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Kinetic Evidence for Two Separate trans-2-Enoyl CoA Reductases in Rat Hepatic Microsomes: NADPH-Specific Short Chain- and NAD(P)H-Dependent Long Chain-Reductase*

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The rat hepatic microsomal conversion of crotonyl- and hexenoyl CoA to butyrate and hexanoate was supported only by NADPH, while both NADH and NADPH were effective cofactors in the conversion of trans-2-hexadecenoyl CoA to palmitate. Experiments using mixtures of long- and short-chain enoyl-CoA substrates and competition experiments support the conclusion that microsomes contain 2 distinct enoyl CoA reductases, (1) a long chain enoyl CoA reductase capable of accepting reducing equivalents from either NADH or NADPH, and (2) a NADPH-specific short chain enoyl CoA reductase.

The endoplasmic reticulum of rat liver possesses the ability to catalyze the elongation of fatty acids (1-3). The terminal step of the elongation reaction is the reduction of trans-2-enoyl CoA to the saturated product catalyzed by a reduced pyridine nucleotide-dependent enoyl CoA reductase (1-5).

Podack and Seubert reported the presence of two hepatic enoyl CoA reductases -- one which is found in the mitochondria, is NADPH-specific and is most active with decenoyl CoA, and the other which is localized in the microsomal fraction, utilizes both NADH and NADPH and obtains optimal activity with hexenoyl CoA (6). However, a careful study of the hepatic microsomal enoyl CoA reductase in our laboratory revealed the presence of at least two enzymes, a short-chain reductase and a long-chain reductase (7). This finding

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was based on the observation that only NADPH supported the microsomal conversion of crotonyl CoA or trans-2-hexenoyl CoA to each respective saturated product, whereas long chain acyl CoA's are effectively elongated in the presence of NADH or NADPH. In actuality, three interpretations are consistent with our results: (1) hepatic microsomes contain an NADPH-specific short-chain reductase, an NADPH-specific long-chain reductase and an NADH-specific long-chain reductase, i.e., three separate enoyl CoA reductases; (2) hepatic microsomes contain an NADPH-specific enoyl CoA reductase which catalyzes the reduction of both long- and short-chain enoyl CoA substrates and an NADH-specific long-chain reductase; and (3) the hepatic microsomes contain an NADPH-specific short-chain reductase and a reduced pyridine nucleotide-dependent long-chain reductase which can utilize NADPH and NADH.

In this communication, we provide evidence which strongly supports the third interpretation, i.e., rat liver microsomes contain an NADPH-specific short-chain enoyl CoA reductase and an NAD(P)H-dependent long-chain enoyl CoA reductase.

Materials and Methods

NADPH, NADH, coenzyme A, bovine serum albumin (fatty acid free, type V) and crotonyl CoA were obtained from Sigma Chemical Co. (St. Louis, MO). The trans-2-hexenoic and octenoic acids were purchased from Aldrich Chemicals (Milwaukee, WI).

[^{14}C]-trans-2-hexadecenoic acid was prepared by condensing myristic aldehyde with [2-^{14}C]-malonic acid according to the procedure of Nugteren (1). The coenzyme A esters of trans-2-hexenoic, octenoic and [^{14}C]-hexadecenoic acids were prepared as described by the method of Fong and Schulz (8).

Microsomes were prepared from the livers of male Sprague-Dawley rats weighing 150-200g, as described previously (9).

The assay mixture for measuring the reduction of crotonyl CoA to butyric acid contained the following components in a total volume of 1 ml (final concentration): 100 μM crotonyl CoA, 100 mM Tris-HCl, pH 7.4, 1.0 μM rotenone, either 1 mM NADPH or NADH and 1 mg/ml microsomal protein. The reaction was initiated with microsomes and was carried out at 37°C for five minutes. When trans-2-hexenoyl CoA or trans-2-octenoyl CoA was used as a substrate, 150 μM final substrate concentration was added to the assay mixture and the reaction was incubated for one or two minutes.

The incubation mixture for the conversion of trans-2-hexadecenoyl CoA to palmitate contained 100 mM Tris-HCl, pH 7.4, 15 μM enoyl CoA, 15 μM albumin (a molar ratio of 1:1 substrate:albumin yielded maximal activity under our conditions), 1 mM KCN (to inhibit desaturase activity), 0.5 - 1 mM NADPH or NADH

and 100 μg microsomal protein in a total volume of 1.5 ml. The reaction was initiated with microsomes, incubated at 37°C for 5 minutes and terminated with 0.4 ml 15% KOH in methanol.

Identification of the products of 4:1, 6:1, and 8:1 was achieved with gas chromatography as described previously (7).

For the identification of palmitate, formed from the reduction of trans-2-hexadecenoyl CoA, 150 μg each of cold palmitic and trans-2-hexadecenoic acids were added to the terminated reaction mixture. Methyl esters of the fatty acids were prepared by using diazomethane (10) and separated on 10% silver nitrate impregnated thin layer chromatography (11), employing the solvent system hexane:diethyl ether (97:3, v/v). The chromatogram was sprayed with 0.2% 2',7'-dichlorofluorescein in ethanol, the methyl esters identified under ultraviolet light and the spots were scraped and placed into counting vials containing scintillation cocktail (12); counts per minute were determined to calculate the percent conversion of trans-2-hexadecenoyl CoA to palmitic acids.

Results and Discussion

Recently we have reported that the trans-2-enoyl CoA's, crotonyl CoA and hexenoyl CoA were converted by rat hepatic microsomes to butyrate and hexanoate, respectively, only in the presence of NADPH (7). No conversion was observed when NADPH was replaced by NADH. When the acyl chain was extended to eight carbons, namely trans-2-octenoyl CoA, NADH was capable of providing reducing equivalents to the substrate (Table I). However, the rate of conversion of the octenoyl CoA to octanoic acid in the presence of NADH was 5 nmols per

TABLE I

Cofactor Requirements and Substrates Employed for the Microsomal Conversion of trans-2-Enoyl CoA to the Saturated Product

Cofactor	Trans-2-Enoyl CoA's			
	Crotonyl CoA	Hexenoyl CoA	Octenoyl CoA	Hexadecenoyl CoA
	nmols saturated product formed/min/mg microsomal protein			
NADPH 1 mM	5	50	32	46
NADH 1 mM	0	0	5	20 (43%)*
NADH 1 mM plus NADPH 1 mM	N.D.	-	-	47

All values are the average of duplicates of three separate experiments.
*Percent of the NADPH-catalyzed activity.
N.D. - Not determined
The reaction mixture used for the conversion of 16:1 CoA contained 1 mM KCN, 1:1 ratio of substrate:bovine serum albumin, 100 mM Tris, pH 7.4, and 40 μM 16:1 CoA. When the short chain substrates were employed, KCN and albumin were omitted. The final concentrations of the short chain substrates used in assay mixture were: crotonyl CoA 100 μM; hexenoyl CoA 150 μM; octenoyl CoA 150 μM.

minute per mg microsomal protein which was approximately one-sixth of the rate observed with NADPH as cofactor. These results are in contrast to those of Podack and Seubert (6) who reported that the microsomal enoyl CoA reductase was equally active with NADPH and NADH when trans-2-octenoyl CoA was the substrate. When a long-chain enoyl CoA substrate (16:1) was employed, the activity with NADH was more significant, i.e., NADH was about one-half as effective as NADPH. These results, however, do not distinguish among the three hypotheses described in the introduction.

To determine whether the long chain enoyl CoA reductase was capable of utilizing both cofactors, NADH and NADPH or whether two reductases exist, one specific for NADH and the other for NADPH, both cofactors were included in the incubation assay used for the conversion of trans-2-hexadecenoyl CoA to palmitate. If two separate reductases existed, then the activity determined in the presence of both NADH and NADPH should equal the sum of the activities obtained with each cofactor alone. Clearly, this was not the case (Table I). The sum of the NADPH-dependent activity and the NADH-dependent activity was 66 nmols/min/mg protein, whereas the activity measured in the presence of both cofactors was only 47. These results suggest the presence of only one long chain enoyl CoA reductase which accepts electrons from both cofactors, although NADPH is preferred. Hence, the data in Table I rule out the first and second hypotheses. Consistent with these results, however, is the third hypothesis.

Assuming that the long chain enoyl CoA reductase is approximately one-half (43%) as active in the presence of NADH (Table I) in contrast to NADPH, one can calculate how much activity can be attributed to the short chain enoyl CoA reductase and long chain enoyl CoA reductase when trans-2-octenoyl CoA is used as the substrate. Since 5 nmol of the 8:1 substrate were converted in the presence of NADH, one would expect to obtain a turnover rate by the long chain enoyl CoA reductase of 11.6 nmols/min/mg microsomal protein in the presence of NADPH, a value based on the 43% conversion by NADH. The remaining 20.4 nmol/min/mg conversion would be attributed to the short chain

TABLE II

Influence of trans-2-Hexenoyl CoA and Octenoyl CoA on the NADPH-Supported Microsomal Conversion of trans-2-Hexadecenoyl CoA to Palmitate*

Short Chain Enoyl CoA	16:1 CoA Substrate 15 μM	30 μM
	Palmitate Formed (% of Control)	
6:1 CoA		
50 μM	95	100
100 μM	100	102
250 μM	96	100
8:1 CoA		
25 μM	97	100
50 μM	90	100
250 μM	80	92

*100 μgs Microsomal protein were pre-incubated at 25°C for 5 minutes with increasing amounts of either 6:1 CoA or 8:1 CoA; the pre-incubation mixture also contained 1 mM KCN, 20 μM bovine serum albumin and 100 mM tris-HCl, pH 7.4. Following pre-incubation, either 15 or 30 μM 16:1 CoA was added and the reaction initiated with the addition of 500 μM NADPH. The total assay volume was 1.0 ml. Controls contained only 15 or 30 μM 16:1 CoA substrate, as indicated.

enoyl CoA reductase. In other words, 36% of the total reduction of trans-2-octenoyl CoA is catalyzed by the long chain enoyl CoA reductase while 64% conversion is attributed to the short chain enzyme. This assumes, of course, that the binding of the substrate does not alter the efficiency of the enzyme to utilize NADH or NADPH as the cofactor.

Conclusive evidence for two separate NADPH-dependent enoyl CoA reductases was obtained by examining the influence of trans-2-hexenoyl CoA on the reduction of the hexadecenoyl CoA. As seen in Table II, the 6:1 CoA, which was reduced at the fastest rate (50 nmols/min/mg, from Table I) had no inhibitory effect on the conversion of 16:1 CoA to palmitate, even at a concentration ratio greater than 15 to 1 for 6:1 CoA to 16:1 CoA. Had the enzyme catalyzed the reduction of both CoA's, then one should have observed a significant inhibition of 16:1 CoA reduction by 6:1 CoA. However, when 6:1 CoA was replaced by 8:1 CoA, a small but significant inhibition of 16:1 CoA conversion was observed (Table II); the inhibition was reversed by a higher substrate concen-

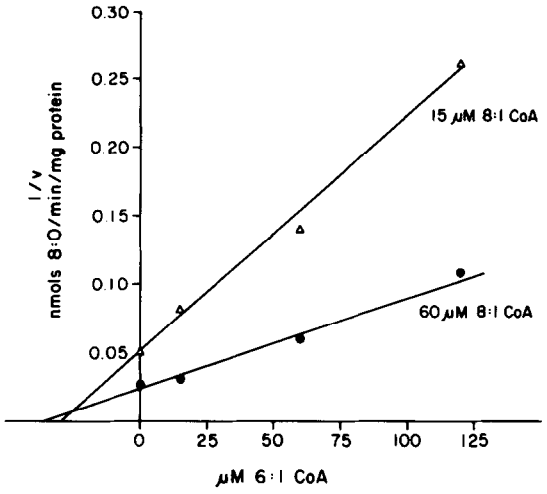

Figure 1. Effect of varying concentrations of 6:1 CoA on the NADPH-supported reduction of 8:1 CoA to octanoate by hepatic microsomes. The incubation mixture contained in a total volume of 1.0 ml, 100 mM Tris-HCl, pH 7.5, 200 μM NADPH, 1 mg microsomal protein from rats on normal diet, 15 μM (-Δ-) and 60 μM (-o-) 8:1 CoA and varying concentrations of 6:1 CoA ranging from 15 μM to 120 μM. The reaction was initiated with the microsomes and seen for 1 minute at 37°C.

tration (30 μM). This small inhibition by 8:1 CoA was expected, since based on our results, 8:1 CoA is preferred by the short chain enoyl CoA reductase. Furthermore, as expected, varying concentrations of 6:1 CoA competitively inhibited the conversion of 8:1 CoA to octanoate (Figure 1). The apparent K_i for 6:1 was 23 μM. Hexenoyl CoA also markedly inhibited the reduction of crotonyl CoA to butyrate, with a K_i of 10 μM for 6:1 (data not shown). A similar competitive inhibition was obtained when 6:1 CoA was the substrate and 8:1 CoA was employed as the inhibitor. These inhibition studies demonstrate the presence of two different enoyl CoA reductases, one acting on the short-chain substrates, 4:1, 6:1, 8:1 enoyl CoAs, and the other preferring long chain substrates, such as 16:1 CoA.

In conclusion, the present report provides strong evidence for the existence of two liver microsomal trans-2-enoyl CoA reductases in the rat. The smallest acyl chain which appears to be a substrate for the long chain reductase is the octenoyl CoA (8:1). Although octenoyl CoA is also a substrate of the short chain trans-2-enoyl CoA reductase, both crotonyl CoA (4:1) and hexenoyl CoA (6:1) are exclusively reduced by the short chain

enzyme, which is unable to utilize reducing equivalents from NADH. Current studies are underway to characterize and isolate each system.

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