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Desaturation of oleoyl groups in envelope membranes from spinach chloroplasts

(ferredoxin/monogalactosyl diacylglycerol/NADPH/*Spinacia oleracea*)

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ABSTRACT **Envelope membranes isolated from chloroplasts of spinach (*Spinacia oleracea*) desaturate oleoyl groups in monogalactosyl diacylglycerol to linoleoyl groups. The desaturation requires NADPH in combination with ferredoxin and is not restricted to monogalactosyl diacylglycerol, since it is also observed in biosynthetic intermediates as, for example, in phosphatidic acid. This indicates a certain degree of unspecificity of the oleate desaturase in isolated envelope membranes. Lipid desaturation is another important function of chloroplast envelopes.**

A common structural feature of different types of plastids is a surrounding envelope that is composed of two different membranes. This membrane system plays an important role in widely divergent processes such as substrate exchange, protein import, and lipid as well as isoprenoid biosynthesis (1–4). Particularly surprising was the discovery that the membrane lipids found in thylakoids are assembled in the envelope before transfer and integration into the acceptor membranes (5). After the first preparation of this membrane system (5, 6), the complete sequence of enzymes catalyzing the conversion of 1-acyl-*sn*-glycerol-3-phosphate via phosphatidic acid to monogalactosyl diacylglycerol (MGD) (7), phosphatidylglycerol (8), digalactosyl diacylglycerol (9), and sulfolipid (10) has been demonstrated in isolated envelope membranes. Immediately after assembly, plastid-made lipids contain oleoyl and palmitoyl residues as acyl groups (11, 12), which subsequently undergo further desaturation to yield linolenic and hexadecatrienoic acid as the predominating acyl groups in chloroplast lipids. All fatty acids are synthesized by soluble enzymes in the stroma (13), where stearoyl acyl carrier protein (ACP) desaturase introduces the first double bond into the C_{18} chains (14). The introduction of further double bonds can occur only after incorporation of palmitic acid (16:0) and oleic acid (18:1) into membrane lipids, from which MGD is a particularly good substrate for the formation of trienoic acids (12, 15, 16). In contrast to its assembly, the desaturation of this glycolipid could only be demonstrated with intact organelles (12, 15–17) and, therefore, the identification of cofactors or a suborganellar localization was not possible.

Only recently, we succeeded in preparing a membrane fraction from detergent-treated chloroplasts that was active in desaturation (18). This fraction contained thylakoids and envelope membranes and required NADPH and ferredoxin (Fd) for desaturation of oleic acid via linoleic acid (18:2) to linolenic acid (18:3). In the present communication, we show that lipid desaturation can also be demonstrated with purified envelope membranes and that these reactions depend on NADPH and Fd as observed before with the mixture of membranes. These results add further competence in an important area of lipid biosynthesis to chloroplast envelopes.

MATERIALS AND METHODS

Biochemicals. An IgG fraction purified from a ferredoxin–$NADP^+$ reductase (FNR; ferredoxin:$NADP^+$ oxidoreductase, EC 1.18.1.2) antiserum (19) was available from a recent investigation (18), acyl-[ACP]:*sn*-glycerol-3-phosphate acyltransferase was a gift from M. Frentzen (University of Hamburg); catalase, bovine serum albumin, FNR, and Fd were from Sigma and [1-^{14}C]oleic acid (2.11 MBq/μmol) was from Amersham.

General Methods. Hydroponic growth of spinach (*Spinacia oleracea* L. cv. Subito), isolation of intact chloroplasts by Percoll gradient centrifugation, extraction of lipids from incubation mixtures, and subsequent separation of individual components by TLC, preparation of fatty acid methyl esters, and their separation by radio-HPLC have been described (16, 18, 20). Protein was determined according to Bradford (21) and chlorphyll was assayed in 80% (vol/vol) acetone (22) by recording the spectra with a Hitachi U 3200 spectrophotometer.

Isolation of Envelope Membranes. Intact chloroplasts (7.5 mg of chlorophyll) in 3 ml of isolation buffer (40 mM Tricine·KOH, pH 8.0/300 mM sorbitol) containing 15 mg of bovine serum albumin were sedimented by centrifugation for 2 min at 3000 × *g*. The supernatant solution was removed and the pellet was mixed with 150 μl of isolation buffer. This suspension was diluted with 6.75 ml of shock buffer (10 mM TAPS·KOH, pH 9.0/10 mM $MgCl_2$; TAPS = *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) for osmotic breakage of chloroplasts. The resultant mixture was placed on a stepped sucrose gradient (in a 14-ml tube) formed by three layers (2 ml each) of increasing sucrose concentrations in 10 mM TAPS·KOH, pH 8.5/4 mM $MgCl_2$. The sucrose concentrations were 0.6, 0.95, and 1.5 M (6). After centrifugation for 20 min at 200,000 × *g*, the yellow envelope membranes were recovered from the 0.6/0.95 M interface. This fraction (0.5–1 ml) was recentrifuged for 1 min at 11,000 × *g* in a Beckman Microfuge. The resultant supernatant fraction with 50–80 μg of protein in 50 μl was immediately used for desaturation assays. For pigment analysis, 200 μl was mixed with 800 μl of acetone followed by a short centrifugation.

Assay for Oleate Desaturation. The envelope fraction (50 μl) was supplemented with various components (in a total vol of 40 μl) to give the following final concentrations or quantities (given in parentheses for the total assay vol of 100 μl): palmitoyl CoA (50 μM), *sn*-glycerol-3-phosphate (0.5 mM), LiCoA (0.25 mM), ATP (2 mM), $MgCl_2$ (7 mM), UDP-galactose (1 mM), spinach Fd (50 μg), NADPH (5 mM),

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Abbreviations: ACP, acyl carrier protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Fd, ferredoxin; FNR, ferredoxin:$NADP^+$ oxidoreductase; MGD, monogalactosyl diacylglycerol.

catalase (5000 units), FNR (20 milliunits), acyl-[ACP]:*sn*-glycerol-3-phosphate acyltransferase (145 ng), and Tricine·KOH buffer (pH 8.0) (12 mM). This solution (90 μl) was mixed with 10 μl of isolation buffer containing 7.5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 150 mM KCl, and 3.77 kBq of [1-^{14}C]oleic acid (final concentration, 17.8 μM). After a 90-min incubation, the reaction was stopped by addition of 2.5 ml of chloroform/methanol (1:1; vol/vol) and 1 ml of 0.45% NaCl (wt/vol) for extraction of lipids.

RESULTS AND DISCUSSION

Our recent experiments with CHAPS-treated chloroplasts had resulted in the preparation of a membrane fraction with high desaturase activity (18). Due to the detergent treatment of the organelles, this fraction was a mixture of thylakoids and envelope membranes, and it was not clear whether or not both types of membranes can carry out desaturation. In these assays, envelope membranes were irreplaceable, because they contain the enzymes required for *in situ* synthesis of [1-^{14}C]oleic acid-labeled MGD (7), which is the most efficient substrate for desaturation (12, 15, 16). Envelope-bound acyl CoA synthetase, acyl-[ACP]:1-acyl-*sn*-glycerol-3-phosphate acyltransferase, phosphatidic acid phosphatase, and UDP-galactose:1,2-diacylglycerol 3-β-D-galactosyltransferase together with soluble, exogenous acyl-[ACP]:*sn*-glycerol-3-phosphate acyltransferase assemble MGD from [1-^{14}C]oleic acid, CoA, ATP, palmitoyl CoA, *sn*-glycerol-3-phosphate, and UDPgalactose (23). The assembly of MGD from preformed fatty acids was the major difference compared with our previous experiments based on acetate labeling of intact chloroplasts (16, 20). By this incubation mode, it is possible to synthesize and retain MGD in the envelope membranes without interference by various effects on lipid equilibration between different membranes, as occurring in intact organelles (16, 24–26). The *in situ* produced MGD with a prokaryotic arrangement (18, 23) of fatty acids (oleic acid at C-1 and palmitic acid at C-2) serves as substrate for desaturation of oleic acid via linoleic acid to linolenic acid in the presence of Fd and NADPH (Fig. 1*a*). This experiment has been repeated to compare the activity of mixed membranes from CHAPS-treated chloroplasts with results obtained in continuation of these experiments.

We now show that osmotically shocked chloroplasts, when assayed in the mode described above, display high desaturase activity and convert MGD-bound oleic acid via linoleic to linolenic acid (Fig. 1*b*). In addition, when the suspension of osmotically shocked chloroplasts was subjected to sucrose gradient centrifugation to obtain an envelope fraction (6), a similar desaturation was observed with this fraction (Fig. 1*c*). The results in Fig. 1 were obtained with fractions prepared in different ways from the same batch of chloroplasts and show that the osmotic sensitivity observed after acetate labeling (16) has been circumvented by the modified incubation mode.

In the experiment shown in Fig. 1*c* even linolenic acid was formed, but at present we are not able to reproducibly demonstrate this linoleic acid desaturase activity in envelope preparations, since in many experiments linolenic acid was not formed despite high desaturation of oleic acid (see Fig. 2 *a* and *b*). We conclude that envelope membranes isolated by the conventional method (6) contain high and rather stable oleic acid desaturase activity ranging from 0.7 to 2.4 nmol per hr per mg of protein, whereas a definite and reliable demonstration of the more labile linoleic acid desaturase requires further optimization.

The assignment of desaturase activity to envelope membranes depends on the purity of this fraction, which was obtained from gradient-purified chloroplasts. The critical contaminations in the present context are thylakoid fragments, traces of which may always be present in the envelope fraction. To further reduce this possible contamination, we routinely recentrifuged the envelope fraction at 11,000 × *g* and sometimes observed a greenish sediment. The chlorophyll content in acetone extracts of the supernatant envelope suspension was always at the limit of detection by conventional photometry and, if present at all, varied between 0.1 and 0.5 μg per mg of protein compared with ≈150 μg per mg of protein in thylakoids. In view of this low (<1%) contamination by thylakoids, we think that the desaturase activity observed in the envelope fraction has to be ascribed to envelope membranes and not to residual thylakoid fragments. This is supported by the observation that the thylakoid fraction recovered from the same gradient from which the envelope was obtained was always less active in desaturation than the envelope fraction. Since the unwashed thylakoid fraction usually contains a high proportion of the original envelope (as evident from the *in situ* synthesis of MGD), we do not know to what extent the desaturation in the thylakoid fraction is actually due to the contaminating envelope membranes. Further experiments are required to show which proportion of the total desaturase capacity in chloroplasts is concentrated in the envelope.

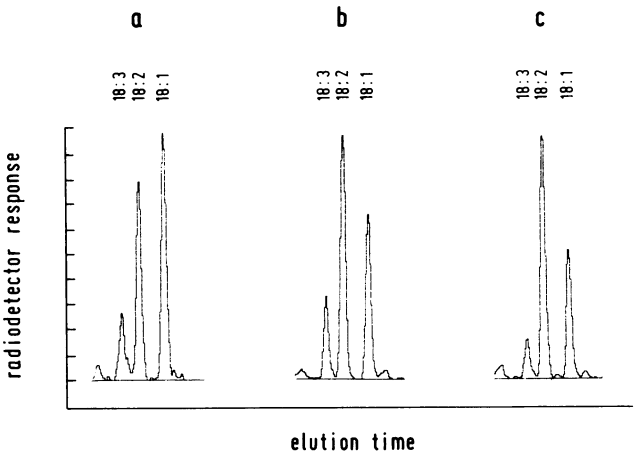

FIG. 1. Desaturation of MGD-bound oleic acid (18:1) in different preparations from chloroplasts. (*a*) Membrane fraction separated by sucrose-gradient centrifugation from CHAPS-treated chloroplasts (18). The fraction contained envelope and thylakoid membranes with 126 μg of protein and 9 μg of chlorophyll. (*b*) Suspension of osmotically shocked chloroplasts containing 53 μg of chlorophyll and 524 μg of protein. (*c*) Purified envelope membranes (78 μg of protein, chlorophyll undetectable) separated by sucrose density-gradient centrifugation from osmotically shocked chloroplasts. All fractions were prepared from the same batch of chloroplasts and were incubated at the same time in parallel with 3.77 kBq of [1-^{14}C]oleic acid and the components required for MGD synthesis and desaturation under identical conditions for 90 min. Extraction of lipids, separation of MGD, preparation of methyl esters, and subsequent resolution by isocratic radio-HPLC were carried out as described (16, 18, 20). Recovery of radioactive fatty acids in MGD (in dpm) and desaturation of [1-^{14}C]oleic acid [given as percent linoleic acid (18:2) plus linolenic acid (18:3) in labeled MGD fatty acids, in parentheses] were 26,900 (52% desaturation) in *a*, 57,700 (66% desaturation) in *b*, and 41,700 (68% desaturation) in *c*.

Next we carried out similar experiments as described for the mixed membranes (18) to identify possible ancillary components involved in desaturation. Incubation of purified envelopes in the absence of electron donors and carriers did not result in desaturation of oleic acid in MGD (Fig. 2*a*), and addition of Fd and FNR did not change this picture (Fig. 2*a*). This also indicates that a gross contamination by thylakoids is absent, which in the light would reduce Fd and support desaturation (18). On the other hand, when NADPH was included (in addition to Fd and FNR) desaturation of oleic

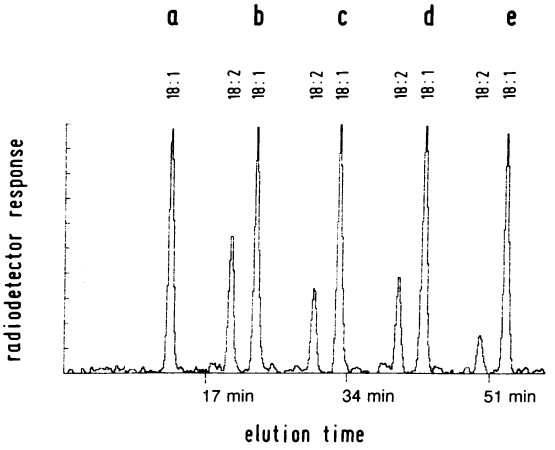

FIG. 2. Involvement of cofactors in envelope-bound desaturation. Aliquots of the envelope membrane fraction (79 μg of protein) recovered from a sucrose gradient were incubated in the light with 3.77 kBq of [1-^{14}C]oleic acid (18:1), additional substrates required for MGD synthesis, and the following components: *a*, no further additions, or plus Fd, or plus FNR, or plus FNR and NADPH, or plus FNR and Fd, none of which supported desaturation; *b*, plus Fd, FNR, and NADPH; *c*, plus Fd and NADPH (omission of FNR); *d*, plus Fd, NADPH, and control IgG (100 μg of protein); *e*, plus Fd, NADPH, and anti-FNR IgG (100 μg of protein). After 90 min of incubation, methyl esters were prepared from MGD for isocratic radio-HPLC. Recovery of radioactive fatty acids in MGD (in dpm) and desaturation of [1-^{14}C]oleic acid [given as percent linoleic acid (18:2) in labeled MGD fatty acids, in parentheses) were 20,900 (no desaturation) in *a*, 37,700 (38% desaturation) in *b*, 30,000 (27% desaturation) in *c*, 27,700 (28% desaturation) in *d*, and 29,000 (16% desaturation) in *e*.

acid to linoleic acid was observed (Fig. 2*b*). Omission of FNR (in the presence of Fd and NADPH) resulted in a partial reduction of desaturation (Fig. 2*c*), whereas after omission of Fd (in the presence of NADPH and FNR) no desaturation was observed (Fig. 2*a*). From these results, we conclude that envelope membranes require NADPH and Fd for desaturation. In analogy to other desaturases (14, 27, 28), it is likely that reduced Fd may be the actual electron donor, although additional carriers between Fd and the desaturase cannot be excluded. When spinach Fd was replaced by Fd from *Spirulina* or *Porphyridium* (all at 26 μg), only a partial reduction in the desaturation of oleic acid was observed (51%, 45%, and 41%, respectively, of linoleic acid), indicating the equivalence of the different Fd in this assay. In view of these results, we imagine that in the light a small proportion of the Fd in chloroplasts will continuously exchange between the surface of envelopes and thylakoids to provide reducing equivalents for desaturation of lipid-bound fatty acids in the envelope. The involvement of Fd could also imply that desaturation in the envelope is limited to its inner membrane. Lipid desaturation completes and extends the competence of envelope membranes in lipid biosynthesis to reactions after the assembly steps. In this context, it may be mentioned that another desaturation sequence in this membrane system converts phytoene in four steps to lycopene. Similar to acyl group desaturation (20), these dehydrogenations also require oxygen, whereas additional electron donors have not yet been identified (29).

Since the reduction of Fd by NADPH can be catalyzed by FNR (30), we tried to find evidence for a possible involvement of this component in desaturation. Omission of FNR from the desaturation assay (Fig. 2*c*) or inclusion of a FNR antibody resulted in a reduction of desaturation (Fig. 2*e*). But as before (18), a complete inhibition was not observed and, therefore, these results can only be considered as preliminary evidence for an involvement of FNR.

Table 1. Detection of radioactive linoleic acid in various lipids after an incubation of envelope membranes with radioactive oleic acid

Component	dpm × 10^{-3} in methyl esters	% 18:2 in methyl esters	dpm × 10^{-3} in 18:2
MGD	38.8	66.2	25.7
Diacylglycerol	19.6	18.3	3.6
Phosphatidic acid	29.9	43.0	12.9
Phosphatidylcholine	17.7	34.0	6.0
Free fatty acids	63.3	2.5	1.6

Envelope membranes (65 μg of protein, $MgCl_2$ increased to 12 mM) were incubated with 3.77 kBq of [1-^{14}C]oleic acid in a desaturase assay for 90 min. The lipid extract was separated by TLC in chloroform/methanol/25% ammonia, 65:25:5 (vol/vol) into individual components, which were used for transmethylation and analysis of fatty acid methyl esters. The low recovery of total radioactivity (77%) is ascribed to the loss of acyl CoA and 1-acyl-*sn*-glycerol-3-phosphate, which are not extracted in our procedure (32, 33) and were not analyzed. MGD was the only lipid that also contained linolenic acid (9.3%), the percentage of which is included in linoleic acid (18:2) of MGD.

Finally, we also looked for oleic acid desaturation in lipid intermediates that are formed during the desaturation assays (Table 1). Apart from free fatty acids, all other lipid components contained linoleic acid, and desaturation was particularly high in phosphatidic acid compared with MGD. This is in contrast to experiments with intact chloroplasts, where desaturation is restricted to MGD as the end product of this assembly sequence (12, 15). It may indicate that the desaturase has no absolute specificity for the lipid headgroup as already concluded from genetic experiments (31). The loss of apparent specificity in our experiments may be due to a change in desaturase accessibility induced during release and isolation of envelope membranes. On the other hand, this may offer the chance to find a suitable desaturase substrate that does not need to be assembled *in situ* and that will further simplify the *in vitro* assays.

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