# The acyl-CoA synthetase and acyl-CoA thioesterase are located on the outer and inner membrane of the chloroplast envelope, respectively

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The chloroplast envelope contains an acyl-CoA synthetase and an acyl-CoA thioesterase which are associated with the outer and inner membrane, respectively.

*Chloroplast envelope* *Outer envelope membrane* *Acyl-CoA thioesterase* *Inner envelope membrane* *Galactosyltransferase* *Acyl-CoA synthetase*

## 1. INTRODUCTION

The localization of both acyl carrier protein and fatty acid synthetase exclusively within the plastids has been taken to indicate that these organelles are the sole site of de novo fatty acid synthesis in plant cells [1–3]. Consequently a special mechanism is probably involved to allow the passage of fatty acids through chloroplast envelope membranes. According to [4], this mechanism involves acyl-CoA. Indeed, the position of both acyl-CoA synthetase [5–7] (EC 6.2.1.3) and acyl-CoA thioesterase [8–10] (EC 3.1.2.2) on envelope membranes suggest that these two enzymes could be involved in the transport of oleic and palmitic acids from the stroma to the cytosol compartment of the leaf cell [7]. To clarify the interacting roles of acyl-CoA thioesterase and acyl-CoA synthetase in leaf glycerolipid metabolism, we have studied the precise localization of these enzymes on the inner and outer envelope membranes from spinach chloroplasts. Some of these findings have been presented in [11].

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## 2. MATERIALS AND METHODS

### 2.1. *Isolation of chloroplasts and of envelope membranes*

Intact chloroplasts were isolated from 3–4 kg spinach leaves by differential centrifugation followed by Percoll density gradient centrifugation as in [12]. Envelope, stroma and thylakoids were prepared from intact purified chloroplasts as in [12,13]. Outer and inner envelope membranes were prepared according to [14]: Intact, purified spinach chloroplasts (150–200 mg chl) were kept on ice for 10 min in 60 ml (2 × 30 ml, final vol.) of hypertonic medium containing 0.6 M mannitol, 4 mM $MgCl_2$ and 10 mM tricine–NaOH (pH 7.9). Under these conditions the outer envelope membrane of the chloroplasts appears to be loosely attached to the inner envelope membrane with large empty spaces in between [15]. Chloroplast suspension (30 ml) was then placed inside a Yeda Press (Linca Scientific Instruments, Tel Aviv). The cell's pressure was raised up to 5 bars using nitrogen. The shrunken chloroplasts were then extruded through the aperture of the Yeda Press at 10 ml/min. The suspension thus obtained was then centrifuged for 10 min at 10000 rev./min (Sorvall

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RC 5, SS 34 rotor). The pellet, containing intact and broken chloroplasts, was discarded and the greenish supernatant diluted 2 times with 4 mM $MgCl_2$ and 10 mM tricine–NaOH (pH 7.9) to give a 0.3 M mannitol suspension (final concentration). Aliquots of 13 ml of the suspension (10–20 $\mu$g chl/ml; 20–40 mg protein/ml) were then layered on top of discontinuous sucrose gradients (1 M, 0.65 M and 0.4 M) and centrifuged for 90 min at 23 000 rev./min (Beckman $L_2$ 65 B, SW 27 rotor). The two bands recovered at the interface 1 M/0.65 M (heavy fraction, $d = 1.13$ g/cm$^3$) and 0.65 M/0.4 M (light fraction, $d = 1.08$ g/cm$^3$) were removed from the tube by using a Pasteur pipette, diluted 4 times with 10 mM tricine–NaOH (pH 7.9), 4 mM $MgCl_2$ and spun for 1 h at 26 000 rev./min (Beckman $L_2$ 65 B, SW 27 rotor). The pellets were suspended in a medium containing 0.3 M sucrose and 10 mM tricine–NaOH (pH 7.9). Analysis of chemical components of these fractions (proteins, lipids, pigments) and of their enzymatic activities led us to the conclusion that the light and heavy fractions were enriched in outer and inner envelope membrane, respectively [14]. For instance, the light fraction was devoid of polypeptide E 30 (a hydrophobic, integral membrane protein involved in the transport of phosphate across the inner envelope membrane [16]) but was strongly enriched in polypeptide E 24 (which is accessible from the cytosolic side of isolated intact chloroplasts to non-penetrating proteases and monospecific antibodies and therefore is localized on the outer envelope membrane [17]). Consequently, the light fraction is enriched in the outer envelope membrane. Conversely, the heavy fraction, which contains a very low amount of polypeptide E 24 and, as one of its major components, polypeptide E 30, is enriched in the inner envelope membrane.

### 2.2. *Assay of acyl-CoA thioesterase*

Activity was routinely assayed at room temperature using palmitoyl-CoA as substrate [8]. The incubation mixture was as follows: 10 mM tricine–NaOH (pH 9.0), 3 $\mu$M [1-$^{14}$C]palmitoyl-CoA (70 000 dpm/nmol); final vol. 400 $\mu$l. The reaction was initiated by addition of enzyme fraction (30–40 $\mu$g protein). At various times (from 15 s to 5 min), 60 $\mu$l aliquots were taken for lipid analysis and determination of the initial velocity of the reaction. The reaction was terminated and the reaction products extracted and analyzed as in [8].

### 2.3. *Assay of acyl-CoA synthetase*

The activity was routinely assayed at room temperature by method C in [7]. This method employed the conversion of [$^{14}$C]oleic acid to [$^{14}$C]oleoyl-CoA. The complete reaction mixture contained: 10 mM tricine–NaOH (pH 8.0); 5 mM ATP; 0.5 mM CoASH; 5 mM phospholenolpyruvate; 200 $\mu$g pyruvate kinase; 10 mM $MgCl_2$; 5 mM dithiothreitol; 1 mM [$^{14}$C]oleic acid (8000 dpm/nmol); 0.5% (w/v) Triton X-100 in 0.6 ml total vol. The reaction was initiated by the addition of the different envelope membrane fractions corresponding to 200–400 $\mu$g protein. At various times (from 20 s to 5 min) 90 $\mu$l aliquots were taken for lipid analysis and determination of the initial velocity of the reaction. The reaction was terminated and the reaction products extracted and analyzed as in [7].

### 2.4. *Assay of UDP-galactose:diacylglycerol galactosyltransferase*

This activity was used as marker for envelope membranes and was measured in chloroplast fractions and envelope subfractions as in [18].

## 3. RESULTS

### 3.1. *Localization of acyl-CoA thioesterase and acyl-CoA synthetase within spinach chloroplasts*

In good agreement with observations on spinach [5–10] and pea [10] chloroplasts, the results presented in table 1 show that acyl-CoA synthetase and thioesterase activities were associated with chloroplast envelope membranes. Indeed, the distribution of these activities follows that of an envelope marker enzyme, UDP-galactose:diacylglycerol galactosyltransferase. In envelope membranes, the specific activity of the acyl-CoA synthetase was 1.5–3 $\mu$mol oleoyl-CoA synthesized.h$^{-1}$.mg protein$^{-1}$, an activity which is ~10-times higher than that of acyl-CoA thioesterase. On the contrary, the activities of acyl-CoA synthetase and of acyl-CoA thioesterase in thylakoid and stroma fractions were very low and could be entirely attributable to contamination of these fractions by envelope fragments. Indeed,

Table 1

Distribution of acyl-CoA thioesterase and acyl-CoA synthetase within spinach chloroplasts

| | Envelope | Stroma | Thylakoids |
|---|---|---|---|
| UDP-galactose:diacylglycerol galactosyltransferase (nmol UDP-[$^{14}$C]galactose incorporated.$h^{-1}$.mg protein$^{-1}$) | 906 | 0.6 | 11 |
| Acyl-CoA thioesterase (nmol [$^{14}$C]palmitic acid released.$h^{-1}$.mg protein$^{-1}$) | 218 | 3 | 10 |
| Acyl-CoA synthetase ($\mu$mol [$^{14}$C]oleoyl-CoA synthesized.$h^{-1}$.mg protein$^{-1}$) | 1.9 | 0.01 | 0.06 |

The chloroplast fractions were prepared as in [12,13]. The activities of the acyl-CoA thioesterase, acyl-CoA synthetase and UDP-galactose:diacylglycerol galactosyltransferase were measured as in section 2. Kinetic experiments were used to determine the initial velocity of the reaction. The values in this table are from a representative experiment

Table 2

Distribution of acyl-CoA thioesterase and acyl-CoA synthetase in membrane fractions deriving from spinach chloroplast envelope

| | Heavy membrane ($d$ = 1.13 g/cm$^3$) | Light membrane ($d$ = 1.08 g/cm$^3$) |
|---|---|---|
| UDP-galactose:diacylglycerol galactosyltransferase (nmol UDP-[$^{14}$C]galactose incorporated.$h^{-1}$.mg protein$^{-1}$) | 880 | 120 |
| Acyl-CoA thioesterase (nmol [$^{14}$C]palmitic acid released.$h^{-1}$.mg protein$^{-1}$) | 350 | 50 |
| Acyl-CoA synthetase ($\mu$mol [$^{14}$C]oleoyl-CoA synthesized.$h^{-1}$.mg protein$^{-1}$) | 0.9 | 3.9 |

The light and heavy fractions were prepared from purified intact spinach chloroplasts as in section 2. Identification of the fractions obtained was as in [11,14]. The activities of acyl-CoA thioesterase, acyl-CoA synthetase and UDP-galactose:diacylglycerol galactosyltransferase were measured as in section 2. Kinetic experiments were used to determine the initial velocity of the reactions. The values in this table are from a representative experiment and have been reproduced at least 3 times

careful washing of thylakoids strongly reduced the activity of these enzymes in the thylakoid fraction (not shown).

### 3.2. *Localization of acyl-CoA thioesterase and acyl-CoA synthetase within envelope membranes*

Light and heavy fractions prepared from chloroplast envelopes as in [14] do not share the same enzymatic activities (table 2). The specific activity of the acyl-CoA thioesterase was very high in the heavy fraction and 300–400 nmol palmitic acid released.$h^{-1}$.mg protein$^{-1}$, a value which was 6–8-times higher than that measured in the light fraction. Conversely, the specific activity of the acyl-CoA synthetase was very high in the light fraction and 3–8 $\mu$mol oleoyl-CoA synthesized.$h^{-1}$.mg protein$^{-1}$, a value which was 4–5-times higher than that measured in the heavy fraction (fig. 1). In good agreement with [11], the UDP-galactose:diacylglycerol galactosyltransferase appeared to be concentrated in the heavy fraction (table 2).

Since biochemical and immunological studies have demonstrated that, on a protein basis, the heavy fraction contained ~80% of inner membrane whereas the light fraction contained ~90% of outer membrane protein ([14], see also [11]), the results presented in table 2 demonstrate that the acyl-CoA synthetase is localized on the outer envelope membrane whereas the acyl-CoA thioesterase, together with the UDP-galactose:diacylgly-

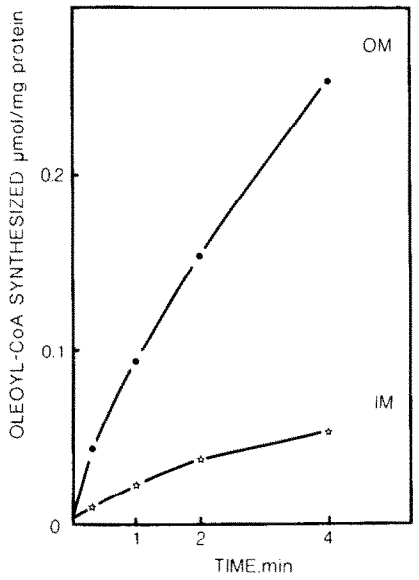


Fig. 1. Synthesis of oleoyl-CoA in membrane fractions deriving from spinach chloroplast envelope. The light and heavy fractions were prepared from purified intact spinach chloroplasts as in section 2. The fractions were identified as in [11,14]. Incorporation of [$^{14}$C]oleic acid into oleoyl-CoA was measured as in section 2. The incubation mixtures (0.6 ml total vol.) contained 350 $\mu$g protein (heavy membrane, enriched in inner envelope membrane, IM) and 330 $\mu$g protein (light membrane, enriched in outer envelope membrane, OM).

cerol galactosyltransferase, is localized on the inner envelope membrane. The low specific activity of acyl-CoA synthetase in the heavy fraction and of the acyl-CoA thioesterase in the light fraction could be entirely attributable to the contamination of each membrane fraction by the other.

## 4. DISCUSSION

The localization of acyl-CoA synthetase and acyl-CoA thioesterase on the outer and inner envelope membrane of spinach chloroplasts, respectively, has major implications in the glycerolipid metabolism in leaf cells.

It was not known whether the true acyl donors for the envelope acyltransferase involved in galactolipid synthesis were acyl-CoA or acyl-ACP (review [19]). The envelope acyl-CoA synthetase and the galactosylation enzyme are localized on two different membranes; we have also shown (in preparation) that the envelope phosphatidic acid phosphatase (making diacylglycerol, the key intermediate in galactolipid synthesis) is localized on the inner membrane. Together these results preclude the use of acyl-CoA as fatty acid donors to galactolipid synthesis via the Kornberg–Pricer pathway. In [20] phosphatidic acid was shown synthesized by acylation of *sn*-glycerol 3-phosphate with acyl-primed acyl carrier protein in the chloroplast envelope.

The presence of acyl-CoA synthetase on the outer envelope membrane readily explains why acyl-CoA synthesis by isolated intact chloroplasts was stimulated by exogenous ATP and CoA [8,21]. Our results also support the view that the acyl-CoA synthetase could be involved in fatty acid export outside the chloroplast [7,19]. Under these conditions, it is likely that fatty acids, synthesized within the plastids [1–3], and destined for further metabolism (phospholipid biosynthesis) in the cytosol, are exported as acyl-CoA, as suggested in [4,21–25].

The physiological significance of the envelope acyl-CoA thioesterase remains unclear. According to [7], a possible role is suggested by the fatty acid specificities of this enzyme: it could be an ultimate control factor to prevent metabolization of fatty acids with carbon chains shorter than C 16. In addition, the acyl-CoA thioesterase could prevent the accumulation of acyl-CoA in the inner membrane since these thioester derivatives behave as detergents and may lead to chloroplast breakage [10].

The interacting roles of each envelope membrane in glycerolipid biosynthesis is complicated by the study of 4 bilayer halves which probably have their own enzymic capabilities.

## REFERENCES

[1] Ohlrogge, J.B., Kuhn, D.N. and Stumpf, P.K. (1979) Proc. Natl. Acad. Sci. USA 76, 1194–1198.
[2] Nothelfer, H.G., Barckhaus, R.H. and Spener, F. (1977) Biochim. Biophys. Acta 489, 370–380.
[3] Vick, B. and Beevers, H. (1978) Plant Physiol. 62, 173–178.
[4] Roughan, P.G., Holland, R. and Slack, C.R. (1980) Biochem. J. 188, 17–24.
[5] Joyard, J. and Douce, R. (1977) Biochim. Biophys. Acta 486, 273–285.

[6] Roughan, P.G. and Slack, C.R. (1977) Biochem. J. 162, 457–459.
[7] Joyard, J. and Stumpf, P.K. (1981) Plant Physiol. 67, 250–256.
[8] Joyard, J. and Stumpf, P.K. (1980) Plant Physiol. 65, 1039–1043.
[9] Liedvogel, B., Kleinig, H., Thompson, J.A. and Falk, H. (1978) Planta 141, 303–309.
[10] Bertrams, M. and Heinz, E. (1980) in: Biogenesis and Function of Plant Lipids (Mazliak, P. et al. eds) pp. 67–71, Elsevier Biomedical, Amsterdam, New York.
[11] Dorne, A.J., Block, M.A., Joyard, J. and Douce, R. (1982) in: Biochemistry and Metabolism of Plant Lipids (Wintermans J.F.G.M. and Liljenberg, C., eds) pp. 153–164, Elsevier Biomedical, Amsterdam, New York.
[12] Douce, R., Block, M.A., Dorne, A.J. and Joyard, J. (1982) in: Chloroplast Molecular Biology (Edelman, M. et al. eds) pp. 239–256, Elsevier Biomedical, Amsterdam, New York.
[13] Douce, R., Holtz, R.B. and Benson, A.A. (1973) J. Biol. Chem. 248, 7215–7222.
[14] Block, M.A., Dorne, A.J., Joyard, J. and Douce, R. (1983) submitted.
[15] Douce, R. and Joyard, J. (1979) Adv. Bot. Res. 7, 1–116.
[16] Heldt, H.W. and Sauer, F. (1971) Biochim. Biophys. Acta 234, 83–91.
[17] Joyard, J., Billecocq, A., Bartlett, S.G., Block, M.A., Chua, N.H. and Douce, R. (1973) J. Biol. Chem. in press.
[18] Douce, R. (1974) Science 183, 852–853.
[19] Douce, R. and Joyard, J. (1980) in: The Biochemistry of Plants vol. 4: Lipids, Structure and Function (Stumpf, P.K., ed) pp. 321–362, Academic Press, New York.
[20] Frentzen, M., Heinz, E., McKeon, T. and Stumpf, P.K. (1982) in: Biochemistry and Metabolism of Plant Lipids (Wintermans, J.F.G.M. and Kuiper, P.J.C. eds) pp. 141–152, Elsevier Biomedical, Amsterdam, New York.
[21] Roughan, P.G., Holland, R. and Slack, C.R. (1979) Biochem. J. 184, 193–202.
[22] Vijay, I.K. and Stumpf, P.K. (1971) J. Biol. Chem. 246, 2910–2917.
[23] Stumpf, P.K., Kuhn, D.N., Murphy, D.J., Pollard, M.R., McKeon, T. and McCarthy, J. (1980) in: Biogenesis and Function of Plant Lipids (Mazliak P. et al. eds) pp. 3–10, Elsevier Biomedical, Amsterdam.
[24] Roughan, P.G. and Slack, C.R. (1982) Annu. Rev. Plant Physiol. 33, 97–132.
[25] Drapier, D., Dubacq, J.P., Trémolières, A. and Mazliak, P. (1982) Plant Cell Physiol. 23, 125–135.