Lipid metabolism in microsomal fraction from photosynthetic tissue

Effects of catalase and hydrogen peroxide on oleate desaturation

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(Received 19 January 1983/Accepted 28 March 1983)

On incubation of microsomal fraction from pea (*Pisum sativum* L.) leaves with ammonium [1-¹⁴C]oleate or [1-¹⁴C]oleoyl-CoA in the presence of ATP, CoA, Mg²⁺ and NADH, the major reactions observed were those catalysed by oleoyl-CoA synthetase, oleoyl-CoA thioesterase, oleoyl-CoA:phosphatidylcholine acyltransferase and oleoyl phosphatidylcholine desaturase. The reaction catalysed by oleoyl phosphatidylcholine desaturase was specifically inhibited by H₂O₂, and this inhibitory effect was overcome by catalase (EC 1.11.1.6).

The biosynthesis *de novo* of palmitate, stearate and oleate in photosynthetic tissues takes place on soluble enzymes of the chloroplast stroma (Ohlrogge *et al.*, 1979; Høj & Mikkelsen, 1982; Shimakata & Stumpf, 1982). Subsequent desaturation of oleate to linoleate, from which linolenate of the chloroplast seems to be formed (Roughan *et al.*, 1979; Jones & Harwood, 1980), occurs on membrane-bound enzymes in the microsomal fraction (Roughan & Slack, 1982). Evidence is now accumulating that phosphatidylcholine containing oleoyl moieties (Slack *et al.*, 1976; Stymne & Appelqvist, 1978; Slack *et al.*, 1979; Stymne & Glad, 1981), rather than oleoyl-CoA (Vijay & Stumpf, 1971; Trémolières *et al.*, 1980), is the substrate for this desaturation reaction.

Attempts to isolate and stabilize membrane-bound desaturases from higher plants have been rather unfruitful (Appelqvist, 1980; Moreau & Stumpf, 1982). Catalase has been reported to have a stimulatory and stabilizing effect on linoleate desaturase in microsomal fraction from developing linseed cotyledons (Browse & Slack, 1981), but so far its effect on the desaturation of either oleate or linoleate in microsomal fraction from leaves has not been examined. It is generally assumed that catalase stimulates the activity of desaturases by scavenging H₂O₂, which is believed to be detrimental to desaturases.

We now report on the metabolism of oleate in pea leaf microsomal fraction, and the actions of catalase and H₂O₂ in respectively stimulating and inhibiting oleate desaturation while the other major reactions catalysed by oleoyl-CoA synthetase, oleoyl-CoA thioesterase and oleoyl-CoA:phosphatidylcholine acyltransferase remain unaffected.

Experimental

Materials

[1-¹⁴C]Oleic acid (56mCi/mmol) and [1-¹⁴C]oleoyl-CoA (56mCi/mmol) were purchased from Amersham-Buchler, Braunschweig, Federal Republic of Germany. ATP, CoA, NADH, catalase and bovine serum albumin (fatty acid-free) were obtained from Sigma Chemie G.m.b.H., München, Federal Republic of Germany. All reagents and adsorbents were from E. Merck A.G., Darmstadt, Federal Republic of Germany. Only distilled solvents were used. Pea seedlings (*Pisum sativum*, cultivar Kleine Rheinländerin) were grown in vermiculite at a 12h photoperiod (continuous white light, 12.5W/m², from Osram-L fluorescent tubes), at 15°C and 60% relative humidity.

Preparation of microsomal fraction

Microsomal fraction was isolated at 4°C. Fresh leaves (5g) from 8-day-old seedlings were added to 25ml of a solution containing (final concentrations) 0.4M-sucrose, 0.1% bovine serum albumin, 10mM-KCl, 3mM-cysteine hydrochloride and 0.1mM-Tris/HCl buffer, pH 7.4, and homogenized by two bursts

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of 2 s each with an Ultra-Turrax apparatus (Janke und Kunkel G.m.b.H., Staufen, Federal Republic of Germany). The homogenate was filtered through two layers of Miracloth, and immediately centrifuged at 12000 *g* for 20 min in order to remove chloroplast and mitochondrial membranes. Microsomal membranes were precipitated by adding MgCl_2 to the supernatant to give a final concentration of 50 mM (Diesperger *et al.*, 1974). The suspension was centrifuged at 18000 *g* for 15 min, and the resulting microsomal pellet was suspended in the basal incubation medium (1–2 ml), containing (final concentrations) 1 mM-NADH, 1% bovine serum albumin, 10 mM- MgCl_2 and 0.1 M-potassium phosphate buffer, pH 7.2. The protein content in a sample part of the microsomal preparation was determined by the method of Lowry *et al.* (1951) (with bovine serum albumin as standard) before the addition of bovine serum albumin.

Incubations

The incubations were performed in loosely fitted screw-cap tubes held on a shaker at 22°C. The reaction mixture contained the basal incubation medium with various supplements plus about 1 mg of microsomal protein and 0.1 μCi of ammonium $[1\text{-}^{14}\text{C}]$ oleate or 0.1 μCi of $[1\text{-}^{14}\text{C}]$ oleoyl-CoA in a total volume of 1.0 ml, to give a final substrate concentration of 3.4 μM . Some incubation mixtures contained, in addition, one or more of the following: 0.3 mM-CoA, 4 mM-ATP, 2000 units of catalase/ml plus various concentrations of H_2O_2 . Incubations were initiated by the addition of microsomal protein.

Lipid extraction and analysis

Incubations were terminated after 1 h by addition of 18 ml of chloroform/methanol (2:1, v/v) to produce a monophasic extract. Water-soluble constituents of the incubation mixture, including acyl-CoA derivatives, were partitioned into the upper phase after the addition of 3.6 ml of 0.7% NaCl to the chloroform/methanol extract. The lower layer, containing the lipids, was recovered, and the solvent was removed in a stream of N_2 . The lipids were redissolved in hexane and stored under N_2 at -20°C.

Radioactivity measurements were made in a Packard Tri-Carb C 2425 liquid-scintillation spectrometer (Packard Instruments Co., Downers Grove, IL, U.S.A.). Lipid samples were measured in toluene scintillator (Packard), and aqueous samples in Aquasol-3 (NEN Chemicals G.m.b.H., Dreieichenhain, Federal Republic of Germany).

Total lipids were fractionated by t.l.c. on silica gel H into neutral lipids and ionic lipids by using hexane/diethyl ether/acetic acid (70:30:1, by vol.) and chloroform/acetone/methanol/acetic acid/water

(10:4:2:2:1, by vol.) respectively as developing solvents. The radioactive lipid fractions were detected by a Berthold LB 2760 radio-t.l.c. scanner (BF-Vertriebsgesellschaft, Wildbad, Federal Republic of Germany), recovered from the chromatoplate and transmethyated (Chalvardjian, 1964). The methyl esters were purified by t.l.c. on silica gel H with hexane/diethyl ether (7:3, v/v) as developing solvent and water-saturated diethyl ether as eluent.

The aqueous phases obtained after partitioning of the crude lipid extracts were concentrated in a rotary evaporator and chromatographed on layers of silica gel H with butan-1-ol/acetic acid/water (5:2:3, by vol.) as developing solvent. The zones corresponding to acyl-CoA, which were distinctly separated from other lipids, were scraped off, and transmethyated in the presence of the adsorbent, and the methyl esters were purified as described above.

Methyl esters were analysed by radio-g.l.c. in a Perkin-Elmer F 22 instrument, equipped with thermal-conductivity detectors (Perkin-Elmer G.m.b.H., Überlingen, Federal Republic of Germany); radioactivity was monitored in a Packard model 894 gas proportional counter. Separations were performed on 10% (w/w) Silar 5CP on Gas-Chrom Q (100–120 mesh) (Applied Science Laboratories, State College, PA, U.S.A.) at 190°C.

Results and discussion

We found that microsomal fraction prepared by a rapid isolation technique (Diesperger *et al.*, 1974) from young pea leaves rapidly metabolized both ammonium oleate and oleoyl-CoA to a wide range of products (Table 1). More than 90–95% of added radioactivity could be recovered in the reaction products. Analysis of the reaction products revealed that the reactions catalysed by oleoyl-CoA synthetase, oleoyl-CoA thioesterase, oleoyl-CoA:phosphatidylcholine acyltransferase and oleoyl phosphatidylcholine desaturase were mainly operative. Oleoyl phosphatidylcholine was rapidly formed from oleoyl-CoA until a steady state was reached after about 12–15 min. Thereafter, the proportion of linoleoyl phosphatidylcholine progressively increased until about 1 h of incubation.

Oleate, added as ammonium salt, was converted into oleoyl-CoA only in the presence of CoA (0.3 mM) and ATP (4 mM) (Table 1). About 0.9 nmol of oleoyl-CoA/h per mg of protein was formed from ammonium oleate. This demonstrates the presence of oleoyl-CoA synthetase activity in leaf microsomal fraction. It cannot be ruled out, however, that the microsomal fraction contained some chloroplast-envelope membranes, which are known to exhibit long-chain acyl-CoA synthetase activity (Roughan & Slack, 1977).

Table 1. *Metabolism of ammonium [1-¹⁴C]oleate and [1-¹⁴C]oleoyl-CoA by microsomal fraction from pea leaves*
 Microsomal fraction was isolated from 8-day-old pea leaves and incubated for 1 h with ammonium [1-¹⁴C]oleate or [1-¹⁴C]oleoyl-CoA, and the products were analysed as described in the Experimental section. Additions were made to give the following final concentrations: CoA (0.3 mM), ATP (4 mM), catalase (2000 units/assay) and H₂O₂ (10 mM). The results are means $\pm$ S.D. for four independent experiments.

Substrate	Additions	Principal products formed* (nmol/h per mg of protein)			
		Oleoyl-CoA	Non-esterified oleic acid	Oleoyl phosphatidylcholine	Linoleoyl phosphatidylcholine
Ammonium [1- ¹⁴ C]oleate	CoA/ATP	0.9 $\pm$ 0.2		0.6 $\pm$ 0.1	0.3 $\pm$ 0.1
Ammonium [1- ¹⁴ C]oleate	None	Trace		0.0	0.0
[1- ¹⁴ C]Oleoyl-CoA	CoA/ATP		0.8 $\pm$ 0.3	3.0 $\pm$ 0.3	1.4 $\pm$ 0.2
[1- ¹⁴ C]Oleoyl-CoA	CoA/ATP/catalase		0.8 $\pm$ 0.3	2.9 $\pm$ 0.3	1.8 $\pm$ 0.3
[1- ¹⁴ C]Oleoyl-CoA	CoA/ATP/H ₂ O ₂		0.8 $\pm$ 0.4	2.8 $\pm$ 0.4	0.0
[1- ¹⁴ C]Oleoyl-CoA	CoA/ATP/H ₂ O ₂ /catalase		0.9 $\pm$ 0.4	3.0 $\pm$ 0.3	1.7 $\pm$ 0.3

* Other minor products detected (max. 0.2 nmol/h per mg of protein) include non-esterified linoleic acid, linoleoyl-CoA, galactosyldioleoylglycerol and galactosyldilinoleoylglycerol.

The conversion of exogenous ammonium oleate into oleoyl-CoA was an absolute prerequisite for its further metabolism and eventual desaturation. Regardless of whether ammonium oleate or oleoyl-CoA was the substrate, in each case about 30–35% of the radioactive pool of oleoyl phosphatidylcholine had been desaturated (Table 1).

Oleoyl-CoA:phosphatidylcholine acyltransferase activity, which presumably is the sum of the activities of oleoyl-CoA:lysophosphatidylcholine acyltransferase (Moreau & Stumpf, 1982) and oleoyl-CoA:phosphatidylcholine transacylase (Stymne & Glad, 1981), was relatively high (Table 1). This activity was hardly affected by the addition of either catalase or H₂O₂ (Table 1).

A reaction competing with the synthesis of oleoyl-CoA and transfer of oleoyl moieties to phosphatidylcholine was that catalysed by oleoyl-CoA thioesterase. The thioesterase activity was found to be about 1.0 nmol of oleic acid released/h per mg of protein in most microsomal preparations, and it was not affected by the addition of either catalase or H₂O₂ (Table 1).

Addition of catalase (2000 units/ml of incubation mixture) resulted in a slight stimulation of oleate desaturation in pea leaf microsomal fraction (Table 1). Considerably higher stimulation of linoleate desaturation by catalase has been reported in microsomal fraction from developing linseed cotyledons (Browse & Slack, 1981). We were interested in finding out whether the effect of catalase on fatty acid desaturations is linked to an interaction with H₂O₂, since catalase is believed to have a protective function against oxidative damage by H₂O₂. In Fig. 1 the effect of increasing concentrations of H₂O₂ on oleate desaturation is shown. The results given are mean values from two sets of incubations with the same batch of microsomal fraction. Exogenous

H₂O₂ had little effect on oleate desaturation in the concentration range 0.1–0.5 mM, but about 50% inhibition of desaturation occurred at approx. 2 mM-H₂O₂ and a complete abolition of desaturation was observed at 10 mM-H₂O₂. The relative lack of susceptibility of oleate desaturase to inhibition at low H₂O₂ concentrations may be due to the presence of an endogenous catalase in the microsomal preparation. The addition of exogenous catalase (2000 units/ml) prevented any inhibition of oleate desaturation by H₂O₂, even at the highest H₂O₂ concentration (10 mM) (Fig. 1). The inhibition of oleate desaturation by H₂O₂ was highly specific, and no effect of H₂O₂ was observed either on oleoyl-CoA thioesterase or oleoyl-CoA:phosphatidylcholine acyltransferase activities over the entire H₂O₂ concentration range employed in the present study (Table 1).

It is conceivable that H₂O₂ affects the oleate desaturase in several ways. It has been shown, for instance, that H₂O₂ is associated with the generation of lipid hydroperoxides in microsomal membranes (Mead, 1976; Dixit *et al.*, 1982), and this may cause membrane changes and loss of activity of membrane-bound enzymes, such as desaturases. Moreover, microsomal lipid peroxidation may increase the orientational order of the acyl chains of membrane lipids (Eichenberger *et al.*, 1982), which would result in diminished membrane protein mobility and a decrease in the polyunsaturated fatty acid complement, with possible effects on membrane-bound enzymes. We found, however, that only traces of oxidized lipids were ever formed with or without the inclusion of H₂O₂ in the incubation mixture.

The second possible effect of H₂O₂ on oleate desaturation could be due to oxidation of NADH, which is one of the cofactors required for the

Fig. 1. Effect of H_2O_2 on the desaturation of oleate by pea leaf microsomal fraction

Incubations with $[1-^{14}C]$ oleoyl-CoA were performed as described in the Experimental section in the presence of ATP (4 mM) and CoA (0.3 mM) and either with (●) or without (○) added catalase (2000 units/ml).

desaturation reaction (Slack *et al.*, 1976; Stymne & Appelqvist, 1978). We tested for a decline in NADH concentrations after the addition of NADH (1 mM) and H_2O_2 (5 mM) to a suspension of the pea leaf microsomal fraction. No such decline was detected.

It is thus obvious that H_2O_2 did not attack either the substrate, oleoyl-CoA, or the cofactor, NADH. The lack of effect of H_2O_2 on such membrane-bound enzymes as oleoyl-CoA thioesterase and oleoyl-CoA:phosphatidylcholine acyltransferase make it unlikely that there was any drastic damage to either the lipid or the protein components of the microsomal membranes. It therefore seems likely that H_2O_2 exerts a specific effect on the oleate-desaturation process itself. Oleate desaturation requires molecular O_2 and NADH and probably proceeds by concerted removal of *D*-hydrogen atoms (Hitchcock & Nichols, 1971). It is possible that H_2O_2 may disrupt this process.

In the present investigation we have demonstrated that pea leaf microsomal fraction is able to form oleoyl-CoA from oleate and convert it into linoleoyl phosphatidylcholine via oleoyl phosphatidylcholine. This may imply that oleate of chloroplastic origin, which is formed by envelope-bound thioesterase (Joyard & Stumpf, 1980), is processed all the way to linoleoyl phosphatidylcholine on the endoplasmic reticulum. Although we detected small

amounts of $[^{14}C]$ linoleoyl-CoA, $[^{14}C]$ linoleate and $[^{14}C]$ linoleoyl galactosylglycerols in the reaction products, phosphatidylcholine was by far the major acceptor for acyl transfer from oleoyl-CoA and also the major point of accumulation of $[^{14}C]$ linoleate. Oleoyl phosphatidylcholine is obviously the major substrate for oleate desaturation in pea leaves.

Financial assistance for this work was provided by Deutsche Forschungsgemeinschaft.

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