Purification and Properties of Acyl Coenzyme A Dehydrogenases from Bovine Liver

FORMATION OF 2-TRANS,4-CIS-DECADIENOYL COENZYME A

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Three straight chain acyl-CoA dehydrogenases were purified to apparent homogeneity from bovine liver using 40–70% (NH$_4$)$_2$SO$_4$ precipitation, gel filtration, DEAE-cellulose column chromatography, and preparative electrophoresis. Separation of the acyl-CoA dehydrogenases by these procedures has been efficiently monitored by two newly developed analytical methods: (i) native staining of acyl-CoA dehydrogenases following separation by electrophoresis in polyacrylamide gels and (ii) determination of general acyl-CoA dehydrogenase by means of a specific substrate, 4-cis-decenoyl-CoA. The three acyl-CoA dehydrogenases were classified into short chain, general, and long chain acyl-CoA dehydrogenases on the basis of their chain length specificities according to the nomenclature proposed by Hall and Kamin (Hall, C. J., and Kamin, H. (1975) J. Biol. Chem. 250, 3470–3486). The enzymes gave single protein bands in polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions, and their subunit and native molecular weights were estimated to be 40,300 and 188,000 for short chain acyl-CoA dehydrogenase, 43,300 and 205,000 for general acyl-CoA dehydrogenase, and 45,200 and 172,000 for long chain acyl-CoA dehydrogenase.

Long chain and general acyl-CoA dehydrogenases markedly differed in their substrate specificities toward unsaturated acyl-CoA esters with a double bond at position 4. The former oxidized 4-cis-decenoyl-CoA at a rate of only 2.7% of that obtained with decanoyl-CoA as substrate, while for the latter enzyme 4-cis-decenoyl-CoA was even a slightly better substrate than decanoyl-CoA. 2-trans,4-cis-Decenoyl-CoA was identified as the product of this reaction.

As most cells degrade a great variety of physiologically occurring fatty acids as well as widely different intermediates arising in the course of β-oxidation, they do need correspondingly flexible enzymatic equipment. Indeed, mitochondria possess sets of β-oxidation enzymes with varying chain length specificities (1–5), whereas in peroxisomes the β-oxidation system appears to be more specific for long chain fatty acyl-CoA esters (6, 7). In the case of straight chain acyl-CoA dehydrogenases, three enzymes were described in mammalian liver (8–13), kidney (14), and heart (10, 12, 15) designated as short chain, long chain, and general (or medium chain) acyl-CoA dehydrogenase (9, 16). Although great effort has been devoted to the elucidation of chain length specificity, the effect of number and positions of double bonds on the rates of individual β-oxidation reactions has been widely neglected so far, although cis-unsaturated intermediates arise in the course of the degradation of all unsaturated fatty acids. Characterization of acyl-CoA dehydrogenases with respect to unsaturated acyl-CoA esters as substrates has not been reported.

Previous studies on oxidation of unsaturated fatty acids from this laboratory led to the conclusion that 2-trans,4-cis-decadienoyl-CoA esters are interesting intermediates of all unsaturated fatty acids possessing cis-double bonds at even-numbered carbon atoms (17–19). They are the substrates of 2,4-dienoyl-CoA reductases (17, 20, 21), whereas their hydration catalyzed by enoyl-CoA hydratase has been questioned (19, 22–24).

The present work was undertaken (i) to investigate which of the different acyl-CoA dehydrogenases from bovine liver mitochondria catalyze the conversion of 4-cis-decenoyl-CoA, a presumed metabolite of linoleate, to 2-trans,4-cis-decadienoyl-CoA and (ii) to obtain purified acyl-CoA dehydrogenases for reconstitution studies with other β-oxidation enzymes.

In this report, we describe analytical methods which allow determination of the individual acyl-CoA dehydrogenases from bovine liver either qualitatively or quantitatively in crude preparations. Furthermore, we report a four-step purification procedure which led to three homogeneous straight chain acyl-CoA dehydrogenases. In addition, we present some molecular and kinetic properties of the individual enzymes with special emphasis on substrate specificities toward unsaturated acyl-CoA esters.

EXPERIMENTAL PROCEDURES

Materials

The following substances used in these investigations were obtained from the sources mentioned in parentheses: iodonitrotetrazolium chloride, Triton X-100, dihydroxyacetone, and reagents for electrophoresis (Serva, Heidelberg, Federal Republic of Germany); FAD, coenzyme A, bovine serum albumin, lactate dehydrogenase, catalase, aldolase, ovalbumin (Boehringer Mannheim, FRG); Sephadex G-150 and polyacrylamide gradient gels PAA 4/30 (Deutsche Pharmacia

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†† Dedicated to Dr. Hans Faillard on the occasion of his sixtieth birthday.

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1 Short chain, general, and long chain acyl-CoA dehydrogenases have previously been referred to us as acyl-CoA dehydrogenase I, II, and III, respectively (17, 19).
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GmbH, Freiburg, FRG; DEAE-cellulose (Whatman DE52; Hermle GmbH, St. Leon-Rot, FRG); saturated fatty acids ( Roth, Karlsruhe, FRG or Fluka AG, Buchs, Switzerland; [1-14C]decanoic acid (Amer- sham Buchler GmbH, Braunschweig, FRG); 8-dimethylamino-2,3-benzenophenazonium chloride (melfolablau; gift from Sandzö AG, Basel, Switzerland). All other chemicals were from E. Merck, Darmstadt, FRG. Glycerophosphorylated fatty acids as well as fatty acyl methyl esters and CoA esters were synthesized in this laboratory as reported previously (17).

Acyl-CoA Dehydrogenase Assay

The spectrophotometric assay was performed as described previously (25). It contained in a volume of 1 ml 0.2 M Bicine* / KOH, pH 8.0, 0.2 M Tris/Cl, pH 7.5, 0.1 mM iodonitrotetrazolium chloride, 0.1 mM acyl-CoA ester, and up to 200 mM of the enzyme solution. The reduction of the tetrazolium salt was monitored at 492 nm at 10 °C.

Protein Determination

Protein was estimated by two procedures. Bovine serum albumin served as protein standard. The spectrophotometric method of Murphy and Smith (28) was used routinely. As an additional proof, the method of Bradford (27) was applied.

Product Analyses

Incubations and the analyses of the formed acyl-CoA esters by means of radiois chromatography were performed as described previously (17).

Analytical Polyacrylamide Gel Electrophoresis

Purity and peptide molecular weights were assessed by sodium dodecyl sulfate-gel electrophoresis in 10% polyacrylamide slab gels formed by generation of formazan. After bands were visible, gels were stained and destained according to Laemmli (28). Gels were stained with 0.25% Coomassie blue R-250 in methanol/acetic acid/water (5:1:5) and destained in methanol/acetic acid/water (4:1:9) (29).

Polycrylamide gel electrophoresis under non-denaturing conditions was done essentially as described in Ref. 30 using slab gels of 4–30% (w/v) acrylamide gradients. Gels were stained and destained as for sodium dodecyl sulfate-gel electrophoresis.

Electrophoresis for specific staining was performed in gel tubes (10 cm, 8-mm inner diameter) as described by Williams and Reifstein (31). The system contained a 2.5% acrylamide stacking gel (0.4 M Tris/phosphate, pH 5.5), a 7.5% acrylamide separating gel (0.56 M Tris/HCl, pH 7.5), and electrode buffer (0.06 M 5,5'-diethylbarbituric acid, 0.084 M Tris, pH 7.0). Electrophoresis was conducted at 20 °C for about 3 h at 2 mA/tube while the proteins migrated in the stacking gel and at 5 mA/tube during separation. Proteins were stained with Amido black.

For locating the acyl-CoA dehydrogenases, the polycrylamide gels were washed in distilled water for 15 min. Then they were incubated at 20 °C in the dark with a freshly mixed staining solution consisting of the following components: 3 ml of 0.2 M Bicine/KOH buffer, pH 8.0, containing 0.15% Triton X-100, 3 ml of an aqueous solution of 2 mM melfolablau, 0.5 ml of an aqueous solution of 5 mM iodonitrotetrazolium chloride, and 15 μl of an aqueous solution of 10 mM acyl-CoA ester. Acyl-CoA dehydrogenases were detected as red bands formed by generation of formazan. After bands were visible, gels were destained by several changes of Bicine buffer until the background was colorless.

Purification of Acyl-CoA Dehydrogenases

Extraction of Acyl-CoA Dehydrogenases—All procedures were performed at 0–4 °C. Beef liver mitochondria were isolated according to the procedure of Brossmann et al. (32). Acetone dry powder from freshly prepared mitochondria was obtained as described by Dahlen and Porter (33). Crude extracts were obtained by stirring 50 g of acetone dry powder in 250 ml of 0.05 M Tris/HCl, pH 7.5, containing 5 × 10−4 M EDTA and 10 −4 M FAD for 30 min. The mixture was centrifuged for 30 min at 100,000 × g. To the supernatant (148 ml, 7,500 mg) was added 5 × 10−3 M dithioerythritol.

Ammonium Sulfate Precipitation—A solution of 3.8 M (NH₄)₂SO₄ (682 g/liter) in 0.05 M Tris/HCl, pH 7.5, containing 5 × 10−3 M dithioerythritol, 5 × 10−3 M EDTA, and 15 mM FAD was used. In the first step (0–40% saturation), 710 ml were added per liter of crude extract. In the second step (40–70% saturation), precipitation of the acyl-CoA dehydrogenases was obtained by addition of 1180 ml/liter. The 40–70% precipitate was homogenized in a small volume of 0.05 M Triss/HCl, pH 7.5, and dialyzed against the same buffer until a clear solution was obtained.

Gel Filtration—The dissolved 40–70% ammonium sulfate precipitate (50 ml, 3750 mg) was applied to a column (10 × 80 cm) of Sephadex G-150, equilibrated with 0.05 M Tris/HCl, pH 7.5. Fractions of 27 ml were collected during elution with the same buffer (flow rate, 150 ml/h). Those fractions containing acyl-CoA dehydrogenase activities greater than 1% (referring to the ammonium sulfate precipitate applied to the column) were pooled (G-150 pool, 500 ml, 1350 mg) and used for further purification without concentration.

Ion Exchange Chromatography—Preswollen DEAE-cellulose (Whatman DE52) was added to the pooled fractions amounting to 100 ml. Binding and elution of proteins was achieved in 0.05 M Tris/HCl, pH 7.5, by a discontinuous NaCl gradient. Equilibration, column loading, and column washing were carried out at 30 mM NaCl. Loading was performed at a flow rate of 400 ml/h followed by washing with 30 mM NaCl at 200 ml/h. Elution was carried out at a flow rate of 100 ml/h with the following NaCl concentrations: 55 mM NaCl, about 12 h; 60 mM NaCl, about 2 h; 90 mM NaCl, about 8 h; 105 mM NaCl, about 2 h; 160 mM NaCl, about 12 h. The fraction obtained with 55 mM NaCl contained mainly short chain acyl-CoA dehydrogenase (i.e. crude short chain acyl-CoA dehydrogenase), the fraction obtained with 90 mM NaCl contained mainly general acyl-CoA dehydrogenase (i.e. crude general acyl-CoA dehydrogenase), and the fraction obtained with 160 mM NaCl contained mainly long chain acyl-CoA dehydrogenase (i.e. crude long chain acyl-CoA dehydrogenase). These crude acyl-CoA dehydrogenase fractions were concentrated to about 10–15 ml by ultrafiltration (diaflow ultrafiltration cell from Amicon Corp., filter membrane size, XM 100A). 10−3 M FAD and 5 × 10−4 M EDTA were added in this step.

Preparative Electrophoresis—Preparative polyacrylamide gel electrophoresis was performed with the system described above for the analytical electrophoresis of native proteins (31). We used a Colora Ultra Phor electrophoretic apparatus equipped with a separating chamber of 7.5-mm thickness and 13.8-cm length. Gels were mixed as for analytical electrophoresis, with an 8 ml-stacking gel and a 32 ml separating gel. The Colora Ultra Phor electrophoretic apparatus was used to continuously elute the proteins leaving the bottom of the gel during electrophoresis (34). The counter chamber was flushed with 0.07 M Tris/HCl, pH 6.7, at a flow rate of 16 ml/h. Elution was carried out with the same buffer, but diluted 1:3 with double distilled water, and 10−3 M FAD and 5 × 10−4 M EDTA were added. The flow rate was 80 ml/h.

The crude acyl-CoA dehydrogenase fractions obtained from ion exchange chromatography were dialyzed over night against the stacking gel buffer (1:8 diluted according to the final gel concentration) and centrifuged for 30 min at 100,000 × g. They were concentrated by ultrafiltration as described above to 3–4 ml.

0.1 ml of bromphenol blue (0.02%) and 0.25 ml of glycerol were added. Preparative electrophoresis was performed at a constant voltage of 400 V over night. 10-ml fractions were collected. Bromphenol blue appeared in the eluate after 3 h.

RESULTS

Detection and Quantitative Determination of Individual Acyl-CoA Dehydrogenases in Crude Extracts

As demonstrated in Fig. 1, it is possible to separate the three straight chain acyl-CoA dehydrogenases from bovine liver by means of polyacrylamide gel electrophoresis. Detection of the acyl-CoA dehydrogenases was achieved by a specific staining procedure based on the same reaction which previously had been developed and employed by us as photometric enzyme test (25). Meldolablau and iodonitrotetrazo-
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FIG. 1. Specific staining of acyl-CoA dehydrogenases. The different acyl-CoA dehydrogenases were separated by analytical polyacrylamide gel electrophoresis according to Williams and Reisfeld (31). Lanes a–d were loaded with crude extract (0.3 mg of protein). Lane a was stained for protein with Amido black. Lanes b–d were stained for acyl-CoA dehydrogenases using different substrates: 40-CoA for general and long chain acyl-CoA dehydrogenases (lane b), 40-CoA for short chain and general acyl-CoA dehydrogenases (unresolved broad band, lane c), 4-10:1-CoA for general acyl-CoA dehydrogenase (lane d). Lanes e and f demonstrate the separation of general and short chain acyl-CoA dehydrogenases in a further purified fraction (DE52 column, 90 mM NaCl fraction, concentrated by ultrafiltration, 10 μg of protein). After staining in the presence of 40-CoA, short chain and general acyl-CoA dehydrogenases are shown as two small bands (lane e). With 4-10:1-CoA, only general acyl-CoA dehydrogenase can be detected (lane f). For the staining technique, see “Experimental Procedures.” G-AD, general acyl-CoA dehydrogenase; SC-AD, short chain acyl-CoA dehydrogenase; LC-AD, long chain acyl-CoA dehydrogenase.

Lithium chloride were used as primary and secondary electron acceptors. The highly colored and poorly water-soluble formazan allowed to localize the zones of acyl-CoA dehydrogenase activities in the polyacrylamide gel after electrophoretic separation.

As both the acyl-CoA dehydrogenases and the staining solution are labile at alkaline pH values, the widely used electrophoretic system of Davis (35) could not be employed for this purpose. Therefore, we applied the system of Williams and Reisfeld (31) which works at neutral pH values. Although under these conditions the separation of proteins is relatively poor (Fig. 1, lane a), it was sufficient to resolve the acyl-CoA dehydrogenases. Especially the general and long chain acyl-CoA dehydrogenases, which due to their overlapping chain length specificities cannot be distinguished in the photometric test with saturated acyl-CoA esters, are easily separated (Fig. 1, lane b). The resolution of the general and the short chain acyl-CoA dehydrogenases is less effective (Fig. 1, lane e). Since the activity of the latter is almost eight times higher in crude extracts from bovine liver mitochondria and both enzymes act on short saturated acyl-CoA esters in crude extracts, these two acyl-CoA dehydrogenases could not be resolved using butyryl-CoA as substrate (Fig. 1, lane c). However, a clear separation is possible from partially purified fractions which contain both enzymes in similar amounts. The specific detection of general acyl-CoA dehydrogenase in all fractions, even in crude extracts, is possible by using 4-cis-decenoyl-CoA (Fig. 1, lanes d and f). The quantitative determination of each of the three acyl-CoA dehydrogenases in crude extracts is possible by exploiting the existence of a specific substrate for at least one of the three acyl-CoA dehydrogenases. Using 4-cis-decenoyl-CoA in addition to butyryl-CoA and decanoyl-CoA as substrate for the photometric test and knowing the relative maximal velocities of the different acyl-CoA dehydrogenases toward the different substrates, the individual activities can be calculated.

Purification of Three Straight Chain Acyl-CoA Dehydrogenases from Bovine Liver

The starting material used to isolate the different straight chain acyl-CoA dehydrogenases from bovine liver was an acetone dry powder of the mitochondrial fraction. Throughout the purification, acyl-CoA dehydrogenase activities were determined using butyryl-, decanoyl-, and 4-cis-decenoyl-CoA esters as substrates. FAD and EDTA were routinely added to all solvents because otherwise the recovery was much lower. Ammonium sulfate precipitation (40–70% saturation) (step 1) and gel filtration on Sephadex G-150 (step 2) were efficiently used to separate more than 80% of the total protein (Table I).

Step 3: DEAE-cellulose Column Chromatography—In the presence of 30 mM NaCl, 50–60% of the applied proteins ran through the DE52 column. The three acyl-CoA dehydrogenases were eluted stepwise by a discontinuous NaCl gradient (Table I) at 55, 90, and 160 mM NaCl. The chosen NaCl concentrations represent the lowest ones, that desorbed corresponding acyl-CoA dehydrogenases. Therefore, elution proceeded very slowly and resulted in large volumes. Two additional elution steps at 60 and 105 mM NaCl were taken to assure that the foregoing step eluting one of the three acyl-CoA dehydrogenases had been exhaustive before the next enzyme was desorbed. These two additional fractions were discarded. The three fractions containing the acyl-CoA dehydrogenases were concentrated by ultrafiltration (Amicon XM 100A) from about 1000 to 10–15 ml with 80% recovery of dehydrogenase activities.
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### TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Substrate = 4:0-CoA</th>
<th>Substrate = 4c-10:1-CoA</th>
<th>Substrate = 10:0-CoA</th>
</tr>
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<tr>
<td></td>
<td>mg</td>
<td>milliunits/mg</td>
<td>units</td>
<td>% purification</td>
</tr>
<tr>
<td>1 Crude extract</td>
<td>7,500</td>
<td>213</td>
<td>1,600</td>
<td>100</td>
</tr>
<tr>
<td>2 40–70% (NH₄)₂SO₄</td>
<td>5,750</td>
<td>341</td>
<td>1,280</td>
<td>80</td>
</tr>
<tr>
<td>3 G-150 pool</td>
<td>1,350</td>
<td>711</td>
<td>960</td>
<td>60</td>
</tr>
<tr>
<td>4 DE52 column</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 55 mM NaCl</td>
<td>120</td>
<td>4,500</td>
<td>540</td>
<td>34</td>
</tr>
<tr>
<td>6 90 mM NaCl</td>
<td>45</td>
<td>1,780</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>6 160 mM NaCl</td>
<td>60</td>
<td>267</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>7 Preparative electrophoresis</td>
<td>17,500</td>
<td>84</td>
<td>1,043</td>
<td>40</td>
</tr>
</tbody>
</table>

| Fraction used to purify short chain acyl-CoA dehydrogenase by means of preparative electrophoresis. |
| Fraction used to purify general acyl-CoA dehydrogenase by means of preparative electrophoresis. |
| Fraction used to purify long chain acyl-CoA dehydrogenase by means of preparative electrophoresis. |
| After preparative electrophoresis, fractions containing enzyme activities were not combined. Therefore, data of total protein and yield of enzyme activity cannot be shown. Data of specific activities refer to the peak fractions of each electrophoresis: short chain acyl-CoA dehydrogenase assayed with 4:0-CoA, general acyl-CoA dehydrogenase assayed with 4c-10:1-CoA, and long chain acyl-CoA dehydrogenase assayed with 10:0-CoA. |

The DE52 column resulted in an almost complete separation of two acyl-CoA dehydrogenases from the respective other two acyl-CoA dehydrogenases (Table I). The short chain acyl-CoA dehydrogenase fraction (55 mM NaCl) was less than 2% contaminated by general acyl-CoA dehydrogenase, and the long chain acyl-CoA dehydrogenase fraction (160 mM NaCl) contained less than 4% general acyl-CoA dehydrogenase activity. The general acyl-CoA dehydrogenase fraction (90 mM NaCl) contained approximately equal amounts of the other two dehydrogenases, but compared to the activities of the three acyl-CoA dehydrogenases in the Sephadex G-150 pool applied to the DE52 column, 85–90% of the short chain and long chain acyl-CoA dehydrogenase activities could be separated from the bulk of the general acyl-CoA dehydrogenase.

Two points were important to obtain this separation. DEAE-cellulose DE52 was superior to DEAE-Sephadex A-50 because from the former all three dehydrogenases eluted at lower NaCl concentrations and with higher recoveries as DEAE-cellulose DE52 was superior to DEAE-Sephadex A-50ase.

The fractions obtained from preparative electrophoresis were used without pooling to study the properties of highly purified acyl-CoA dehydrogenases. Therefore, no recovery data could be presented for this purification step (Table I, row 7).

**Determination of Molecular Weights**

Peptide molecular weights were determined by polyacrylamide-gel electrophoresis under denaturing conditions according to Laemmli (28). Only one band was observed in each purified acyl-CoA dehydrogenase fraction (Fig. 3). Molecular weights were estimated by comparison with known peptide markers (Fig. 4) to be 40,500, 43,300, and 45,200 for the short chain, general, and long chain acyl-CoA dehydrogenases, respectively. Native molecular weights were obtained by gel electrophoresis in a polyacrylamide gradient (4–30%). Comparison with standard proteins (Fig. 5) resulted in molecular weights of 188,000 ± 5,600 (short chain acyl-CoA dehydrogenase), 205,000 ± 5,500 (general acyl-CoA dehydrogenase), and 172,000 ± 5,600 (long chain acyl-CoA dehydrogenase). These values are in close agreement with the expected molecular weights of acyl-CoA dehydrogenases as tetramers of identical subunits. The elution pattern of the acyl-CoA dehydrogenases from Sephadex G-150 (data not shown) confirmed the native molecular weights to be very similar and to be in the range of 160,000–200,000. The elution volumes of the activity peaks measured with butyryl-CoA, decanoyl-CoA, and 4-cis-decenoyl-CoA differed only by minimal values, allowing recognition of the fact that general acyl-CoA dehydrogenase eluted...
change chromatography was finally purified by this method.  

with 4:O-CoA 

ion (160 mM NaCl). Acyl-CoA dehydrogenase activities were assayed using mel dolablau instead of 

crude general acyl-CoA dehydrogenase fraction (90 mM NaCl); C, elution patterns (280 nm). Among the three substrates, short chain acyl-CoA dehydrogenase, oxidizes only 4:O-CoA, long chain acyl-CoA dehydrogenase, 2 μg; purified general acyl-CoA dehydrogenase, 2 μg; purified long chain acyl-CoA dehydrogenase, 2 μg.

slightly in front of long chain acyl-CoA dehydrogenase and the latter shortly before the short chain acyl-CoA dehydrogenase.

Although the subunit molecular weights as well as the native molecular weights are very, similar polyacrylamide gel electrophoresis under nondenaturing and denaturing conditions allows the resolution of the different acyl-CoA dehydrogenases from bovine liver.

Substrate specificities of Acyl-CoA Dehydrogenases

Substrate specificities of the three purified acyl-CoA dehydrogenases were determined using mel dolablau instead of phenazine methosulfate or electron-transferring flavoprotein as primary and iodonitrotetrazolium chloride as terminal electron acceptor (25). The assay mixture contained 0.15% Triton X-100 and the substrate concentration was 100 μM. As shown in Table II, the apparent $K_m$ values for short chain and general acyl-CoA dehydrogenases were considerable lower, indicating that 100 μM substrate represents saturation conditions, whereas the measured activities for long chain acyl-CoA dehydrogenase had to be extrapolated in order to obtain values reflecting substrate saturation of this enzyme. As expected, apparent maximal velocities measured with saturated substrates of different chain lengths (Table II) revealed marked differences for the three acyl-CoA dehydrogenases. Apparent maximal velocity for the optimal substrates were 53.8 units/mg for long chain acyl-CoA dehydrogenase, 17.9 units/mg for...
short chain acyl-CoA dehydrogenase, but only 1.5 units/mg for general acyl-CoA dehydrogenase. The patterns of relative maximal velocities versus chain length for the three acyl-CoA dehydrogenases from bovine liver (Fig. 5) justified the terminology short chain, general, and long chain acyl-CoA dehydrogenases adapted from Ref. 9. The first enzyme showed the highest activity with butyryl-CoA as substrate but had no activity towards acyl-CoA esters with more than eight carbon atoms. The second one exhibited high activities toward substrates with a rather broad range of different chain lengths, whereas the last dehydrogenase had a distinct optimum with dodecanoyl-CoA.

To determine the influence of cis-double bonds and their positions within the carbon skeleton on the activities of acyl-CoA dehydrogenases, we chose acyl-CoA esters with 10 carbon atoms and one cis-double bond at position 6, 5, or 4. The apparent $K_m$ and apparent maximal velocities of general and long chain acyl-CoA dehydrogenases for these unsaturated substrates and decanoyl-CoA are shown in Table III. The data clearly demonstrate that in comparison to decanoyl-CoA, the cis-double bonds at all three positions had only minor effects on the $K_m$ values of the two acyl-CoA dehydrogenases. However, a very pronounced alteration of the $V_{\max}$ value was observed when long chain acyl-CoA dehydrogenase reacted with 4-cis-decanoyl-CoA. The apparent maximal activity was only 2.7% of that of decanoyl-CoA. The drastic effect of multiple bonds in position 4 on the activity of this acyl-CoA dehydrogenase was confirmed with 4-cis,7-cis-decadienoyl-CoA (a metabolite of linolenic acid) and with 4-decenoyl-CoA (Table III). On the other hand, general acyl-CoA dehydrogenase exhibited no markedly different activities between saturated and unsaturated $C_{10}$ substrates regardless of multiple bond position. The reaction rate with 4-cis-decenoyl-CoA was even 10% higher than with decanoyl-CoA.

Comparative investigations on $C_{16}$ substrates revealed a corresponding behavior of general and long chain acyl-CoA dehydrogenases on hexadecanoyl-CoA and all-cis-4,7,10,13-hexadecatetraenoyl-CoA (Fig. 6). Relative activities measured with the tetraenoyl-CoA (first double bond in position 4) compared to the saturated substrate were 110% for the general acyl-CoA dehydrogenase but only 22% for the long chain acyl-CoA dehydrogenase. The apparent $K_m$ values, however, were in the same range for the unsaturated and saturated CoA esters.

Substrate Specificity and Product Identification of General and Long Chain Acyl-CoA Dehydrogenases

Both purified general and long chain acyl-CoA dehydrogenases oxidized decanoyl-CoA as shown in Table II, but the latter exhibited only a residual activity with 4-cis-decenoyl-CoA (Table III). These results could be confirmed when the reaction mixtures of incubations containing either [1,14C]decanoyl-CoA or 4-cis-[4,5-3H]decanoyl-CoA in the presence of general acyl-CoA dehydrogenase or long chain acyl-CoA dehydrogenase were analyzed by radiogas chromatography (Fig. 7). Both enzymes converted decanoyl-CoA to 2-trans-decanoyl-CoA, whereas the oxidation of 4-cis-decanoyl-CoA to trans,4-cis-decadienoyl-CoA was only catalyzed by general acyl-CoA dehydrogenase. When the incubation time was largely extended from 10 min to 12 h, a minor formation (3%) of 2-trans,4-cis-decadienoyl-CoA was also observed in the presence of long chain acyl-CoA dehydrogenase (data not shown).

The identification of the oxidation product of 4-cis-decenoyl-CoA as a 2,4-dienoyl-CoA ester could also be based on the absorption spectrum (data not shown). It exhibited an absorption maximum at 296 nm. The same maximum has been described for 2-trans,4-trans-hexadienoyl-CoA and reported to be characteristic of 2,4-dienoyl-CoA esters (30). Both 2-trans,4-cis- and 2-trans,4-trans-decadienoyl-CoA esters (23) show the same maximum in their absorption spectra. Based on this property of 2,4-dienoyl-CoA esters, it was possible to determine specifically the activity of the general acyl-CoA dehydrogenase at 296 nm with 4-cis-decenoyl-CoA as substrate.

**DISCUSSION**

Due to the overlapping chain length specificities of acyl-CoA dehydrogenases, it has not been possible so far to deter-
purpose, the enzymes had first to be separated. Traditionally, individual dehydrogenases in crude preparations. For this reason, a visual method, following the yellow or green color of the acyl-CoA dehydrogenases, has been employed to monitor separation in the isolation procedures (9, 10, 14–16, 37–39). The development of a specific staining procedure for acyl-CoA dehydrogenases following electrophoresis in polyacrylamide gels (Fig. 1) and the use of a specific substrate, 4-cis-decenoyl-CoA, for the general acyl-CoA dehydrogenase from bovine liver (Table III) have solved this problem. These analytical methods permitted unambiguous detection of the individual acyl-CoA dehydrogenases and thus enabled us to test extensively various purification procedures. It will be of practical importance to determine whether acyl-CoA dehydrogenases from other sources have also substrate specificities which allow their selective determinations.

By the four-step purification procedure described in this report, we have been able to separate and purify three different acyl-CoA dehydrogenases from bovine liver to apparent homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). These enzymes contained no activities of any other β-oxidation enzymes. In contrast to purification methods described for acyl-CoA dehydrogenases from other sources (8, 9, 15, 40), the procedure described above does not use ammonium sulfate precipitation and gel filtration to separate the different acyl-CoA dehydrogenases because it has never been successfully used for this purpose. Instead, these two purification steps were employed by us to separate efficiently other proteins. However, ion exchange chromatography on DEAE-cellulose led to complete separation of two of the three acyl-CoA dehydrogenases (short chain and long chain acyl-CoA dehydrogenases) from the corresponding other two. Furthermore, general acyl-CoA dehydrogenase was separated from about 80% of the short chain and long chain acyl-CoA dehydrogenase activities. This separation was especially difficult as the acyl-CoA dehydrogenase pool applied to the DE52 column contained the latter two acyl-CoA dehydrogenases (short and long chain acyl-CoA dehydrogenases) in 10-fold excess (Table I). Thus, chromatography on DE52 column is the essential step of the present purification procedure. The final purification was achieved by subsequent preparative electrophoresis.

Recently, Davidson and Schulz (12) described a two-step procedure which was designed to separate but not to purify the three acyl-CoA dehydrogenases from bovine heart and liver. Their method was based on the separation of short chain acyl-CoA dehydrogenase from the other two enzymes by means of chromatography on DEAE cellulose, whereas subsequently a hydroxyapatite column was used to resolve general and long chain acyl-CoA dehydrogenases. Very recently, a combination of DEAE-Sephadex A-50 column chrom
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Fig. 7. Radiogas chromatographic analyses of incubation products of general and long chain acyl-CoA dehydrogenases. 100 munits of purified dehydrogenase (determined with decanoyl-CoA as substrate) were incubated for 10 min with 250 μmol of acyl-CoA in the presence of 0.1 mM meldolablau and 0.25 mM iodonitrotetrazolium chloride in 1 ml of 0.2 M Bicine/KOH buffer, pH 8.0, containing 0.15% Triton X-100. Fatty acyl-CoA esters were converted to methyl esters, and aliquots were assayed by radiogas chromatographic analysis as described previously (17). A, general acyl-CoA dehydrogenase and [1-14C]decanoyl-CoA; B, long chain acyl-CoA dehydrogenase and [1-14C]decanoyl-CoA; C, general acyl-CoA dehydrogenase and 4-cis-[4,5-2H3]decanoyl-CoA; D, long chain acyl-CoA dehydrogenase and 4-cis-[4,5-2H3]decanoyl-CoA. Peak a, methyl decanoate; peak b, methyl 4-cis-decanolate; peak c, methyl 2-trans-decenoate; peak d, methyl 2-trans,4-cis-decadienoate; peak e, methyl 2-trans,4-cis-decadienoate (an artifact, derived from the 2-trans,4-cis-compound by isomerization during preparation of methyl esters).

Matography, hydroxyapatite column chromatography, and isoelectric focusing was reported by Ikeda et al. (13) to separate three straight chain and two branched chain acyl-CoA dehydrogenases from rat liver. The data presented in the present paper together with those of Davidson and Schulz (12) and Ikeda et al. (13) strongly suggest that chromatography on DEAE cellulose and on hydroxyapatite are the most efficient methods for the separation of individual acyl-CoA dehydrogenases from different sources.

The purified acyl-CoA dehydrogenases from bovine liver showed similar molecular weights in the range of \( M_w = 170,000-205,000 \) (Fig. 4). These values are consistent with those reported for acyl-CoA dehydrogenases from other sources (10-12, 14, 13). As expected, the bovine liver acyl-CoA dehydrogenases are tetramers consisting of four identical subunits. Although molecular weights are very similar, the different acyl-CoA dehydrogenases can be readily distinguished by electrophoretic techniques under denaturing as well as non-denaturating conditions (Figs. 3 and 4). Surprisingly, the \( K_m \) values for purified long chain acyl-CoA dehydrogenase for all substrates tested were still higher than those published by Davidson and Schulz (12). Although the reason is not known, it seems likely that the different assay conditions, especially the use of meldolablau as primary electron acceptor and 0.15% Triton X-100 in this study, account for the observed differences. As discussed by Hall et al. (15), it might be that without Triton X-100 the limiting factor is the critical micellar concentration of acyl-CoA esters and thereby the availability of an active subunit form.

However, the relative specific velocities toward different substrates are obviously not affected by the assay conditions as the chain length specificity patterns determined with our assay (Fig. 5) are very similar to those published by others. The short chain acyl-CoA dehydrogenase has a narrow chain length specificity (<C10), whereas the general and long chain acyl-CoA dehydrogenases show broad ranges, but differ in their chain length optima. Only minor differences from this general scheme are observed in all mammalian species and tissues investigated so far (1, 11-13).

The apparent maximal velocities obtained with the unsaturated series of C10 substrates together with the considerable different \( K_m \) values for general and long chain acyl-CoA dehydrogenases suggest a specific function of the former in the metabolism of unsaturated fatty acids and their metabolites possessing a cis-double bond in position 4. 4-cis-Decenoyl-CoA at a concentration of \( 5 \times 10^{-4} \) M was selectively converted to 2-trans,4-cis-decadienoic acid by general, but not by long chain acyl-CoA dehydrogenase as judged by specific staining after gel electrophoresis (Fig. 1) as well as product analysis (Fig. 7). That this might be a general feature is further supported by the results obtained with hexadecanoyl-CoA and 4,7,10,13-hexadecatetraenoyl-CoA (Fig. 6). Exploiting the pronounced differences of the \( K_m \) values between general and long chain acyl-CoA dehydrogenases as well as the large difference of the relative specific velocities of the latter toward decanoyl-CoA and 4-cis-decenoyl-CoA, it is possible to select assay conditions rendering 4-cis-decenoyl-CoA an entirely specific substrate for general acyl-CoA dehydrogenase.

There are no relevant experimental data indicating the conversion of 4-cis-decenoyl-CoA to 2-cis-octenoyl-CoA, a reaction sequence generally assumed to be an essential part of the degradation of linoleic acid (42). On the contrary, there is accumulating evidence that the second step of this particular \( \beta \)-oxidation cycle does not proceed (19, 22-24). The substrate specificity data for the general acyl-CoA dehydrogenase presented here (Table III and Fig. 7) demonstrate that the first step, however, does occur in bovine liver, yielding 2-
trans,4-cis-decadienoyl-CoA from 4-cis-decenoyl-CoA. This in turn is a prerequisite for the physiological role of 2,4-dienoyl-CoA reductase in mitochondrial β-oxidation. Whether or not these results mean that long chain acyl-CoA dehydrogenase in vivo has no or only a limited function in the metabolism of acyl-CoA esters containing a cis-double bond in position 4 cannot yet be answered. Essential data concerning the effective concentration of acyl-CoA intermediates in vivo, their poses. First, we have presented analytical methods which cannot yet be answered. Essential data concerning the effect of chain on the reaction rate are unknown. The data presented in this paper serve three major purposes. First, we have presented analytical methods which allow the determination of the individual acyl-CoA dehydrogenases in crude preparations and thereby greatly improving the monitoring in separation. Second, we have described for the first time the substrate specificities of purified acyl-CoA dehydrogenases toward unsaturated acyl-CoA esters. These results provided evidence that cis-double bonds in position 4 may alter drastically the activity of long chain acyl-CoA dehydrogenase toward a substrate of a given chain length. Third, the purification and characterization of general and long chain acyl-CoA dehydrogenases from bovine liver enable us to use them for reconstituted β-oxidation systems to study the degradation of unsaturated fatty acids in more detail.

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