2,4-Dienoyl Coenzyme A Reductases from Bovine Liver and Escherichia coli

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2,4-Dienoyl-CoA reductases, enzymes of the \( \beta \)-oxidation of unsaturated fatty acids which were purified from bovine liver and oleate-induced cells of Escherichia coli, revealed very similar substrate specificities but distinctly different molecular properties. The subunit molecular weights, estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were 32,000 and 73,000 for the mammalian and the bacterial enzyme, respectively. The native molecular weights, calculated from sedimentation coefficients and Stokes radii yielded 124,000 for the bovine liver and 70,000 for the bacterial enzyme. Thus, bovine liver 2,4-dienoyl-CoA reductase is a tetramer consisting of four identical subunits. The \( E. coli \), 2,4-dienoyl-CoA reductase, however, possesses a monomeric structure. The latter enzyme contains 1 mol of FAD/mol of enzyme, whereas the former reductase is not a flavoprotein. The bovine liver reductase catalyzed the reduction of the same two substrates but in contrast yielded 2-trans-decenoyl-CoA as reaction product. Certain other properties of the two 2,4-dienoyl-CoA reductases are also presented. The localization of the reductase step within the degradation pathway of 4-cis-decenoyl-CoA, a metabolite of linoleic acid, is discussed.

EXPERIMENTAL PROCEDURES

Materials

The following substances used in these investigations were obtained from the sources mentioned in parentheses: NAD, NADH, NADP, NADPH, FAD, FMN, CoASH, catalase, fumarase, malate dehydrogenase, and lactate dehydrogenase (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany); Sephadex G-50, blue Sepharose CL-6B, and 2',5'-ADP-Sepharose 4B (Deutsche Pharmacia GmbH, Freiburg, FRG); Matrex gel red A (Amicon GmbH, Witten, FRG); DEAE-cellulose (Whatman DE52; Hermle GmbH, St. Leon-Rot, FRG); dithioerythritol (Biomol GmbH, Ilvesheim, FRG); p-chloromercuribenzoate (Ega-Chemie GmbH, Steinheim, FRG); Triz-35 (Serva, Heidelberg, FRG); Matrex gel red A (Amicon GmbH, Witten, FRG); dithioerythritol (Biomol GmbH, Ilvesheim, FRG); p-chloromercuribenzoate (Ega-Chemie GmbH, Steinheim, FRG). The preparation of 2-trans-decen-4-ynoic acid was synthesized according to Crambe (14). Other unsaturated carboxylic acids were prepared as reported previously (1). For the synthesis of acyl-CoA thioesters, the mixed anhydride method was used (16), and for their purification the full man method was employed (17).

Growth of E. coli on Different Carbon Sources

For induction experiments, \( E. coli \) cells were grown to late exponential phase in M9 mineral salts medium (18) with either oleate (0.1%) plus Brij-35 (0.4%), acetate (0.25%), glucose (0.5%), or oleate (0.1%) plus Brij-35 (0.4%) as carbon sources. Cell-free extracts were prepared as published elsewhere (10).
Purification of the 2,4-Dienoyl-CoA Reductases

From E. coli—E. coli cells (strain K12Ymel, gift from Dr. P. Overath, Max-Planck-Institut, Tübingen, Federal Republic of Germany) were grown on 0.1% olate, cell-free extracts (about 100 units of 2,4-dienoyl-CoA reductase activity) were prepared in 25 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.5 mM dithioerythritol (buffer A), and the DEAE-cellulose column (2.2 × 12 cm, Whatman DE52) was performed as described previously (10). The DE52 fraction was applied to a 2',5'-ADP-Sepharose 4B column containing 1 mM EDTA and 0.5 mM dithioerythritol (buffer B), and the enzyme was eluted with 500 ml of 2 mM 2,4-trans-decadienoyl-CoA in buffer B. Concentration was most easily achieved by coupling a small DE52 column (0.9 × 4 cm) to the 2',5'-ADP-Sepharose 4B column. The enzyme first eluted from the 2',5'-ADP-Sepharose and subsequently bound to the small DEAE-cellulose column was eluted with 5 ml of 400 mM potassium phosphate buffer containing 1 mM EDTA and 0.5 mM dithioerythritol and stored at −18 °C. This procedure yielded a homogeneous enzyme preparation as demonstrated previously (10).

From Bovine Liver—Bovine liver was obtained from the local slaughterhouse. The mitochondria were isolated according to Brosnan et al. (19). Acetone dry powder was prepared as described by Dahlen and Porter (20) and stored at −18 °C. Crude extracts of soluble mitochondrial proteins were prepared as described previously (1). 20 g of acetone dry powder were extracted with 100 ml of buffer B and centrifuged at 100,000 × g, the supernatant contained about 100 units of 2,4-dienoyl-CoA reductase activity. It was applied to a blue Sepharose CL-6B column (5.0 × 8.0 cm) which was equilibrated with buffer B. After washing, the enzyme was eluted with 1 M KCl in buffer B. The desalted eluate (Sephadex G-50) was applied to a Matrex gel red A column (2.6 × 20.0 cm) and washed with buffer B containing 330 mM KCl, and the 2,4-dienoyl-CoA reductase was eluted in a sharp peak with 5 mM NADP and 330 mM KCl in buffer B. The fractions containing the enzyme were pooled and separated from NADP and KCl by chromatography on Sephadex G-50. Then the sample solution was applied to a 2',5'-ADP-Sepharose 4B column (2.2 × 3.0 cm) and eluted with 500 ml of 5 mM 2,4-trans,4-trans-decadienoyl-CoA. Concentration was achieved by coupling a small Matrex gel red A column (0.9 × 4.0 cm) directly to the 2',5'-ADP-Sepharose 4B column. The enzyme eluted from the 2',5'-ADP-Sepharose and subsequently bound to the small Matrex gel red A column, was eluted with 5 ml of 2 M KCl in buffer B, desalted on a small Sephadex G-25 column, and stored at −18 °C. This purification procedure yielded a homogeneous enzyme as demonstrated previously (10).

Enzyme Assays

Determinations of 2,4-dienoyl-CoA reductase as well as of the usual β-oxidation enzymes were performed as described previously (1). Activities of catalase, fumarase, malate, and lactate dehydrogenases were measured according to established procedures described in Ref. 31.

Absorption Spectra and Flavin Determination

Absorption spectra of 2,4-dienoyl-CoA reductases were recorded with a Shimadzu spectrophotometer, Model UV 300, at a scan speed of 150 nm/min.

The nature of the flavin moiety dissociated from the enzyme protein by heating at 100 °C for 3 min was analyzed by thin layer chromatography on precoated cellulose plates (0.1-mm thickness). The solvent system used was 1-butanol-acetic acid-water (4:3:3). The FAD content was quantitatively calculated from the spectrum of the flavin protein after heating using a molar extinction coefficient at 445 nm of 11,300 M−1 cm−1 (22).

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Protein Determination

Protein was assayed using two procedures. Bovine serum albumin served as standard. The spectrophotometric method of Murphy and Kies (23) was used routinely. As an additional proof, the method of Bradford (24) was applied.

Molecular Weight Determination

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was carried out essentially by the method of Laemmli (25). The running gel contained 10% (w/v) acrylamide and the stacking gel was 5%.
2,4-Dienoyl-CoA Reductases

**Fig. 2.** Sucrose density gradient centrifugation of 2,4-dienoyl-CoA reductases according to Martin and Ames (32). A linear gradient of 5–20% sucrose in buffer B was used. Centrifugation was performed in a Beckman rotor SW 41 at 40,000 rpm for 15 h at 4 °C. Malate dehydrogenase, \( M_r = 70,000 \) (1), lactate dehydrogenase, \( M_r = 134,000 \) (2), fumarase, \( M_r = 194,000 \) (3), and catalase, \( M_r = 250,000 \) (4) were used as protein markers. Fractions (10 drops each) were collected from the bottoms of the tubes and assayed for enzyme activities of reductases and protein markers as described under "Experimental Procedures." Migration distances were calculated from the elution volumes and plotted against \( M_r^2 r \). \( r \) = migration distance through the gradient.

**Fig. 3.** Molecular weight determinations by 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to Laemmli (25). Protein standards used were: bovine serum albumin, \( M_r = 67,000 \) (1), ovalbumin, \( M_r = 45,000 \) (2), lactate dehydrogenase, subunit \( M_r = 35,000 \) (3), and chymotrypsinogen, \( M_r = 25,000 \) (4).

Sedimentation velocity experiments in an analytical ultracentrifuge were 6.43 for the bovine liver reductase and 5.21 for the *E. coli* enzyme. The Stokes radii were determined from gel filtration on Sephadex G-200 (Fig. 1B) to be 4.65 and 3.25 nm, respectively. The results yielded a native \( M_r = 124,000 \) for the bovine 2,4-dienoyl-CoA reductase and of 70,000 for the enzyme from *E. coli*.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed one protein band for each of the two purified reductases with an apparent \( M_r = 32,000 \) and 73,000 for the mammalian and bacterial enzymes, respectively (Fig. 3).

Mizugaki *et al.* (12) recently published a native molecular weight for the *E. coli* enzyme of 50,000 (determined by gel filtration of Sephacryl S-200 superfine) but did not report a molecular weight determined by electrophoresis under denaturing conditions. This value for the bacterial enzyme is even smaller than the one of 60,000 found in the present study by gel filtration (Fig. 1A).

**Absorption Characteristics and Flavin Content**—The absorption spectra for the 2,4-dienoyl-CoA reductases from *E. coli* and from bovine liver are shown in Fig. 4. The bacterial (0.25 mg/ml) and the mammalian reductase (0.35 mg/ml) were dissolved in 50 mM potassium phosphate buffer, pH 7.4, at 25 °C; the light path was 1 cm. The reduced state of the *E. coli* enzyme was obtained by adding NADPH (2). The reduced state was reoxidized by the substrate 2-trans,4-trans-decadienoyl-CoA (3).

**Fig. 4.** Absorption spectra of purified *E. coli* 2,4-dienoyl-CoA reductase (1–3) and bovine liver 2,4-dienoyl-CoA reductase (4). The bacterial (0.25 mg/ml) and the mammalian reductase (0.35 mg/ml) were dissolved in 50 mM potassium phosphate buffer, pH 7.4, at 25 °C; the light path was 1 cm. The reduced state of the *E. coli* enzyme was obtained by adding NADPH (2). The reduced state was reoxidized by the substrate 2-trans,4-trans-decadienoyl-CoA (3).

**Fig. 5.** Identification of the flavin moiety of 2,4-dienoyl-CoA reductase from *E. coli*. 42 μl of purified *E. coli* enzyme containing 250 milliunits were heated in a boiling water bath for 3 min to release the flavin cofactor. The supernatant, which still contained potassium phosphate buffer, was applied to a thin layer chromatography plate coated with cellulose. FAD and FMN (2 nmol each, dissolved in potassium phosphate buffer) were co-chromatographed in 1-butanolacetic acid:water (4:3:3). 1, FAD; 2, flavin moiety of 2,4-dienoyl-CoA reductase from *E. coli*; 3, FMN.
sium phosphate buffer, pH 7.4, exhibits two absorption maxima (441 and 360 nm), a pattern very similar to those of other flavin-containing dehydrogenases in the oxidized state (33). The flavin moiety can be reduced by NADPH and reoxidized by substrate. In contrast, the spectrum of the mammalian enzyme revealed no indication of flavin as prosthetic group. These interpretations of the spectra could be supported by experiments to determine the flavin content. When solutions of the bovine liver 2,4-dienoyl-CoA reductase were boiled, the supernatant was colorless. No flavin absorption or fluorescence was detected (data not shown), nor was there yellow color in the precipitate. The E. coli 2,4-dienoyl-CoA reductase released flavin upon incubation at 100 °C for 3 min. The flavin was identified by thin layer chromatography as FAD (Fig. 5) and quantitatively calculated from the spectrum of the supernatant using as extinction coefficient of ε_{452} = 11,300 $\text{M}^{-1} \text{cm}^{-1}$ (22). For a sample containing 4.37 nmol of enzyme/ml (based on $M_r = 71,500$), the FAD content was found to be 4.38 nmol/ml, indicating 1 mol of FAD bound/mol of enzyme.

Product Analyses—Product analyses were performed using tritium-labeled substrate (2-trans,4-cis-[4,5-3H]decadienoyl-CoA), tritium-labeled coenzyme (45)-[4-3H]NADPH, or tritiated water. The results of the product analyses by gas chromatography (Table I) clearly demonstrate different properties of the bacterial reductase as compared to the mammalian enzyme. Experiments using tritiated 2-trans,4-trans-decadienoyl-CoA led to 2-trans-decenoyl-CoA with the E. coli reductase and to 3-decenoyl-CoA with the bovine liver enzyme. Tritiated 3-decenoyl-CoA was also obtained when the reduction catalyzed by the mammalian enzyme was carried out in the presence of (4S)-[4-3H]NADPH. In contrast, reduction catalyzed by the bacterial enzyme in the presence of either (4S)-[4-3H]NADPH or (4R)-[4-3H]NADPH did not lead to incorporation of radioactivity into the reduction product. Instead, the tritium could be detected in water (data not shown). As expected from the latter result, reduction of 2-trans,4-cis-decadienoyl-CoA with E. coli reductase in the presence of tritiated water yielded labeled 2-trans-dienoyl-CoA.

It was not possible to determine by gas chromatography on a packed column the geometry of the double bond in the reduction product (3-decenoyl-CoA) of the bovine liver reductase. However, thin layer chromatography (Fig. 6) employing silica gel plates impregnated with silver nitrate (20%) and the solvent system hexane-diethyl ether (95:5) allowed complete separation of the 3-cis- and 3-trans-isomers. The results of Fig. 6 unequivocally demonstrate that, irrespective of whether 2-trans,4-cis- or 2-trans,4-trans-decadienoyl-CoA was reduced in the presence of (4S)-[4-3H]NADPH, the product of the bovine reductase was 3-trans-decenoyl-CoA.

Kinetic Properties and Substrate Specificities—The dependence of the activities of 2,4-dienoyl-CoA reductases purified from bovine liver and E. coli on substrate and coenzyme concentrations showed for both enzymes a strong inhibition. This is shown in Fig. 7 for 2-trans,4-trans-decadienoyl-CoA as substrate. This implies that there is for both enzymes only a small concentration range of the substrate as well as the coenzyme in which one can expect linearity of the initial reaction rates with time. As a practical consequence, the concentrations of substrate and coenzyme used for the enzyme assays are critical.

Both 2,4-dienoyl-CoA reductases reduced all three different 2,4-dienoyl-CoA esters used as substrates (Table II). They exhibited similar affinities (K_m) for the three substrates; however, they had significantly lower activities toward 2-trans,4-trans-hexadienoyl-CoA than for the two decadienoyl-CoA esters. 2-trans-decenoyl-CoA and 4-cis-decenoyl-CoA were not reduced (data not shown). Both reductases are strictly NADP-dependent; NADH could not substitute for NADPH (data not shown).

NADH is a noncompetitive inhibitor of E. coli 2,4-dienoyl-CoA reductase with an inhibitor constant (K_i) of 1.1 $\times 10^{-4}$ M (Fig. 7D), but it does not inhibit the bovine liver enzyme. The effect of 2-trans-decen-4-ynoyl-CoA on the activities of the reductases was tested. Lineweaver-Burk plots (Fig. 7, A

**TABLE I Identification of the reaction products by tritium-labeling experiments**

Incubations were performed and controlled photometrically like normal enzyme tests. The tritium label was in the substrate 2-trans,4-cis-[4,5-3H]decadienoyl-CoA (2.2 $\times 10^6$ cpm/μmol) (type 1), in the coenzyme (45)-[4-3H]NADPH (6.3 $\times 10^6$ cpm/μmol) (type 2), or in water (2 $\times 10^6$ cpm/μmol) (type 3). When the consumption of NADPH had slowed down markedly, the incubations were stopped by 0.2 ml of 2 N NaOH. Fatty acid CoA esters were converted into methyl esters, and aliquots were subjected to gas chromatographic analyses as described previously (1).

| Type of incubation | Enzyme source | $^3$H radioactivity |
|--------------------|---------------|---------------------|
|                    |               | Methionyl 2,4-decadienoyl-CoA | Methionyl 3-trans-decenoyl-CoA | Methionyl 2-trans-decenoyl-CoA |
| 1 Bovine liver     | E. coli 1,200 | 13,000              | 4,700               | 500         |
| 2 Bovine liver     | E. coli 170   | 25,300              |                      |             |
| 3 E. coli          | E. coli 6,700 | 68,400              |                      |             |

*As isomerization of 2-trans,4-cis-decadienoyl-CoA occurs during preparation of the methyl esters for gas chromatographic analysis, the counts given here are the sum of radioactivity detected in methyl 2-trans,4-cis- and methyl 2-trans,4-trans-decadienoyl-CoA.

**Fig. 6. Identification of the reaction product of bovine liver 2,4-dienoyl-CoA reductase by thin layer chromatography.** Silica gel-coated alumina sheets were impregnated with 20% silver nitrate and chromatographed in hexane-diethyl ether (95:5). 2-trans,4-trans-Decadienoyl-CoA (1) or 2-trans,4-trans-decadienoyl-CoA (3) were incubated with 2,4-dienoyl-CoA reductase in the presence of tritiated (4S)-[4-3H]NADPH. The radioactively labeled reaction products were transformed to methyl esters. They were applied to the thin layer chromatography plate together with a mixture of the marker substances methyl 3-trans-decenoyl-CoA (3t) and methyl 3-cis-decenoyl-CoA (3c) in the starting positions (1 and 2). After the run, the alumina sheet was cut into pieces of 0.5-cm height for each trace and the pieces were put into scintillation vials and counted for radioactivity. The results are shown for the reaction product of 2-trans,4-cis-decadienoyl-CoA at the left and for the product of 2-trans,4-trans-decadienoyl-CoA at the right.
FIG. 7. Double reciprocal plots demonstrating the dependence of 2,4-dienoyl-CoA reductases from bovine liver (A and C) and from E. coli (B and D) on substrate or coenzyme concentrations in the presence and absence of inhibitors. A, bovine liver enzyme, 100 μM NADPH, 2-trans,4-trans-decadienoyl-CoA (2-400 μM) (●—●) and with addition of 2-trans-decen-4-ynoyl-CoA (Δ—Δ, 50 μM, and ○—○, 2.5 μM). B, E. coli enzyme, 100 μM NADPH, 2-trans,4-trans-decadienoyl-CoA (2-500 μM) (●—●) and with addition of 2-trans-decen-4-ynoyl-CoA (Δ—Δ, 50 μM). C, bovine liver enzyme, 40 μM 2-trans,4-trans-decadienoyl-CoA, NADPH (1.8-500 μM) (●—●). D, E. coli enzyme, 40 μM 2-trans,4-trans-decadienoyl-CoA, NADPH (1.8-400 μM) (○—○), and with addition of NADH (Δ—Δ, 100 μM).

TABLE II
Kinetic properties of 2,4-dienoyl-CoA reductases

The assay mixtures (1 ml) contained 2,4-dienoyl-CoA reductase from bovine liver or E. coli in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 0.5 mM dithioerythritol, and 1% bovine serum albumin. Substrate dependencies were measured with 100 μM NADPH in the enzyme test, whereas coenzyme dependency was determined with 40 μM 2-trans,4-trans-decadienoyl-CoA.

| Substrate                        | Inhibitor       | 2,4-Dienoyl-CoA reductase from | Bovine liver | E. coli |
|----------------------------------|----------------|--------------------------------|-------------|--------|
|                                  |                | Km (μM) | kcat (nmol·min⁻¹·μg⁻¹) | Vmax (μM) | Km (μM) | kcat (nmol·min⁻¹·μg⁻¹) | Vmax (μM) |
| 2-trans,4-trans-Hexadienoyl-CoA  |                | 3.9     | 1.8                        | 100.0      | 6.5     | 11.2                        | 100.0      |
| 2-trans,4-cis-Decadienoyl-CoA    |                | 3.0     | 38.2                       | 4.1        | 67.5    |                            |           |
| 2-trans,4-trans-Decadienoyl-CoA  |                | 6.7     | 100.0                      | 8.8        | 100.0   |                            |           |
| 2-trans-Decen-4-ynoyl-CoA        | 2-trans-Decen-4-ynoyl-CoA (2.5 μM) | 14.7   | 72.1                      | 6.65       | 98.6    |                            |           |
| 2-trans-Decen-4-ynoyl-CoA        | 2-trans-Decen-4-ynoyl-CoA (50 μM) | 24.2   | 20.7                      | 75.8       | 102.0   |                            |           |
| NADPH                            | NADPH          | 93.7    | 109.5                      | 10.1       | 109.5   | 110                        | 50.8       |

* Kᵢ, inhibitor constant.

NADPH: nicotinamide adenine dinucleotide phosphate.

and B) in the presence and absence of this inhibitor revealed competitive inhibition (Kᵢ = 6.7 × 10⁻⁶ M) with respect to 2-trans,4-trans-decadienoyl-CoA for the bacterial enzyme and a mixed inhibition type for the bovine liver reductase. This particular unsaturated acyl-CoA ester was originally chosen by us as a potential substrate to study the influence of a triple bond in position 4. However, neither the bacterial nor the mammalian enzyme reduced 2-trans-decen-4-ynoyl-CoA (data not shown). The effect of three different sulfhydryl inhibitors on the activity of the two reductases is shown in Table III.

Induction of E. coli 2,4-Dienoyl-CoA reductase—To determine whether and to which extent 2,4-dienoyl-CoA reductase from E. coli is being induced, cell-free extracts from cells grown on different carbon sources were assayed for reductase activity. As shown in Table IV, activity of 2,4-dienoyl-CoA reductase prepared from cells grown on oleate was highest, about 10 times higher than those obtained from cells grown on acetate medium. The addition of glucose to the synthetic medium either as sole carbon source or in addition to oleate yielded cells whose extracts had depressed levels of 2,4-dienoyl-CoA reductase activity.

DISCUSSION

Not until very recently has it been possible to purify 2,4-dienoyl-CoA reductases from two different sources to appar-
ent homogeneity (10). In the present studies, these two 2,4-dienoyl-CoA reductases, from E. coli and bovine liver, have been characterized with respect to their molecular and kinetic properties. Although some similarities have been noted between the two enzymes, comparison of their molecular properties (Table V) reveals considerable differences.

First, the two 2,4-dienoyl-CoA reductases possess entirely different structures. The native molecular weights calculated from the sedimentation coefficients and the Stokes radii were in very good agreement with four times and one time the subunit molecular weights of bovine liver and E. coli reductase, respectively. Therefore, the mammalian enzyme has a tetrameric structure with four identical subunits whereas the bacterial enzyme consists of one polypeptide chain. Second, the reductase from bovine liver contains no flavin, while the enzyme purified from E. coli is a flavoprotein containing 1 mol of FAD/mol of enzyme. As it is known that hydrogen from reduced FAD readily exchanges with that of water, the absence of flavin might explain why the mammalian enzyme catalyzes the transfer of tritium from tritiated NADPH to the

substrate, while the flavin-containing bacterial reductase does not.

A third major difference, and perhaps the most surprising aspect of this comparative characterization, concerns the reaction products. Bovine liver 2,4-dienoyl-CoA reductase catalyzes the reduction of 2-trans,4-cis-dienoyl-CoA and 2-trans,4-trans-dienoyl-CoA to 3-trans-decenoyl-CoA (Table I). Thus, the overall reaction is a 1,4-addition of hydrogen across the 2,4-diene system. In contrast, the bacterial enzyme catalyzes a 1,2-addition of hydrogen to the double bond in position 4 of 2,4-dienoyl-CoA esters, yielding 2-trans-enoyl-CoA esters as reaction products (Table I). The latter result is in agreement with a recent report of Mizugaki et al. (12) who described 2-trans-decenoyl-CoA as the reduction product of the E. coli reductase reaction. This property of the bacterial reductase seems to be a unique feature among the ubiquitously occurring 2,4-dienoyl-CoA reductases. This is because, in addition to the bovine liver enzyme described in this study, the peroxisomal 2,4-dienoyl-CoA reductase from rat liver (4) as well as the peroxisomal 2,4-dienoyl-CoA reductase from C. tropicalis (3), all form 3-enoyl-CoA esters. These results seem to indicate that eucaryotic 2,4-dienoyl-CoA reductases form 3-enoyl-CoA esters. Therefore, it is unlikely that the reaction product of the mitochondrial 2,4-dienoyl-CoA reductase from rat liver is the 2-trans-enoyl-CoA ester as assumed by others (7, 8, 34). The different mechanisms of reduction might be reflected in the different types of inhibition evoked by 2-trans-decen-4-ynoyl-CoA on the reactions of E. coli and bovine liver 2,4-dienoyl-CoA reductases (Table II). This interesting point clearly needs further investigations to be established.

In contrast to the differences described above, the substrate specificities of both 2,4-dienoyl-CoA reductases are very similar (Table II). They are strictly NADPH-dependent and require a 2,4-dienoyl-CoA structure as part of their substrate molecules. The corresponding monounsaturated acyl-CoA esters with a cis-double bond in either position 4 or 2 are not reduced. 2-trans-Decen-4-ynoyl-CoA is not a substrate but inhibits both enzymes (Fig. 7, A and B). Both reductases are inhibited by substrate and coenzyme at higher concentrations (Fig. 7). The chain length of the substrate seems less critical. Both reductases reduced 2,4-dienoyl-CoA esters with 10 and 6 carbon atoms although at different rates. Borrebaek et al. (6) as well as Hiltunen and Davis (7) showed indirectly that 2,4-dienoyl-CoA reductase(s) from rat liver reduces 2,4-pen-

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**Table III**

**Effects of SH reagents on 2,4-dienoyl-CoA reductases**

The assay mixtures (1 ml) contained 100 mM NADPH and 2,4-dienoyl-CoA reductase from bovine liver or E. coli in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, and 1% bovine serum albumin. Inhibitors were dissolved in the same buffer and added as indicated. After 3 min of preincubation, the reactions were started by adding 40 nmol of 2-trans,4-trans-decadienoyl-CoA.

| Inhibitor concentration | Activity of 2,4-dienoyl-CoA reductase from | Bovine liver | E. coli |
|-------------------------|-------------------------------------------|-------------|--------|
| mM                      | units/mg %                                |             |        |
| None                    | 3.9 100 10.9 100                          |             |        |
| Iodoacetamide           | 3.9 100 10.9 100                          |             |        |
| N-ethylmaleimide        | 1.0 100 7.4 68                            |             |        |
|                         | 2.5 3.35 86 4.47 41                       |             |        |
|                         | 5.0 2.35 59 2.62 24                       |             |        |
|                         | 7.5 1.33 34 1.31 12                       |             |        |
|                         | 1.0 0.94 24 0.76 7                        |             |        |
| p-Chloromercuribenzoate | 0.25 3.9 100 9.05 83                      |             |        |
|                         | 0.5 3.9 100 7.30 67                       |             |        |
|                         | 0.75 3.9 100 2.07 19                      |             |        |
|                         | 1.0 3.9 100 0                            |             |        |

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**Table IV**

**Specific relative activities of 2,4-dienoyl-CoA reductase in cell-free extracts of E. coli grown on various carbon sources**

The media used for growing E. coli consisted of a synthetic medium without a carbon source according to Lengeler (18) and the indicated carbon sources. NB-medium is an optimal medium with 8 g of Nutrient Broth (Difco)/liter. The cells were grown to the late exponential phase. Cell-free extracts were prepared as published previously (10).

| Carbon source | 2,4-Dienoyl-CoA reductase activity | milliunits/mg % |
|--------------|----------------------------------|----------------|
| Oleate (1 g/liter) | 41.2 100.0                       |                |
| Acetate (2.5 g/liter) | 4.5 10.9                        |                |
| Glucose (5.0 g/liter) | 1.2 2.9                          |                |
| Oleate + glucose (1 g/liter + 5 g/liter) | 1.6 3.9                 |                |
| NB-medium | 2.5 6.1                           |                |

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**Table V**

**Comparison of properties**

| Properties | 2,4-Dienoyl-CoA reductase from |
|------------|--------------------------------|
| Bovine liver | E. coli |
| Native molecular weight | 124,000 70,000 |
| Subunit molecular weight | 32,000 73,000 |
| Number of subunits | 4 1 |
| Sedimentation coefficient (s20,w) | 6.43 5.21 |
| Stokes radius (nm) | 4.65 3.25 |
| Flavin content | None 1 mol FAD/mol enzyme |
| Substrates | 2,4-Dienoyl-CoA esters 2,4-Dienoyl-CoA esters |
| Products | 3-trans-Enoyl-CoA esters 2-trans-Enoyl-CoA esters |
| Hydrogen donor | NADPH NADPH |
| Inhibition by | p-Chloromercuribenzoate |
| Enzyme activity (units/mg) | 3.9 10.9 |
| Turnover number (units/μmol) | 500 780 |
tadienoyl-CoA by measuring in crude extracts NADPH consumption in the presence of this acyl-CoA ester. The reduction of the same compound has also been observed by Schulz in extracts from E. coli' and by Schulz (9) and Hiltunen and Davis (7) in rat heart mitochondria. Previous experiments from this laboratory (35-38) on partial degradation of highly unsaturated fatty acids with their first double bond in position 4 (e.g. 4,7,10,13,16-docosapentaenoic acid) and data recently published by Osmundsen et al. (34) suggest that 2,4-dienoyl-CoA reductase also participates in the metabolism of polysaturated fatty acids with chain lengths of more than 18 carbon atoms.

The 2,4-dienoyl-CoA reductase from E. coli is induced when the cells are grown on oleate as sole carbon source and depressed by glucose in the growth medium (Table IV). The extent of induction was essentially the same as reported by Overath et al. (39, 40) and Weeke et al. (41) for the four "classical" β-oxidation enzymes. Since oleate, a fatty acid without a double bond at an even-numbered position, does not require 2,4-dienoyl-CoA reductase for its degradation, but induces its activity together with those of the other β-oxidation enzymes, some form of unit control is suggested. The same induction pattern has been found for the β-oxidation enzymes of C. tropicalis (3).

The determination of the reaction products allows us to complete the reaction sequence which we have previously published (1) for the degradation of 4-cis-decenoyl-CoA, a key metabolite of linoleic acid, in bovine liver, and to compare it to the catalytic route in E. coli (Fig. 8). In bovine liver, 2,4-decadienoyl-CoA is reduced to 3-trans-decenooyl-CoA which in turn is isomerized to 2-trans-decenooyl-CoA. Thus, two reactions are necessary between the first and the second step of the β-oxidation cycle in order to metabolize the double bond in position 4 (Fig. 8A). In E. coli, in contrast, the isomerization is not necessary as 2-trans-decenoyl-CoA is the direct reduction product of 2-trans,4-cis-decadienoyl-CoA (Fig. 8B).

There is growing evidence (1-4, 34) that these reaction sequences of the reductase pathway are obligatory for removal of all cis-double bonds located at even-numbered carbon atoms of polyunsaturated fatty acids. The molecular reason seems to be that 2-trans,4-cis-dienoyl-CoA esters cannot serve as substrates for enoyl-CoA hydratase (2, 3, 5, 8) required for the epimerase pathway proposed by Stoffel and Caeser (13).

As, however, 3-hydroxyacyl-CoA epimerase activity in E. coli is located on one of the two multifunctional polypeptides comprising five β-oxidation activities (42-44), the interesting question is raised as to the physiological function of the 3-hydroxyacyl-CoA epimerase in the degradation of the various naturally occurring fatty acids. At present no relevant experimental data are available.

In conclusion, although 2,4-dienoyl-CoA reductases from bovine liver and E. coli reduce the same substrates and serve the same metabolic function, their molecular structures and the reactions they catalyze are very different. That makes these enzymes not only interesting as parts of the β-oxidation systems but also from the point of view of reaction mechanisms.

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REFERENCES
1. Kunau, W.-H., and Dommes, P. (1978) Eur. J. Biochem. 91, 533-544
2. Cuebas, D., and Schulz, H. (1982) J. Biol. Chem. 257, 14140-14144
3. Dommes, P., Dommes, V., and Kunau, W.-H. (1983) J. Biol. Chem. 258, 10846-10862
4. Dommes, V., Baumgart, C., and Kunau, W.-H. (1981) J. Biol. Chem. 256, 8259-8262
5. Borrebaek, B., Osmundsen, H., and Bremer, J. (1980) Biochem. Biophys. Res. Commun. 85, 1173-1180
6. Borrebaek, B., Osmundsen, H., Christiansen, E. N., and Bremer, J. (1980) FEBS Lett. 121, 23-24
7. Hiltunen, J. K., and Davis, E. J. (1981) Biochem. J. 194, 427-432
8. Mizugaki, M., Nishimaki, T., Yamamoto, H., Sagi, M., and Yanamaka, H. (1982) J. Biochem. (Tokyo) 92, 2501-2504
9. Schulz, H. (1983) Biochemistry 22, 1827-1832
10. Dommes, V., Luster, W., Cvetanovic, M., and Kunau, W.-H. (1982) Eur. J. Biochem. 125, 335-341
11. Mizugaki, M., Nishimaki, T., Yamamoto, H., Nishimura, S., Sagi, M., and Yanamaka, H. (1982) J. Biochem. (Tokyo) 91, 1453-1456
12. Mizugaki, M., Kimura, C., Nishimaki, T., Yamamoto, H., Sagi, M., Nishimura, S., and Yanamaka, H. (1982) J. Biochem. (Tokyo) 92, 1671-1674
13. Stoffel, W., and Caeser, H. (1966) Hoppe Seyler's Z. Physiol. Chem. 342, 76-83
14. Crombie, L. (1955) J. Chem. Soc. 1007-1025
15. Lindlar, H., and Rubins, R. (1966) Org. Synth. 46, 89-92

FIG. 8. Pathway of linoleic acid degradation. A, pathway in bovine liver. B, pathway in E. coli. Reactions catalyzed by: 1, acyl-CoA dehydrogenase; 2, 2,4-dienoyl-CoA reductase of bovine liver; 3, 3,2-enoyl-CoA isomerase; 4, 2,4-dienoyl-CoA reductase of E. coli 5, enoyl-CoA hydratase.
16. Goldman, P., and Vagelos, P. R. (1961) *J. Biol. Chem.* **236**, 2620–2623

17. Pullman, M. E. (1973) *Anal. Biochem.* **54**, 188–198

18. Lengeler, J. (1966) *Z. Vererbungsbiol.* **98**, 203–229

19. Brosnan, J. T., Kopec, B., and Fritz, I. B. (1973) *J. Biol. Chem.* **248**, 4075–4082

20. Dahlén, J. V., and Porter, J. W. (1965) *Arch. Biochem. Biophys.* **127**, 207–223

21. Bergmeyer, H. U. (ed) (1974) *Methoden der enzymatischen Analyse*, 3rd ed., Verlag Chemie, Weinheim

22. Beinert, H. (1960) in *The Enzymes* (Boyer, P. D., Lardy, H., and Myrbäck, K., eds) 2nd ed., Vol. 2, pp. 339–416, Academic Press, New York

23. Murphy, J. B., and Kies, M. W. (1960) *Biochim. Biophys. Acta* **45**, 382–384

24. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254

25. Laemmli, U. K. (1970) *Nature (Lond.)* **227**, 680–685

26. Weber, K., and Osborn, M. (1961) *J. Biol. Chem.* **244**, 4406–4412

27. Andrews, P. (1965) *Biochem. J.* **96**, 595–606

28. Siegel, L. M., and Monty, K. J. (1966) *Biochim. Biophys. Acta* **112**, 346–362

29. Cantor, C. R., and Schimmel, P. R. (eds) (1980) *Biophysical Chemistry*, Part II, pp. 549–655, W. H. Freeman and Company, San Francisco

30. Schachman, H. K. (1957) *Methods Enzymol.* **4**, 32–103

31. Stoffel, W., and Ecker, W. (1969) *Methods Enzymol.* **14**, 99–105

32. Martin, R. G., and Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379

33. Kearney, E. B., and Kennedy, W. C. (1974) in *Horizons in Biochemistry and Biophysics* (Quagliariello, E., Palmieri, F., and Singer, T. P., eds) Vol. 1, pp. 62–96, Addison-Wesley Publishing Co., Reading, MA

34. Osmundsen, H., Cervenka, J., and Bremer, J. (1982) *Biochem. J.* **208**, 749–757

35. Kunau, W.-H., and Couzens, B. (1971) *Hoppe-Seyler’s Z. Physiol. Chem.* **352**, 1397–1395

36. Kunau, W.-H. (1971) *FEBS Lett.* **16**, 54–56

37. Kunau, W.-H., and Bartnik, F. (1974) *Eur. J. Biochem.* **48**, 311–318

38. Kunau, W.-H. (1977) in *Polyunsaturated Fatty Acids* (Kunau, W.-H., and Holman, R. T., eds) American Oil Chemists Society Symposium Monograph No. 4, pp. 51–67, American Oil Chemists Society, Champaign, IL

39. Overath, P., Raufuss, E. M., Stoffel, W., and Ecker, W. (1967) *Biochem. Biophys. Res. Commun.* **29**, 28–33

40. Overath, P., Pauli, G., and Schairer, H. U. (1969) *Eur. J. Biochem.* **7**, 559–574

41. Weeks, G., Shapiro, M., Burns, R. O., and Wakil, S. J. (1969) *J. Bacteriol.* **97**, 827–836

42. Pramanik, A., Pawar, S., Antonian, E., and Schulz, H. (1979) *J. Bacteriol.* **137**, 469–477

43. Pawar, S., and Schulz, H. (1981) *J. Biol. Chem.* **256**, 3894–3899

44. Binstock, J., and Schulz, H. (1981) *Methods Enzymol.* **71**, 403–411
2,4-Dienoyl coenzyme A reductases from bovine liver and Escherichia coli.
Comparison of properties.
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J. Biol. Chem. 1984, 259:1781-1788.

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