Electron-transport components of the 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ^{12} -desaturase (Δ^{12} -desaturase) in microsomal preparations from developing safflower (*Carthamus tinctorius* L.) cotyledons

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The major cytochrome in microsomal membrane preparations from developing seeds of safflower (*Carthamus tinctorius*, var High Linoleate), has a reduced-minus-oxidized difference spectrum characteristic of a *b*-type cytochrome, and was identified from its midpoint-potential ($E'_{7.2}$) value as cytochrome b_5 . Cytochromes *P*-450 and *P*-420 were also present. The cytochrome b_5 content of microsomal preparations from a number of oilseed species was found to be in the order of 200–300 pmol/mg of protein. The cytochrome b_5 was reduced in the membrane preparations by NADH, demonstrating the presence of an NADH:cytochrome b_5 reductase; NADPH was a less effective donor. Microsomal membranes catalysed the NAD(P)H-dependent conversion of radioactive oleate into linoleate, indicating acyl-CoA:lysophosphatidylcholine acyltransferase and 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ^{12} -desaturase (Δ^{12} -desaturase) activity. Desaturation of oleate to linoleate was unaffected by CO, but inhibited by CN^- . The addition of oleoyl-CoA to the NADH-reduced membranes resulted in the CN^- -sensitive partial re-oxidation of cytochrome b_5 , indicating that electrons from NADH were transferred to the site of desaturation via this cytochrome. The Δ^{12} -desaturase in safflower, therefore, is CN^- -sensitive and appears to require cytochrome b_5 and NADH:cytochrome b_5 reductase for activity.

INTRODUCTION

C_{18} -polyunsaturated fatty acids are widely distributed in the Plant Kingdom, particularly in photosynthetic membranes and in some commercially important vegetable oils. There is, therefore, interest in their biosynthesis and the regulatory mechanisms involved. Linoleic acid ($C_{18:2,\Delta^9,12}$) and α -linolenic acid ($C_{18:3,\Delta^9,12,15}$) are synthesized sequentially (Harris & James, 1964) from oleic acid ($C_{18:1,\Delta^9}$), through the activity of Δ^{12} - and Δ^{15} -desaturase enzymes respectively (Roughan & Slack, 1982; Jaworski, 1987). Work on plant desaturases has been limited by the lability of the enzymes and difficulties in obtaining preparations for study *in vitro*. More amenable to investigation has been the Δ^9 -desaturase involved in the synthesis of oleate from stearate ($C_{18:0}$). This stearyl-acyl carrier protein (ACP) desaturase requires molecular O_2 and a source of electrons (Nagai & Bloch, 1968) and has been purified 200-fold from safflower (*Carthamus tinctorius*) (McKeon & Stumpf, 1982). Reduced ferredoxin was thought to be the immediate electron donor of the desaturase (Jaworski & Stumpf, 1974; McKeon & Stumpf, 1982), and in photosynthetic tissue this may arise through the activity of Photosystem I (Jaworski, 1987). There is no evidence that cytochrome b_5 reductase or cytochrome b_5 is a component of the chloroplast system, although these are part of the stearyl-CoA desaturase in animals (Enoch *et al.*, 1976).

In oilseeds, the substrate for the Δ^{12} -desaturase is the oleate of phosphatidylcholine (Roughan, 1970; Slack *et al.*, 1978; Stymne & Appelquist, 1978). Oleate, from oleoyl-CoA, is channelled largely into position *sn*-2 of phosphatidylcholine (Murphy *et al.*, 1984) through the activity of an acyl-CoA:lysophosphatidyl-

choline acyltransferase (Stymne & Stobart, 1984a, 1987). The Δ^{12} -desaturase and the acyltransferase are particularly active in microsomal preparations from developing seed cotyledons of some oilseed species in which they are associated with the biosynthesis of triacylglycerols (Stymne & Stobart, 1984b; Stobart & Stymne, 1985; Stobart *et al.*, 1983). The Δ^{12} -desaturase requires oxygen and NAD(P)H as reductant (Vijay & Stumpf, 1972). Attempts to define its electron-transport components were unsuccessful (Gennity & Stumpf, 1985), although CN^- , but not CO, inhibited desaturation (Vijay & Stumpf, 1972), suggesting that cytochrome *P*-450 was not involved. Here we identify major electron-transport components which appear to be associated with Δ^{12} -desaturase activity in microsomal membranes from the developing seeds of safflower (*Carthamus tinctorius* var. High Linoleate).

MATERIALS AND METHODS

Chemicals

[1- ^{14}C]Oleic acid (octadeca-9-enoic acid) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). BSA (fraction V, fatty-acid-free), CoA and reduced nicotinamide nucleotides were purchased from Sigma. [1- ^{14}C]Oleoyl-CoA (sp. radioactivity 1930 d.p.m./nmol) was synthesized from the mixed anhydrides as described by Sanchez *et al.* (1973).

Plant material and microsomal-fraction preparation

Safflower, borage (*Borago officinalis*) and sunflower (*Helianthus annuus*) were grown from seed in a 16 h photoperiod at 25 °C and

Abbreviations used: Δ^{12} -desaturase, 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ^{12} -desaturase; $E'_{7.2}$, midpoint potential at pH 7.2; ACP, acyl carrier protein; d.a.f., days after flowering; pCMB, *p*-chloromercuribenzoate.

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an 8 h night at 18 °C. Seeds were harvested 14–18 days after flowering (d.a.f.) and the cotyledons were removed and placed in cold 0.1 M-phosphate buffer, pH 7.2. All further procedures were carried out at 1–4 °C. The cotyledons were ground in a mortar with 2 parts (w/v) of 0.1 M-potassium phosphate buffer, pH 7.2, containing 0.1 % BSA and 0.33 M-sucrose. The homogenate was filtered through a double layer of Miracloth, diluted 5-fold with fresh grinding medium and centrifuged at 18000 *g* for 20 min. The supernatant was again filtered through Miracloth to remove floating oil droplets and centrifuged at 105000 *g* for 90 min. The microsomal pellet was resuspended in 0.1 M-phosphate buffer, pH 7.2, and stored at –80 °C. Microsomal membranes from adult male guinea-pig liver were prepared similarly after homogenization in extraction medium by using a Polytron overhead blender.

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 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 determined by using a difference absorption coefficient ($\Delta E_{558-575}$) of 21 mm⁻¹·cm⁻¹ (Estabrook & Werringloer, 1978). Cytochrome *P*-450 was determined from the (reduced+CO)-minus-reduced difference spectra, using an $\Delta E_{450-490}$ value of 91 mm⁻¹·cm⁻¹ (Omura & Sato, 1964).

Potentiometric titrations were carried out in a stirred cuvette fitted with platinum and calomel electrodes under an atmosphere of argon (Dutton, 1978). Microsomal membranes were suspended in 0.1 M-phosphate buffer, pH 7.2, containing 100 mM-KCl in a final volume of 1 ml. Redox mediators added to the suspension were 3,6-diaminodurene (25 μ M), phenazine methosulphate (25 μ M), phenazine ethosulphate (25 μ M), duroquinone (25 μ M), pyocyanin (12.5 μ M) and anthroquinone (25 μ M). The potentials investigated were from +200 mV to –200 mV, and the spectra were obtained using a split-beam rapid-scanning spectrophotometer with data-storage facilities.

Changes in the reduction state of cytochrome *b*₅ after the addition of oleoyl-CoA were also monitored by dual-wavelength spectroscopy ($\Delta A_{558-575}$). Microsomes were suspended in 0.1 M-phosphate buffer, pH 7.2, at 25 °C, and substrates were added in small volumes of buffer.

Enzyme assays

Microsomal Δ^{12} -desaturase activity was assayed at 25 °C in a water bath with constant shaking. The incubation mixtures contained 10 mg of BSA, substrates and the microsomal preparation (at concentrations stated in the Tables and Figures), dissolved in 0.1 M-potassium phosphate buffer, pH 7.2, in a final volume of 1 ml. Reactions were terminated by the addition of 1 ml of 0.15 M-acetic acid and the lipids extracted by a modified procedure of Bligh & Dyer (1959). The chloroform phase was removed, evaporated to dryness at 45 °C under a stream of N₂, and the residue taken up in a small volume of cold chloroform. Polar lipids were purified by t.l.c. on pre-coated silica-gel plates (Merck; silica-gel 60) with chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Lipid areas, located by lightly staining with I₂ vapour, were removed from the plates and either assayed for radioactivity or methylated *in situ* with methanolic HCl (3.5 %, w/w) (Kates, 1964). The fatty acid methyl esters were separated by t.l.c. with benzene on precoated silica-gel

plates impregnated with AgNO₃ (4 %, w/v). The methyl esters, located by lightly spraying with water, were removed for radioactive assay in PPO(2,5-diphenyloxazole)/POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] in toluene (4 g of PPO + 0.3 g of POPOP/litre toluene). Radioactivity was counted in a Searle Isocap 300 liquid-scintillation counter with correction for background and quenching.

NADH:ferricyanide reductase (diaphorase activity) was assayed by a modification of the method of Mihara & Sato (1978). Reaction mixtures contained potassium ferricyanide (1 μ mol) and microsomal membrane preparation in a final volume adjusted to 1 ml with 0.1 M-phosphate buffer, pH 7.2. NADH was added (final concn. 1 mM) after baseline stabilization, and the reduction of the ferricyanide was determined at 420 nm by using an ϵ value of 1.02 mm⁻¹·cm⁻¹. In inhibitor studies, microsomal preparations were incubated with the appropriate inhibitor in the reaction mixture for 10 min before the addition of reductant.

Protein determination

Protein was determined by the BCA (Pierce Ltd.) method, with incubation for 30 min at 37 °C and BSA as standard

RESULTS

Dithionite-reduced-minus-oxidized difference spectra of microsomes from developing safflower seeds indicated the presence of a *b*-type cytochrome having characteristic α , β and γ bands at 558, 528 and 425 nm respectively (Fig. 1, trace B). The spectra were almost identical with those obtained for similarly treated guinea-pig liver microsomes (Fig. 1, trace A). CO binding spectra of dithionite-reduced microsomes indicated the presence of cytochromes *P*-450 and *P*-420 (Fig. 2), cytochrome *P*-450 being the predominant species, with a concentration of some 54 pmol/mg of protein. The *b*-type cytochromes, however, were the major cytochromes present, and were over four times more abundant than cytochrome *P*-450.

Potentiometric titrations of the safflower *b*-type cytochrome indicated the presence of two components. The major one had a midpoint potential at pH 7.2 ($E'_{7,2}$) of -24 ± 2.2 mV, with an α

Fig. 1. Reduced-minus-oxidized difference spectra of microsomal membranes from safflower (var. High Linoleate) and guinea-pig liver

Microsomes (equivalent to 0.5 mg of protein) were suspended in 100 mM-potassium phosphate buffer, pH 7.2, and the dithionite-reduced-minus oxidized absorbance spectra was recorded. For experimental details, see the Materials and methods section. Trace A, guinea pig; trace B, safflower.

Fig. 2. CO binding difference spectrum of dithionite-reduced safflower microsomal preparations

Microsomes (equivalent to 0.5 mg of protein), prepared from the High Linoleate variety, were suspended in 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.

Table 1. *b*-Type cytochrome content of microsomal preparations from the developing cotyledons of oilseed species

The *b*-type cytochrome was determined from the absorbance of the α band of steady-state reduced-minus-oxidized difference spectra obtained with either NADH or sodium dithionite as the reductant (see the Materials and methods section). The value in parentheses is the cytochrome b_5 reduced in the presence of NADPH. Abbreviations: nd, not detectable; tr, trace.

Species/variety	Reductant (1 mM)...	<i>b</i> -Type cytochrome (pmol/mg of microsomal protein)	
		NADH	Dithionite
Safflower			
High Linoleate		214 (28)	262
Intermediate Linoleate		242	317
High Oleate		207	327
High Linoleate, 30 d.a.f.		nd	tr
Sunflower		217	272
Borage		175	273

peak at 558.8 nm. The spectral characteristics and $E'_{7.2}$ values of this component were similar to those of animal cytochrome b_5 (Lemberg & Barrett, 1973), and those reported for this cytochrome in cauliflower (*Brassica oleracea*) and mung-bean (*Phaseolus aureus*) microsomal membranes (Rich & Bendall, 1975; Hendry *et al.*, 1981). The cytochrome b_5 comprised some

90 % of the *b*-type cytochromes present. The minor component (~ 10 %) had an $E'_{7.2}$ of $+110 \pm 47$ mV and an α peak at 561 nm.

The spectral properties of microsomal preparations from the developing seeds of other safflower varieties and oilseed species were examined. All displayed reduced-minus-oxidized difference

Table 2. Electron-transport and enzymic activity in microsomal preparations from the developing seeds of safflower (var. High Linoleate)

(a) Microsomal membranes (equivalent to 0.5 mg of protein) were suspended in phosphate buffer and the reduction of ferricyanide was determined spectrophotometrically after the addition of the electron donor (see the Materials and methods section). Results are expressed as a percentage of the activity found with NADH as the electron donor ($3.8 \mu\text{mol}$ ferricyanide reduced $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$). (b) Microsomal membranes (equivalent to 1 mg of protein) were suspended in phosphate buffer and the cytochrome b_5 determined from steady-state reduced-minus-oxidized difference spectra (see the Materials and methods section). Results are expressed as a percentage of the activity found with NADH as the electron donor (200 pmol/mg of protein). (c) Microsomal membranes (equivalent to 0.5 mg of protein) were incubated with [^{14}C]oleoyl-CoA (100 nmol), BSA (10 mg) and NADH (1 mM). After incubation for 40 min the reactions were stopped by the addition of acetic acid, and the lipids extracted and purified as described in the Materials and methods section. Results are expressed as a percentage of the total radioactivity found in the phosphatidylcholine with NADH as the electron donor (after incubation, 70 nmol of radioactive fatty acid was in phosphatidylcholine). (d) Microsomal membranes (equivalent to 0.5 mg of protein) were incubated with [^{14}C]oleoyl-CoA (100 nmol), BSA (10 mg) and NADH (1 mM). After incubation for 40 min the reactions were stopped by the addition of acetic acid, and the lipids extracted and purified (see the Materials and methods section). Results are expressed as a percentage of the control desaturase activity with NADH as the electron donor (after incubation, some 40 nmol of radioactive linoleate was in phosphatidylcholine). Where inhibitors were used the microsomal membranes were incubated with the inhibitor 10 min before the addition of substrates and NADH.

	Activity (% of control)			
	(a) Ferricyanide reductase	(b) Cytochrome <i>b</i> reduced	(c) Acyltransferase	(d) Desaturase
Electron donors				
NADH (1 mM)	100	100	100	100
NADPH (1 mM)	12	13	98	102
Inhibitors				
(with NADH as the electron donor)				
CO (bubbled through)	—	—	100	99
KCN (0.5 mM)	—	—	102	26
KCN (1 mM)	97	100	104	15
<i>p</i> CMB (0.5 mM)	8	—	89	98
<i>p</i> CMB (2 mM)	—	45	64	28
Mycobactin (1 mM)	—	100	90	81
<i>o</i> -Phenanthroline (1 mM)	—	—	85	83
8-Hydroxyquinoline (1 mM)	—	—	88	76
2,2'-Bipyridyl (0.1 mM)	—	—	108	94

spectra characteristic of *b*-type cytochrome. From these, the total *b*-type cytochrome was some 200–300 pmol/mg of protein for all the species examined (Table 1). The cytochrome *b* contents of the safflower varieties were similar irrespective of whether or not they produced highly polyunsaturated oils. As the oilseeds matured (see safflower var. High-Linoleate) the cytochrome *b* content declined, and at 30 d.a.f. (days after flowering) it was negligible.

Ferricyanide reductase activity was determined in the microsomal membranes from safflower var. High-Linoleate. The results show that NADH was a more efficient electron donor than NADPH (Table 2). In membranes of mature safflower seed, the NADH:ferricyanide reductase activity was low (results not shown). CN⁻ had no effect on NADH:ferricyanide reductase activity, whereas *p*-chloromercuribenzoate (*p*CMB) was an efficient inhibitor at 0.5 mM (Table 2). The effect, therefore, of these compounds on the NADH:cytochrome *b*₅ reductase was investigated. Reduced-minus-oxidized difference spectra of microsomal membranes in the presence of NADH showed that cyanide had no effect on the reduction of endogenous cytochrome *b*₅ (Table 2). Higher concentrations of *p*CMB (2 mM) inhibited quite considerably the reduction of cytochrome *b*₅ by NADH (Table 2). The inhibition by *p*CMB, however, was not complete, even at high inhibitor concentrations (results not shown), and some reduction of the cytochrome *b*₅ always occurred.

The safflower microsomal membranes possessed Δ^{12} -desaturase activity and readily catalysed the synthesis of linoleate from oleoyl-CoA in the presence of NAD(P)H (Table 2). The substrate for the Δ^{12} -desaturase in microsomal membranes from oilseeds is the acyl group in *sn*-phosphatidylcholine, in particular the oleate esterified to position *sn*-2 (Roughan & Slack, 1982; Stymne & Stobart, 1987). The transfer of oleate from oleoyl-CoA to position *sn*-2 of the complex lipid is catalysed by acyl-CoA:lysophosphatidylcholine acyltransferase, an enzyme active in the microsomal membranes of most oilseeds (Stymne & Stobart, 1987). Incubation mixtures that contained microsomes (microsomal fractions), [¹⁴C]oleoyl-CoA and NAD(P)H readily incorporated label into phosphatidylcholine, with approx. 55% of it being desaturated to [¹⁴C]linoleate within 40 min (Table 2).

Fig. 3. Effect of oleoyl-CoA on the NADH-reduced spectrum of safflower microsomal preparations

Microsomes (equivalent to 0.5 mg of protein), prepared from the High Linoleate variety, were reduced by the addition of NADH (1 mM), and the absorbance spectrum recorded before and after the addition of oleoyl-CoA (70 nmol). The data presented is a difference spectrum of the steady-state NADH-reduced minus NADH-reduced + oleoyl-CoA spectra measured 1 min after addition.

Desaturation of oleate was unaffected by pretreatment of the microsomes with CO. CN⁻ (1 mM) had little effect on the transfer of oleate to phosphatidylcholine, but inhibited the desaturase by some 80%. *p*CMB had some effect at high concentrations (3 mM) on the activity of the acyltransferase and also inhibited desaturation quite considerably. The metal chelators *o*-phenanthroline, 8-hydroxyquinoline and 2,2'-bipyridyl had little effect on the activity of the acyltransferase and the desaturase. Similarly mycobactin, a potent scavenger of iron in hydrophobic environments (Snow, 1970), had little effect.

Microsomal membranes from safflower var. High-Linoleate catalysed, therefore, the reduction of cytochrome *b*₅ using the electron donor NADH (or, less effectively, NADPH) and the NAD(P)H-dependent desaturation of 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine i.e. Δ^{12} -desaturase activity. Experiments were conducted to determine whether the desaturase utilized reducing potential from NADH through the mediation of cytochrome *b*₅. Microsomal NADH-reduced cytochrome *b*₅ was 20% oxidized in the steady state upon addition of oleoyl-CoA (Fig. 3). Further evidence for the involvement of the cytochrome *b*₅ in desaturation was obtained by monitoring the NADH-reduced cytochrome *b*₅ by dual-wavelength spectroscopy ($\Delta A_{558-575}$). The addition of limiting oleoyl-CoA to the microsomal suspension caused a significant decrease in absorbance, indicating that a rapid re-oxidation of the reduced cytochrome

Fig. 4. Effect of oleoyl-CoA on the steady-state reduction of cytochrome *b*₅ of safflower microsomal preparations

Microsomes (equivalent to 0.5 mg of protein), prepared from the High Linoleate variety, were reduced by the addition of NADH (50 μ M), and the absorbance ($\Delta A_{558-575}$) monitored by dual-wavelength spectroscopy. (a) Absorbance before and after the addition of oleoyl-CoA (22 nmol); (b) before and after the addition of KCN (1 mM) and oleoyl-CoA (22 nmol) respectively.

Fig. 5. Re-oxidation of microsomal cytochrome b_5 after reduction by limiting NADH

Safflower (var. High Linoleate) microsomal membranes (equivalent to 1 mg of protein) were suspended in phosphate buffer, pH 7.2, and the cytochrome reduced by the addition of NADH (15 μ M). Re-oxidation of the cytochrome was monitored by dual-wavelength spectroscopy ($\Delta A_{558-575}$) after the addition of acyl-CoA. Trace A, minus acyl-CoA; trace B, plus linoleoyl-CoA (30 nmol); trace C, plus oleoyl-CoA (30 nmol).

b_5 had taken place (Fig. 4a). The absorbance returned to its former level after a short period if the amount of oleate added was limiting relative to the NADH available to reduce the cytochrome b_5 . The transitory oxidation of the cytochrome b_5 was not observed with linoleoyl-CoA (results not shown) and was abolished if the microsomes were incubated in CN^- (1 mM) before the addition of the oleoyl substrate (Fig. 4b). Microsomal preparations from the High Oleate variety of safflower, which have no Δ^{12} -desaturase activity (Stymne *et al.*, 1983), but exhibit good electron-transport activity (Tables 1 and 2), did not show this rapid re-oxidation of reduced cytochrome b_5 on the addition of oleoyl-CoA (results not shown). In all of the microsomal membranes studied, the NADH reducible cytochrome b became completely re-oxidized after a few minutes, even in the absence of exogenously supplied acyl substrate. The cytochrome was restored to the reduced state by the further addition of NADH. If greater concentrations of NADH were added, the cytochrome b_5 remained in the reduced state for a longer time. This cycle could be repeated a number of times. The subsequent complete re-oxidation of reduced cytochrome b_5 also occurred in microsomal preparations from rat liver (Oshino *et al.*, 1971).

Limiting concentrations of NADH in the presence of acyl substrate caused immediate reduction of the cytochrome b_5 , followed by rapid and complete re-oxidation. The rate of re-oxidation was always greater in the presence of oleoyl-CoA (trace C), whereas linoleoyl-CoA (trace B) had little effect (Fig. 5).

DISCUSSION

Reduced-minus-oxidized difference spectra of microsomal preparations from the developing cotyledons of safflower (var. High Linoleate) showed the presence of b -type cytochromes of which the major component had an $E'_{7,2}$ of -24 mV. This is similar to that reported for cytochrome b_5 in other membrane

preparations from plants (Rich & Bendall, 1975; Hendry *et al.*, 1981; Asard *et al.*, 1989; Askerlund *et al.*, 1989). Mammalian cytochrome b_5 has similar spectral properties and redox values (Lemberg & Barrett, 1973). The data strongly indicate that the microsomal fraction from safflower has a substantial cytochrome b_5 content. CO binding spectra indicated that cytochromes $P-450$ and $P-420$ were also present. Although microsomal preparations are heterogeneous in membrane composition, the cytochrome profiles from oilseed preparations were less complex than might be anticipated (see Rich & Bendall, 1975; Askerlund *et al.*, 1989), and appear relatively rich in cytochrome b_5 . The concentration of b -type cytochrome in microsomal membranes from the oilseed species investigated (200–300 pmol/mg of protein) was similar to that in similar preparations from *Arum* (cuckoo-pint) spadix (Yahiel *et al.*, 1974), potato (*Solanum tuberosum*) tuber (Bonnerot *et al.*, 1985), pea (*Pisum sativum*) (Jollie *et al.*, 1987), and in plasmalemma membranes (Askerlund *et al.*, 1989).

Previous reports on the electron-transport components in microsomal preparations from safflower cotyledons (Gennity & Stumpf, 1985) require comment. These workers found no evidence of a cytochrome b_5 . Their membrane preparations, however, were capable of some NADH:ferricyanide reductase and Δ^{12} -desaturase activity, albeit lower than observed in the present study. The stage of cotyledon maturation is paramount in studies on fatty acid and triacylglycerol synthesis in oilseeds, optimally active membrane preparations being obtained only over a narrow time window during development (Stymne & Stobart, 1987). In safflower this is 16–20 d.a.f., when 70% of the oil is laid down (Ichihara & Noda, 1980; Slack *et al.*, 1985). In seeds advanced in maturity, and certainly when dehydration commences, the enzyme activities are low and, as shown here, the cytochrome b_5 almost disappears completely (see Table 1).

The microsomal preparations of safflower catalysed the formation of linoleate from oleate at rates comparable with those found in previous studies (Stymne *et al.*, 1983; Stobart *et al.*, 1983). Hence it was possible to test the relationship between Δ^{12} -desaturase activity and the state of the electron-transport components. The possible involvement of the cytochrome b_5 in desaturation was demonstrated by recordings of the steady-state reduced-minus-oxidized difference spectra and by dual-wavelength spectroscopy. The addition of small amounts of oleoyl-CoA to the microsomes in the presence of NADH brought about the transient re-oxidation of the cytochrome b_5 (Figs. 3 and 4) and strongly indicated that this cytochrome was providing electrons for the desaturase enzyme. Preparations from the High Oleate variety of safflower, which lack Δ^{12} -desaturase activity (Stymne *et al.*, 1983), had an active NADH:cytochrome b_5 reductase, but here the addition of oleoyl-CoA had no effect on the oxidation state of the cytochrome b_5 (results not shown).

The addition of CN^- to the membranes substantially inhibited the desaturation of oleate to linoleate (Table 2; see Vijay & Stumpf, 1972) and prevented the transient re-oxidation of the cytochrome b_5 on the addition of oleoyl-CoA (Fig. 4). CN^- , however, did not affect the reduction of endogenous cytochrome b_5 by NADH. The CN^- -sensitive site is, therefore, on the oxidizing side of cytochrome b_5 . The mammalian stearoyl desaturase consists of three major, membrane-bound, protein components: NADH:cytochrome b_5 reductase, cytochrome b_5 and a terminal desaturase enzyme which requires O_2 (Holloway, 1983). The site of CN^- -sensitivity is the terminal desaturase (Oshino *et al.*, 1971; Strittmatter *et al.*, 1974), an enzyme which has an integral non-haem iron component. The stearoyl-ACP desaturase in *Euglena*, which utilizes reduced ferredoxin as an electron donor, is also inhibited by CN^- (Nagai & Bloch, 1968), and it appears that here, too, the CN^- -sensitive site is the desaturase enzyme. It is interesting, however, that iron-chelating

Scheme 1. Proposed 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ^{12} -desaturase complex in microsomal membranes from developing safflower seeds

Oleate, from oleoyl-CoA, is transferred to position *sn*-2 of *sn*-phosphatidylcholine, through the action of acyl-CoA:lysophosphatidylcholine acyltransferase, for its subsequent desaturation to linoleate. The desaturation reaction requires molecular O_2 and a primary reductant, NAD(P)H. Electron transport, between NADH and the desaturase enzyme, is mediated through cytochrome b_5 and NADH:cytochrome b_5 reductase. The C_{18} polyunsaturated fatty acid product is returned to the acyl-CoA pool, through acyl exchange, and is utilized in the assembly of triacylglycerol. Abbreviations: Enz., enzyme (Δ^{12} -desaturase); ox., oxidized; red., reduced.

reagents, such as 2,2'-bipyridyl and *o*-phenanthroline, had only slight inhibitory effects on the mammalian stearoyl-CoA desaturase (Oshino *et al.*, 1966). In the present study, such chelators, and even mycobactin, were only slightly inhibitory towards the desaturase and had little effect on the reduction by NADH of cytochrome b_5 or ferricyanide (Table 2).

The thiol inhibitor *p*CMB inhibited desaturase activity in safflower microsomes (Vijay & Stumpf, 1972), and it was concluded that the enzyme required intact thiol groups. In the present investigation the site of *p*CMB action was largely at the reduction of the cytochrome b_5 by NADH (Table 2). The inhibitory effect of the *p*CMB was also apparent on microsomal NADH dehydrogenase activity when measured with ferricyanide as the acceptor (Table 2). The inhibition of the steady-state NADH reduction of cytochrome b_5 by *p*CMB was, however, never complete, and sufficient residual electron transfer from NADH remained to bring about some oleate desaturation. Rates of activity of the oleoyl desaturase and the NADH:ferricyanide reductase in the microsomal membranes were in the order of 2–3 and 4000 nmol·min⁻¹·mg of protein⁻¹ respectively (results not shown). It is reasonable to assume that a good proportion of the ferricyanide reductase activity reflects the NADH:cytochrome b_5 reductase. A 90% decrease in reductase activity in these preparations (see *p*CMB; Table 2) may have little effect, therefore, on desaturation (Table 2). In highly active microsomal membranes, therefore, the NADH:cytochrome b_5 reductase activity, and even the relatively poorer NADPH:cytochrome b_5 reductase activity (Table 1), were well in excess of that required for the observed activity of the Δ^{12} -desaturase (Table 2). In much less active safflower preparations the relative discrimination towards NADPH as an electron donor to the desaturase (Vijay & Stumpf, 1972) could be accounted for by the much lower electron-transport capacity of the membranes (see Gennity & Stumpf, 1985).

In summary, the data presented here for the activity of safflower microsomal 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ^{12} -desaturase is not incompatible with the involvement of an electron-transport chain (Scheme 1) similar to that described for the stearoyl-CoA desaturase in mammalian systems (Enoch *et al.*, 1976).

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