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Characterization of an Acyl-Coenzyme A Thioesterase Associated with the Envelope of Spinach Chloroplasts[1]

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ABSTRACT

The enzymic hydrolysis of acyl-coenzyme A occurs in intact and purified chloroplasts. The different components of spinach chloroplasts were separated after a slight osmotic shock and the purified envelope membranes were shown to be the site of very active acyl-CoA thioesterase activity (EC 3.1.2.2.). The enzyme, which had a pH optimum of 9.0, was not affected by sulfhydryl reagents or by serine esterase inhibitors. However, the acyl-CoA thioesterase was strongly inhibited by unsaturated fatty acids, especially oleic acid, at concentrations above 100 micromolar. In marked contrast, saturated fatty acids had only a slight effect on the thioesterase activity. Substrate specificities showed that the velocity of the reaction increased with the chain length of the substrate from decanoyl-CoA to myristoyl-CoA and then decreased with the chain length from myristoyl-CoA to stearoyl-CoA. Interestingly, oleoyl-CoA was only slowly hydrolyzed. These results suggest that the envelope acyl-CoA thioesterase coupled with an envelope acyl-CoA synthetase may be involved in a switching system which indirectly allows acyl transfer from acyl carrier protein derivatives to unsaturated acyl-CoA derivatives and ensures the predominance of unsaturated 18 carbon fatty acids in plants. Furthermore, the position of both acyl-CoA thioesterase and synthetase in the envelope membranes suggest that these two enzymes may be involved in the transport of oleic acid from the stroma phase to the cytosol compartment of the leaf cell.

The chloroplast is the major site of lipid metabolism in leaf cells since the stroma is the sole site for *de novo* fatty acid synthesis in the cell (18) and the envelope is the site of synthesis of galactolipids, the major plant polar lipids (4, 5). The interaction between the stroma phase and envelope membranes allows the transfer of acyl moieties from ACP[3]-linked reactions (located in the stroma) to CoA-linked reactions (located in the envelope) so that acyl transfers of acyl-CoA to suitable acceptors for galactolipid and/or phospholipid synthesis can occur. This important switching system probably involves a soluble acyl-ACP thioesterase, which converts the stromal oleoyl-ACP to oleic acid and regenerates free ACP (18). In turn, free oleic acid is converted to oleoyl-CoA by an envelope-bound acyl-CoA synthetase (10, 19).

This paper describes the properties of an acyl-CoA thioesterase, associated with the envelope of spinach chloroplasts, which catalyzes the hydrolysis of acyl-CoA thioesters to free fatty acids and CoA. We also discuss the relationship between the acyl-CoA thioesterase and the acyl-CoA synthetase, both of which are associated with the envelope membranes.

MATERIALS AND METHODS

Isolation of Chloroplasts and of Envelope Membranes. Chloroplasts were isolated from 1 to 2 kg spinach leaves obtained from the local markets. Deveined leaves were homogenized for 2 s in a 4-liter Waring Blendor in the following medium: 0.3 M sucrose, 30 mM Tricine-NaOH (pH 7.6) and 0.1% defatted BSA (volume: 2-liters medium/kg leaves). A crude chloroplast fraction was obtained from the leaf homogenate as described by Douce *et al.* (6). In order to avoid "microsomes" and swollen grana lamellae in the chloroplast fraction, the intact chloroplasts were purified by the usual sucrose density gradient procedure (6). Envelope, stroma and thylakoids were prepared from intact purified chloroplasts as described by Douce *et al.* (6). From 1 kg of spinach leaves, the yield of envelope membranes was 2–3 mg protein.

The essential absence of both NADH:Cyt *c* oxidoreductase and of phosphatidylethanolamine precludes significant contamination of the envelope fraction by extrachloroplastic membranes. Furthermore, since the Chl content of the envelope fraction was less than 0.02 μg/mg protein, thylakoid fragments were negligible components.

Assay of Acyl-CoA Thioesterase. The activity was routinely assayed at room temperature (20–23 C) using palmitoyl-CoA as substrate. The incubation mixture was: 10 mM Tricine-NaOH (pH 9.0), 3 μM palmitoyl-CoA (70,000 dpm/nmol); final volume: 400 μl. The reaction was initiated by addition of envelope membranes corresponding to 30–40 μg protein. At various times (from 15 s to 5 min), 60-μl aliquots were taken for lipid analysis and determination of the initial velocity of the reaction. The reaction was terminated by the addition of 1 ml isopropyl alcohol and 1 ml water (containing 30 μl acetic acid) to the aliquots. Free fatty acids were extracted with 3 × 2 ml of petroleum ether (boiling point 35 to 60 C) saturated with 50% isopropyl alcohol. In good agreement with Mancha *et al.* (15), we have verified that this procedure allows a rapid and accurate separation of free fatty acids from acyl-CoA. The radioactivity in the petroleum ether extract (containing the free fatty acids) and the aqueous phase (containing the acyl-CoA thioesters) was counted with 10 ml PCS/xylene (2:1, v/v) scintillation mixture (Amersham/Searle) using a Beckman LS 230 liquid scintillation counter.

To determine whether the acyl-CoA thioesterase was also able to hydrolyze other coenzyme A derivatives, envelope membranes were incubated in presence of radioactive butyryl-CoA, acetyl-CoA or malonyl-CoA. The reaction was initiated by adding envelope membranes (corresponding to 40 μg protein) to the incubation medium (see above) containing 0.2 mM substrate (final volume: 200 μl). The reaction was terminated by addition of 50 μl glacial acetic acid at 3 C. A 10-μl aliquot of the reaction mixture was then applied to silica gel-impregnated glass fiber sheets (ITLC-SG, Gelman Instrument Company), dried under N_2 and

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[3] Abbreviations: ACP: acyl carrier protein; FFA: free fatty acids; PCS: phase combining system.

chromatographed in the following solvent system: water saturated diethyl ether-formic acid (7:1, v/v) according to the procedure described by Huang (8). The radioactivity was located by autoradiography (Kodak, C-Omat XR5 films) and the different products identified by comparison with standards; the CoA derivatives remained at the origin while the corresponding acids moved to the front (8). The radioactivity in the reaction mixture and in the products separated by chromatography was determined as described above.

Identification of the Reaction Products. To rule out a possible transfer of substrate to polar lipids by an acyl transferase (21) the reaction products were analyzed after incubation of envelope membranes with different acyl-CoAs. First, an aliquot of the incubation mixture was chromatographed directly on silica gel-precoated TLC sheets (Baker-Flex) impregnated with potassium oxalate (20) in the following solvent system: chloroform-methanol-water (50:50:8, v/v). This chromatographic system allows a good separation of fatty acids and acyl-CoA thioesters. Second, an aliquot of the incubation mixture was extracted according to the procedure of Bligh and Dyer (3), and the chloroform phase was then chromatographed on silica gel-precoated TLC plates (Analtech) in the following solvent system: chloroform-methanol-water (65:25:4, v/v). In both cases, the radioactivity was located by autoradiography (Kodak, C-Omat XR5 film). The separated lipids were identified by comparison with standards. The only labeled products found in the incubation mixture were free fatty acids and acyl-CoA thioesters. Labeled polar lipids were barely detectable (less than 5% of the total activity) thus indicating that if an acyltransferase was present in the envelope membranes, its activity was negligible compared to that of the thioesterase.

Appearance of CoA during the incubation was followed spectrophotometrically at 412 nm, in presence of 5,5'-dithiobis(2-nitrobenzoic acid) according to Ellman (7). The amount of thiol release was calculated from the M absorption coefficient E_{412} = 13,600 M^{-1} cm^{-1} (7).

Protein determination. Protein concentration was determined by the method of Lowry *et al.* (14) with BSA as standard.

Chemicals. [1-^{14}C]Decanoyl-CoA (58 mCi/mmol), [1-^{14}C]lauroyl-CoA (58 mCi/mmol), [1-^{14}C]myristoyl-CoA (50 mCi/mmol), [1-^{14}C]stearoyl-CoA (57 mCi/mmol), [1,3-^{14}C]malonyl-CoA (58 mCi/mmol), and [1-^{14}C]acetyl-CoA (58 mCi/mmol) were purchased from Rosechem Products; [1-^{14}C]palmitoyl-CoA (53 mCi/mmol), [1-^{14}C]oleoyl-CoA (43 mCi/mmol), and [1-^{14}C]butyryl-CoA (21.2 mCi/mmol) were obtained from New England Nuclear. The principal substrate, [1-^{14}C]palmitoyl-CoA, used in this study, was diluted to the required specific activity with nonlabeled palmitoyl-CoA purchased from Sigma. Since acyl-CoA thioesters form micelles at critical concentrations (from 2 to 10 μM, according to the chain length [1]) and adsorb strongly to proteins and at the air-water interface (2), the concentrations of acyl-CoA used in this investigation represented only the amount added to the reaction mixture and not the actual concentration in the bulk phase.

Free fatty acids were obtained from Applied Science Laboratories or Sigma. All solvents used were reagent grade.

RESULTS

Localization of Acyl-CoA Thioesterase Activity in the Chloroplast. When purified and intact chloroplasts were incubated with acyl-CoA thioesters (lauroyl-CoA, myristoyl-CoA, palmitoyl-CoA) the substrates were very rapidly hydrolyzed, with a stoichiometric appearance of labeled free fatty acids and CoA. To localize the site of acyl-CoA thioesterase activity, a slight osmotic shock was used for the preparation and isolation of the different chloroplast subfractions (6). In marked contrast with the stroma fraction, and to a lesser extent, with the thylakoid fraction, the envelope membranes fraction very rapidly hydrolyzed palmitoyl-CoA to free palmitic acid and CoA. The kinetics of palmitic acid production by the different chloroplast subfractions are shown in Figure 1. These findings demonstrate that a very active acyl-CoA thioesterase was present in the envelope fraction.

CHARACTERIZATION OF THE ENVELOPE ACYL-COA THIOESTERASE

General Properties. With an assay time of 5 min, the formation of free palmitic acid was proportional to the amount of protein added over a limited range (Fig. 2). When purified envelope membranes were incubated at pH 7.0, only a slow hydrolysis of acyl-CoA occurred; at a higher pH, hydrolysis of acyl-CoA occurred readily (Fig. 3). The maximum rate of free palmitic acid formation was obtained at pH 9.0 (the average value for 35 experiments was 225 ± 45 nmol palmitic acid released/mg protein · h). Furthermore, the enzyme retained full activity within a wide range of temperatures. Above 20 C, the activation energy of the reaction was 8,000 cal/mol (Fig. 4). The acyl-CoA thioesterase was highly stable; its activity at 45 C was two times higher than at room temperature and most preparations did not lose any activity on storage at −20 C for 1 month.

The envelope acyl-CoA thioesterase does not require any cat-

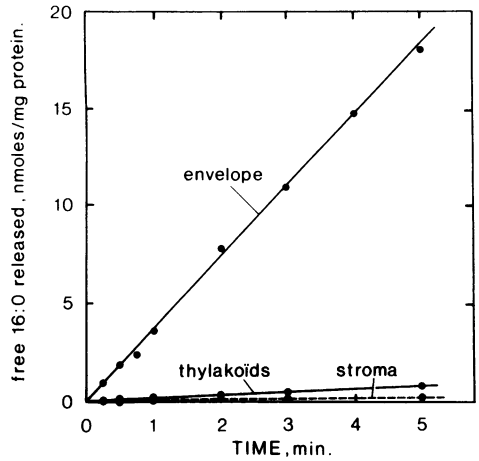

FIG. 1. Hydrolysis of palmitoyl CoA by different fractions separated from intact and purified chloroplasts after a slight osmotic shock. The incubation medium and the lipid extraction were as described under "Materials and Methods." One mg stroma protein, 0.96 mg thylakoid protein, and 0.04 mg envelope protein were used.

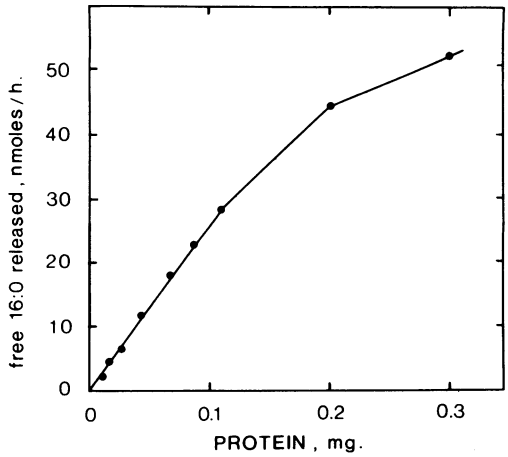

FIG. 2. Effect of protein concentration on the release of free palmitic acid by chloroplast envelope membranes incubated in presence of palmitoyl-CoA. Incubation medium and lipid extraction were as described. Envelope membranes equivalent to 10–300 μg protein were added to the incubation medium.

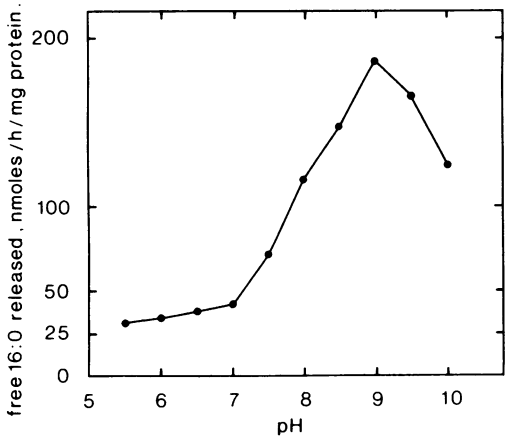

FIG. 3. Effect of pH of incubation medium on the release of free palmitic acid by chloroplast envelope membranes incubated in presence of palmitoyl-CoA. The incubation medium was as described under "Materials and Methods" except that the buffer used below 7.5 was 10 mM Tris-maleate-NaOH. The lipids were extracted as described. Envelope membranes equivalent to 35 μg protein were used.

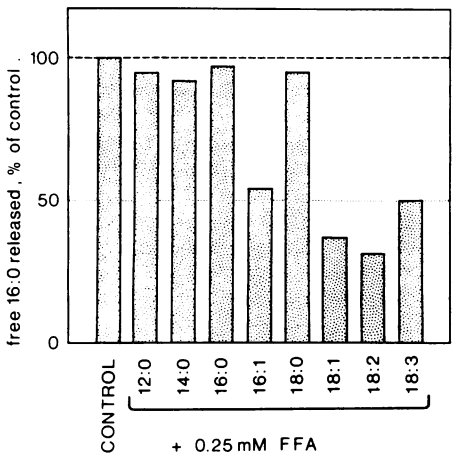

FIG. 5. Effect of free fatty acids on the release of free palmitic acid by chloroplast envelope membranes incubated in presence of palmitoyl-CoA. The incubation medium was as described except that 0.25 mM free fatty acids were added. The lipid extraction was as described. Envelope membranes equivalent to 30 μg protein were used.

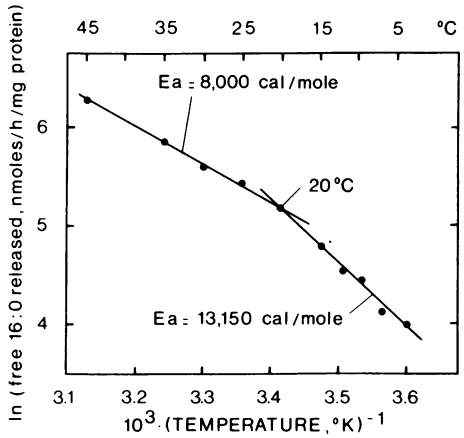

FIG. 4. Effect of the temperature of the incubation medium on the release of free palmitic acid by chloroplast envelope membranes incubated in presence of palmitoyl-CoA. Data are presented by Arrhenius plots. The incubation medium and the lipid extraction were as described. Envelope membranes equivalent to 31 μg protein were used.

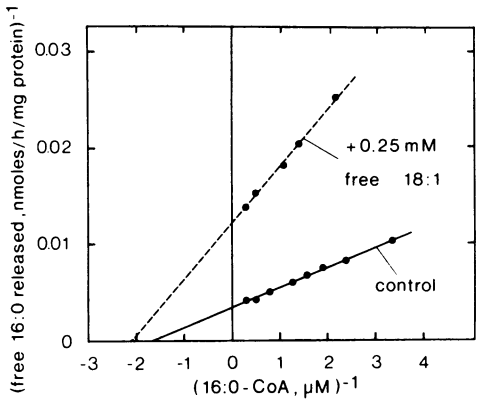

FIG. 6. Effect of free oleic acid on the release of palmitic acid by chloroplast envelope membranes incubated in presence of various concentrations of palmitoyl-CoA. Data are presented according to Lineweaver and Burk. The incubation medium was as described except that 0.25 mM oleic acid was added in one series of experiments and that palmitoyl-CoA concentration used was in the range of 0.3–3 μM. The lipid extraction was as described. Envelope membranes equivalent to 35 μg proteins were used.

ions since the activity was very high in the absence of exogenously added cations. However, the surface of the envelope membranes is strongly negatively charged (16) and is able to bind large amounts of cations such as Mg^{2+} or Ca^{2+} (9, 16). Consequently, it is possible that bound Ca^{2+} or Mg^{2+} could be sufficient to activate the enzyme. However, since the addition of 10 mM Ca^{2+} or Mg^{2+} to the incubation medium inhibited the thioesterase by 30–35%, it was more likely that divalent cations slightly inhibit the envelope acyl-CoA thioesterase.

Effect of Inhibitors. The envelope acyl-CoA thioesterase is insensitive to the sulfhydryl reagents, *N*-ethylmaleimide and iodoacetamide, and to the serine esterase inhibitor, phenylmethylsulfonyl fluoride, two common types of thioesterase inhibitors (12, 18).

In our assay conditions, *i.e.* at an acyl-CoA concentration level of 0.1–30 μM, the envelope thioesterase was not inhibited by the products of the reaction, namely CoA and free fatty acids. Addition of BSA (1 mg/ml) only slightly stimulated the activity of the thioesterase. However, at higher concentrations (above 100 μM) unsaturated fatty acids were found to be effective inhibitors. In marked contrast, saturated fatty acids had only a slight effect on the palmitoyl-CoA thioesterase activity (Fig. 5). This difference remained after increasing the concentration of free fatty acids in the incubation medium up to 1 mM. For instance, we have shown that 1 mM palmitic acid inhibits the palmitoyl-CoA thioesterase only by 30% whereas the same concentration of oleic acid inhibits this activity by more than 90%. This behavior cannot be entirely attributable to the solubility properties of the different fatty acids since oleic and linoleic acids were more effective than palmitoleic and linolenic acids (Fig. 5). Analysis of the reaction kinetics of the enzyme on Lineweaver-Burk plots, in absence and presence of oleic acid, presents a mixed type of inhibition (uncompetitive/noncompetitive) with an apparent inhibition constant value (K_i) of approximately 90–100 μM (Fig. 6).

Substrate Specificity. The specificity of the envelope acyl-CoA thioesterase was investigated with different CoA and ACP thioesters. First, the envelope thioesterase was unable to hydrolyze either malonyl-CoA, acetyl-CoA or butyryl-CoA. Second, the acyl-CoA thioesterase hydrolyzes only very slowly palmitoyl-ACP and stearoyl-ACP, whereas the stroma fraction, in good agreement with previous reports (18) readily hydrolyzed the acyl-ACP thioesters. Third, we investigated the specificity of the acyl-CoA thioesterase with CoA derivatives of saturated fatty acids (from decanoic

acid to stearic acid) and of an unsaturated fatty acid (oleic acid). The velocity of the reaction was determined for each substrate in a wide range of concentration (from 0.1 to 30 μM). The results (Fig. 7), and especially the apparent K_m values, show very clearly the effect of micelle formation on the velocity of the reaction. For each substrate, the velocity remained constant above the critical micellar concentration, thus indicating that only free monomeric acyl molecules served as substrate. Furthermore, myristoyl-CoA was the most effective substrate for the enzyme. The level of activity obtained with oleoyl-CoA was much lower although this acyl-CoA is a relatively soluble molecule. The comparative order of reactivity of the different acyl-CoA substrates remained constant over the concentration range of 0.1–10 μM: the velocity of the reaction increased with the chain length of the substrate from decanoyl-CoA to myristoyl-CoA and then decreased from myristoyl-CoA to stearoyl-CoA and oleoyl-CoA.

DISCUSSION

The results reported here demonstrate the presence of an active acyl-CoA thioesterase associated with the envelope of purified spinach chloroplasts. It has been shown recently (13) that chromoplasts from *Tropaeolum majus* also contain an acyl-CoA thioesterase "which sometimes seems to be very active in envelope membranes" (11). Unfortunately, the standard incubation time (30 min) used for these experiments was too long to allow a precise localization of the acyl-CoA thioesterase within the chromoplast and it was suggested that this enzyme was probably soluble "possessing, however, high affinities to lipid rich structures" (13). In purified envelope membranes from spinach chloroplasts, the high specific activity of the acyl-CoA thioesterase and the absence of acyl-ACP thioesterase rule out the possibility of a contaminating soluble enzyme in this fraction. The acyl-CoA thioesterase is therefore membrane-bound and the very low level of activity found in the stroma fraction could be entirely attributable to a contamination by small envelope vesicles. In support of this hypothesis, we have observed that some stroma preparations were entirely devoid of acyl-CoA thioesterase activity.

At present, the role of acyl-CoA thioesterase in plant lipid metabolism is not clear. The function of the envelope acyl-CoA thioesterase is probably very different from that of the acyl-ACP thioesterase located in the stroma since both enzymes have a very different substrate specificity; oleoyl-CoA is slowly hydrolyzed by the acyl-CoA thioesterase while oleoyl-ACP is the major substrate for the acyl-ACP thioesterase (18). In addition to the acyl-CoA thioesterase, the chloroplast envelope contains an acyl-CoA synthetase which synthesizes acyl-CoA in presence of ATP-Mg, free fatty acids and CoA (10, 19). It may be that these two enzymes function together in a tightly coupled manner. It is likely that during galactolipid synthesis (which occurs on the chloroplast envelope and requires the functioning of different enzymes including especially acyl-CoA synthetase, (4, 5)) the acyl-CoA thioesterase should have only a low level of activity to allow the transfer of fatty acids to polar lipids via acyl-CoA derivatives. Recently, Kleinig and Liedvogel (11) have suggested that the very high levels of ATP (and CoA) required for the incorporation of fatty acids into chloroplast polar lipids facilitate the acyl-CoA synthetase at the expenses of the thioesterase. Furthermore, Roughan and Slack (19) have shown that the velocity of palmitoyl-CoA biosynthesis by isolated envelope membranes is very high (~ 500 μmol/mg protein·h) although this acyl-CoA constitutes a good substrate for the thioesterase. From these observations, it seems that when active lipid synthesis occurs in the chloroplast, especially in envelope membranes, the acyl-CoA thioesterase must be controlled.

However, the peculiar substrate specificity of the enzyme and the inhibition of its activity by free unsaturated fatty acids may suggest a role for this enzyme in the control of the chain length of the fatty acids synthesized in the chloroplast. In this organelle, the stromal enzymes involved in fatty acid synthesis, and especially the acyl-ACP thioesterase, play a predominant role to ensure the predominance of unsaturated 18 carbon fatty acids in plants (18). From our data, it is possible to suggest that the acyl-CoA thioesterase (coupled to the acyl-CoA synthetase) could also be involved in controlling fatty acid chain length prior to their incorporation into polar lipids. Indeed, these two enzymes appropriately positioned in the envelope membranes may be directly involved in the transport of oleic acid from the stroma phase to the cytosolic compartment of the leaf cell. In these conditions, it would be of a great interest to localize more precisely the acyl-CoA thioesterase and the acyl-CoA synthetase on the inner or the outer membrane of the chloroplast envelope. Unfortunately, up to now, all the attempts made to try to separate the two membranes of the envelope from each other have failed. To clarify the interacting roles of acyl-CoA thioesterase and acyl-CoA synthetase, techniques must be developed to allow the independent measurement of these two enzymes with respect to their substrate specificity.

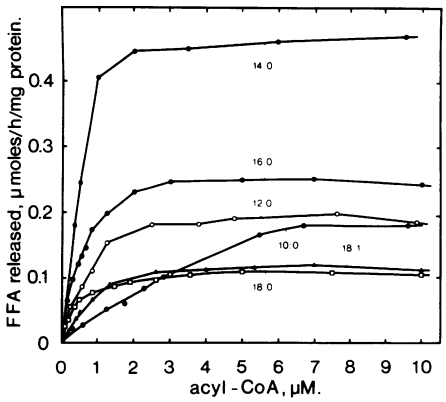

FIG. 7. Release of free fatty acids by chloroplast envelope membranes incubated in presence of various concentrations of different acyl-CoA derivatives. The incubation medium was as described except that the acyl-CoA concentrations used were in the range of 0.1–30 μM. The different acyl-CoA used were as indicated. The lipid extraction was as described. Envelope membranes equivalent to 30–40 μg protein were used. Results for acyl-CoA concentrations between 10 and 30 μM are not shown, since there was no change in the velocity of the reactions above 10 μM. The apparent K_m values were determined for each substrate from Lineweaver-Burk plots as follows: decanoyl-CoA, 2.9 μM; lauroyl-CoA, 1.3 μM; myristoyl-CoA, 1 μM; palmitoyl-CoA, 0.6 μM; stearoyl-CoA, 0.33 μM, and oleoyl-CoA, 1.3 μM.

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