaturase and cytochrome *c* reductase in castor microsomes

Δ^{12}-Hydroxylase and desaturase activities were measured in microsomes prelabelled with *sn*-2-[1-^{14}C]oleoylphosphatidylcholine. The membranes were incubated with anti-(cytochrome b_5) IgG, after which NADH was added. Labelled linoleate and ricinoleate were determined in the total lipids. For experimental details, see the Materials and methods section. The results are expressed as percentages of the activity in incubations without serum (Δ^{12}-desaturase, 10 pmol of [1−^{14}C]oleate desaturated · min^{-1} · mg of protein^{-1}; Δ^{12}-hydroxylase, 16 pmol of [1-^{14}C]oleate hydroxylated · min^{-1} · mg of protein^{-1}). Cytochrome *c* reductase activity was measured in microsomal preparations (equivalent to 51 μg of protein) that had been incubated for 15 min with anti-(cytochrome b_5) IgG in potassium phosphate buffer (0.1 M, pH 7.2; final volume 80 μl) in a cuvette on ice, after which the reaction mixture [KCN (10 mM), cytochrome *c* (0.04 mM) and NADH (0.4 mM) in phosphate buffer to give a final volume of 1 ml] was added. Reduction of cytochrome *c* was followed spectrophotometrically at 550 nm. The results are expressed as percentages of the activity in incubations without serum (350 nmol of cytochrome *c* reduced · min^{-1} · mg of protein^{-1}).

Antibody (μg of protein)	Activity (% of control)		
	Δ^{12}-Desaturase	Δ^{12}-Hydroxylase	Cytochrome *c* reductase
Anti-IgG			
0	100	100	100
30	63	14	25
60	0	0	11
120	0	0	10
Pre-immune serum			
30	125	101	101

cytochrome b_5 in electron transport during hydroxylation was not practicable because of the concomitant Δ^{12}-desaturase activity. Polyclonal antibodies were, therefore, raised in rabbit against the hydrophilic cytochrome b_5 fragment from cauliflower floret microsomes, and the purified IgG was used in experiments against hydroxylase activity. The results (Table 1) show that the anti-(cytochrome b_5) IgG was an efficient inhibitor of both the castor-bean microsomal Δ^{12}-desaturase and Δ^{12}-hydroxylase activities. At a microsomal to IgG preparation protein ratio of almost 2:1, over 85% inhibition in hydroxylation was observed. Pre-immune serum did not affect the hydroxylase activity. Catalase activity was also unaffected by the anti-(cytochrome b_5) IgG (results not shown).

The affinity of the anti-(cytochrome b_5) IgG was further characterized by examining its effect on cytochrome *c* reductase activity in the castor-bean microsomes. In these preparations the microsomal cytochrome *c* reductase activity, with NADH as donor, was some 350 nmol of cytochrome *c* reduced · min^{-1} · mg of microsomal protein^{-1} and was antimycin-insensitive. The anti-(cytochrome b_5) IgG was an efficient inhibitor of cytochrome reductase activity (Table 1), and at microsomal to IgG preparation protein ratios of some 2:1 and 1:1 decreased reductase activity by 75% and 90% respectively. Pre-immune serum had little effect on the cytochrome *c* reductase activity. The antimycin-insensitive reduction of cytochrome *c* by microsomal membranes is considered to be due mainly to the passage of electrons from NAD(P)H through cytochrome b_5 (Estabrook & Werringloer, 1978; Noshiro & Omura, 1978). Thus the anti-(cytochrome b_5) IgG appears to prevent either the transfer of electrons from cytochrome b_5 to cytochrome *c* or possibly the reduction of cytochrome b_5 by NADH via NADH:cytochrome b_5 reductase.

The latter possibility is unlikely, since the anti-(cytochrome b_5) IgG had no effect on the microsomal NADH reduction of ferricyanide (results not shown). The rabbit anti-(cauliflower cytochrome b_5) IgG, used in the above experiments, therefore appears to be specific for cytochrome b_5.

In summary, the Δ^{12}-hydroxylation of oleate to form ricinoleate in developing castor bean has the properties of a mixed-function oxygenase and requires the channelling of electrons from NAD(P)H to the hydroxylase (1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ^{12}-hydroxylase) via cytochrome b_5. In this respect the electron-transport chain appears similar to that described for the mammalian stearoyl-CoA desaturase (Enoch *et al.*, 1976) and the Δ^{12}-desaturase (1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ^{12}-desaturase) in oilseeds (Smith *et al.*, 1990*b*).

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REFERENCES

Achaya, K. T., Craig, B. M. & Youngs, C. G. (1964) J. Am. Oil Chem. Soc. **41**, 783–784

Bafor, M., Smith, M. A., Jonsson, L., Stobart, A. K. & Stymne, S. (1991) Biochem. J. **280**, 507–514

Bligh, E. G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917

Donaldson, R. P. & Luster, D. G. (1991) Plant Physiol. **96**, 669–674

Enoch, H. G., Catala, A. & Strittmatter, P. (1976) J. Biol. Chem. **251**, 5095–5103

Estabrook, R. W. & Werringloer, J. (1978) Methods Enzymol. **52**, 212–220

Galliard, T. & Stumpf, P. K. (1966) J. Biol. Chem. **241**, 5806–5812

Harlow, E. & Lane, D. (1988) Antibodies: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Harwood, J. L. (1988) Annu. Rev. Plant Physiol. Plant Mol. Biol. **39**, 101–138

Kajihara, T. & Hagihara, B. (1968) in Structure and Function of Cytochromes (Okunuki, K., Kamen, N. & Sekuzu, I., eds.), pp. 581–593, University of Tokyo Press, Tokyo

Kates, M. (1964) J. Lipid Res. **5**, 132–135

Kearns, E. V., Hugly, S. & Somerville, C. R. (1991) Arch. Biochem. Biophys. **284**, 431–435

Lemberg, R. & Barrett, J. (1973) Cytochromes, Academic Press, New York

Moore, A. L. & Proudlove, M. O. (1983) in Isolation of Membranes and Organelles from Plants (Hall, J. L. & Moore, A. L., eds.), pp. 153–184, Academic Press, London

Moreau, R. A. & Stumpf, P. K. (1981) Plant Physiol. **67**, 672–676

Morris, L. J. (1967) Biochem Biophys. Res. Commun. **29**, 311–315

Noshiro, M. & Omura, T. (1978) J. Biochem. (Tokyo) **83**, 61–77

Omura, T. & Sato, R. (1964) J. Biol. Chem. **239**, 2370–2385

Reid, L. S. & Mauk, A. G. (1982) J. Am. Chem. Soc. **104**, 841–845

Schägger, H. & von Jagow, G. (1987) Anal. Biochem. **166**, 368–379

Smith, M. A., Cross, A. R., Jones, O. T. G., Griffiths, W. T., Stymne, S. & Stobart, A. K. (1990*a*) in Plant Lipid Biochemistry: Structure and Utilization (Quinn, P. J. & Harwood, J. L., eds.), pp. 138–141, Portland Press, London

Smith, M. A., Cross, A. R., Jones, O. T. G., Griffiths, W. T., Stymne, S. & Stobart, A. K. (1990*b*) Biochem. J. **272**, 23–29

Stobart, A. K. & Stymne, S. (1990) in Methods in Plant Biochemistry (Harwood, J. L. & Bowyer, J. R., eds.), vol. 4, pp. 19–46, Academic Press, London

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

White, R. E. & Coon, M. J. (1980) Annu. Rev. Biochem. **49**, 315–356

Wilson, G. S. (1978) Methods Enzymol. **54**, 396–410

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