Vol. 140, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1986 Pages 74-80

DO RAT HEPATIC MICROSOMES CONTAIN MULTIPLE NADPH-SUPPORTED FATTY ACID CHAIN ELONGATION PATHWAYS OR A SINGLE PATHWAY?*

Mahmoud N. Nagi, Lynda Cook, M. Renuka Prasad, and Dominick L. Cinti

Department of Pharmacology,
University of Connecticut Health Center,
Farmington, CT 06032

Received August 20, 1986

[2-^{14}C]-trans-2-hexadecenoyl CoA (16:1) and [2-^{14}C]-trans-2-cis-8,11,14-eicosatetraenoyl CoA (20:4) were chemically synthesized and employed as competitive substrates for the liver microsomal trans-2-enoyl CoA reductase component of the fatty acid chain elongation system. Both 7.5 μM and 15 μM 20:4 competitively inhibited the reduction of 16:1 CoA to palmitoyl CoA. In addition, the reduction of both substrates was identically inhibited to the same extent by the acetylenic derivative, dec-2-ynoyl CoA. Furthermore, trypsin, chymotrypsin and subtilisin inhibited trans-2-enoyl CoA reductase activity when three different substrates were employed--16:1, 20:4 and trans-2-cis-11-octadecadienoyl CoA (18:2). These results are consistent with the hypothesis of multiple condensing enzymes connected to a single elongation pathway.

The endoplasmic reticulum of mammalian liver possesses the capability of catalyzing fatty acid chain elongation through the involvement of the sequential activities of four enzymes: 1) condensing enzyme; 2) β-ketoacyl CoA reductase; 3) β-hydroxyacyl CoA dehydrase; and 4) trans-2-enoyl CoA reductase (1-3). Employing hepatic microsomes from rats on a high carbohydrate, fat-free diet, Sprecher (4) reported results which suggested the existence of more than one fatty acid chain elongation pathway. Similarly, Bourre et al. (5) demonstrated the presence of more than one elongation pathway in brain microsomes from both normal and quaking mice; this was corroborated and extended by Block and co-workers (6). Recently, using four experimental procedures, namely, competitive substrates in the form of coenzyme A, dietary alteration, administration of di-(2-ethylhexyl) phthalate and the proteolytic enzyme, chymotrypsin, our laboratory (7) reported that rat

*This work was supported by U.S. Public Health Service Grant AM21633.

0006-291X/86 $1.50

liver microsomes contain three separate condensing enzymes, one for saturated acyl-CoAs, a second for monounsaturated acyl-CoAs and a third for polyunsaturated acyl-CoAs. These results are consistent with two interpretations, that 1) hepatic microsomes contain multiple (at least 3) elongation pathways, or that 2) hepatic microsomes contain multiple condensing enzymes, all of which, are linked to a single elongation system.

In this communication, studies on the terminal enzyme, trans-2-enoyl CoA reductase, with 1) competitive substrates, trans-2-hexadecenoyl CoA and trans-2-cis-8,11,14-eicosatetraenoyl CoA, 2) the inhibitor, dec-2-ynoyl CoA, and 3) the proteases, trypsin, chymotrypsin and subtilisin, provide evidence which supports the second interpretation.

Methods

The synthesis of [2-^{14}C]-trans-2-hexadecenoic acid was prepared by the condensation of myristic aldehyde (Aldrich, Milwaukee) with [2-^{14}C]-malonic acid (NEN, Boston) according to the procedure of Nugteren (1). The method of Stoffel and Pruss (8) was employed to synthesize [2-^{14}C]-trans-2-cis 8,11,14-20:4 acid via the tosylation of γ-linolenoyl alcohol (Nu-Chek-Prep, Elysian, MN) and oxidation to the corresponding aldehyde followed by the condensation with [2-^{14}C]-malonic acid; the same procedure was used to synthesize cold trans-2-cis-11-octadecadienoyl CoA (18:2) beginning with palmitoleoyl alcohol (Nu-Chek-Prep). The acetylenic acid, $\Delta^{2}10\equiv1$, was prepared by the method of Stoffel and Echer (9). The CoA esters were prepared as described by the method of Fong and Schulz (10). The proteases -- trypsin, Type XI, bacterial subtilisin, Type VII, α-chymotrypsin, Type II and PMSF and soybean trypsin inhibitor, Type I-S -- were obtained from Sigma (St. Louis).

Liver microsomes from male Sprague-Dawley rats (150-200g) on either Purina rat chow or a fat-free diet (Nutritional Biochemicals, OH) were obtained as described previously (11).

The assay mixture for measuring the enoyl-CoA reductase activity by the radioisotope method contained the following components in a total volume of 1 ml (final concentration): 0.1 M Tris-HCl buffer, pH 7.4, 40 μM enoyl CoA, 40 μM bovine serum albumin, 1 mM KCN (to inhibit desaturase activity), and 500 μM NADPH. The reaction was initiated by addition of 100 μg microsomal protein and allowed to proceed at 37°C for 5 minutes. The reaction was terminated with 0.5 ml of 15% methanolic KOH and the contents saponified for 45 minutes at 65°C. The aqueous phase was extracted with two-3 ml volumes of pentane after acidification with 0.5 ml of 4N HCl. Fatty acid methyl esters were prepared by using diazomethane (12). Radio-gas liquid chromatographic identification of fatty acids was carried out in a Packard 894 proportional counter connected to a Varian 3700 gas chromatograph. Separation was achieved on 10% Silar 10C column (183 cm x 0.2 cm i.d.) (Applied Science, State College, PA) with helium as carrier gas at a flow rate of 50 ml/min. For products of the enoyl CoA reductase-catalyzed reaction employing trans-2-16:1 CoA as substrate, the temperature was programmed as follows: 150°C for 10 minutes, then increased to 200°C at a rate of 5°C per minute and maintained at 200°C for 5 minutes. When trans-2-cis 8,11,14-20:4 CoA was used, the temperature was programmed as follows: 180°C for 10 minutes, 180°C-220°C at a rate of 5°C per minute and then maintained at 220°C for 10 minutes.

For the proteolytic studies, microsomes were incubated with varying amounts of protease (trypsin, chymotrypsin, subtilisin) for 1.5 hrs at 25°C in a total volume of 1.25 ml containing 100 mM potassium phosphate buffer, pH 7.2. The reaction was terminated by the addition of a 2:1 ratio of trypsin inhibitor:trypsin or with 1.0 mM PMSF in ethanol when chymotrypsin or subtilisin were employed. Samples were diluted with 7.0 ml of 0.25 M sucrose, centrifuged for 45 minutes at 105,000 xg and the pellets resuspended in 1.0 ml of 100 mM phosphate buffer, pH 7.2. Enoyl CoA reductase activity was then measured spectrophotometrically by following the ratio of oxidation of NADPH at 340 nm. The assay misture contained 100 mM Tris-HCl, pH 7.4, 400 µg protease treated microsomes, 10 µM bovine serum albumin, 100 µM NADPH and 5 µM substrate in a final reaction volume of 1.0 ml. Controls included boiled microsomes and preincubations with both protease and inhibitor.

Results and Discussion

Recently, our laboratory (7) provided evidence for multiple condensing enzymes present in rat liver microsomes catalyzing the condensation of saturated, monounsaturated and polyunsaturated acyl CoA. In attempt to determine whether the other components of the elongation pathway also existed in multiple forms, the terminal step was studied. To this end, two commercially unavailable substrates were successfully synthesized, namely, [2-^{14}C]-trans-2-16:1 CoA and [2-^{14}C]-trans-2-cis 8,11,14-20:4 CoA, the elongated products of saturated myristoyl CoA (14:0) and polyunsaturated γ-linolenyl CoA (cis 6,9,12-18:3).

As demonstrated by the Lineweaver-Burk plot in Figure 1, in the presence of 7.5 µM and 15 µM trans-2-cis 8,11,14-20:4 (20:4), the NADPH-dependent reduction of trans-2-16:1 CoA (16:1) to 16:0 was competitively inhibited. The apparent V_{max}, which was calculated to be 50 nmol/min/mg microsomal protein, remained unaffected by the presence of the substrate inhibitor, whereas the apparent K_m for the trans-2-16:1 CoA increased from 21 µM in the absence of inhibitor to 57 µM in the presence of 15 µM 20:4. These results strongly suggest that the saturated and polyunsaturated substrate intermediates are utilized by the same NADPH-dependent trans-2-enoyl CoA reductase.

Consistent with this interpretation is the similar inhibition of NADPH-supported enoyl CoA reductase activity by the acetylenic derivative, $\Delta^2$10≡1 CoA, in the presence of 16:1 and 20:4. We have recently reported that dec-2-ynoyl CoA markedly inhibited the NADPH-dependent reduction of 16:1

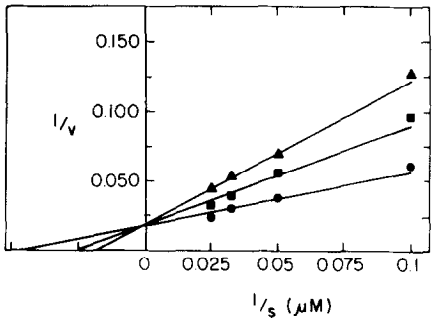

Figure 1. Lineweaver-Burk plot of the effect of trans-2-cis-8,11,14 20:4 CoA on the rate of reduction of trans-2-16:1 CoA. The enzymatic activity was measured by radio-gas liquid chromatography (glc) as described in "Materials and Methods." The reaction mixture contained in a total volume of 1 ml, varying amounts of [2-^{14}C]-trans-2-16:1 CoA and 7.5 μM (■ - ■), 15 μM (▲ - ▲) or 0 μM (● - ●) trans-2-cis 8,11,14-20:4 CoA, 1 nmol BSA per nmol total fatty acyl CoA, NADPH, 500 μM and 100 μg/ml microsomal protein. The reaction was carried out at 37°C for 5 minutes.

CoA, with an apparent K_i of 2.5 μM (13). As seen in Figure 2, incubation of liver microsomes with varying concentrations of $\Delta^2$10≡1 CoA resulted in a marked inhibition of the reduction of trans-2-cis 8,11,14-20:4 to

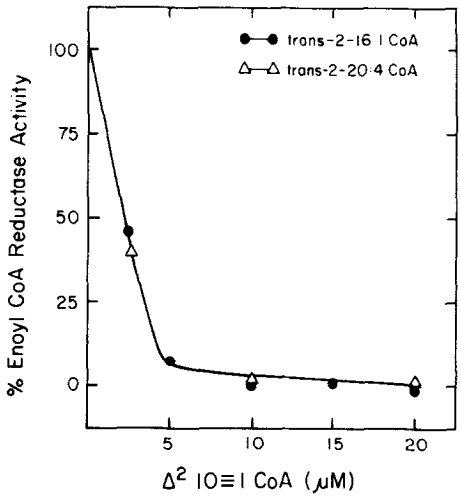

Figure 2. Effect of varying concentrations of the acetylenic CoA, $\Delta^2$10≡1 CoA, on hepatic microsomal trans-2-enoyl CoA reductase activity. The reductase activity was measured by radio-g.l.c. as described in "Materials and Methods." The final concentration of microsomal protein was 100 μg/ml, of [2-^{14}C]-trans-2-16:1 CoA, 40 μM (40,000 cpm), of [2-^{14}C]-trans-2-cis 8,11,14 20:4 CoA, 40 μM, and of NADPH, 500 μM. The specific activity of trans-2-enoyl CoA reductase in the absence of inhibitor was 40 nmol/min/mg protein when trans-2-16:1 CoA was used, and 45 nmol/min/mg when trans-2-cis 8,11,14-20:4 CoA was used. These values are represented in the figure as 100%.

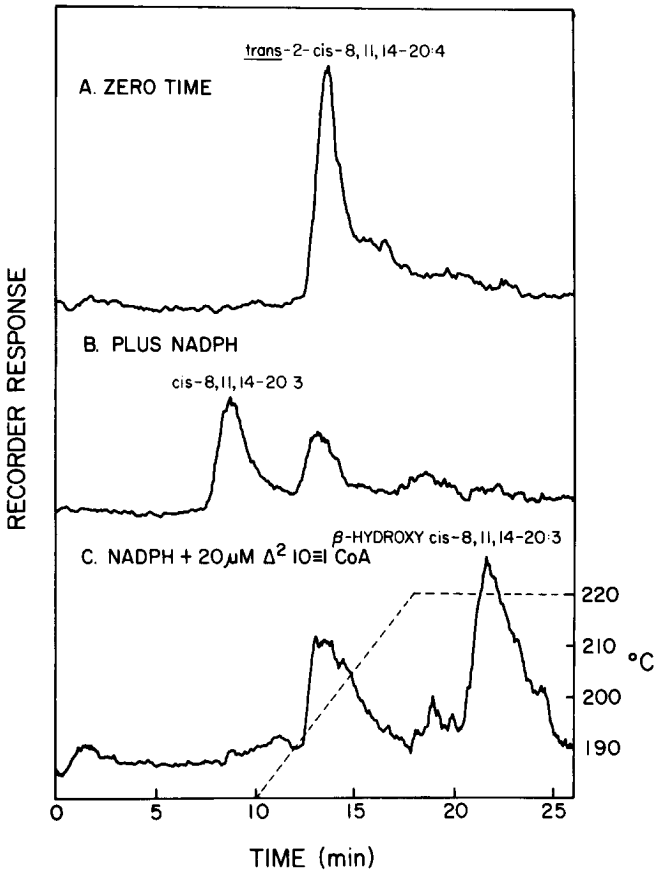

Figure 3. Measurement of liver microsomal trans-2-enoyl CoA reductase activity. Radio-gas chromatographic tracing of reaction products obtained following incubation of rat liver microsomes with [2-^{14}C]-trans-2-cis-8,11,14-20:4 CoA and NADPH in the presence and absence of dec-2-ynoyl CoA. The assay conditions were as described in "Materials and Methods." The microsomal protein concentration was 100 μg per ml; NADPH, 500 μM; trans-2-cis 8,11,14-20:4, 40 μM (30,000 cpm); when present, $\Delta^2$10≡1 CoA was 20 μM.
(A) Zero time incubation; the radioisotope peak in the tracing represents trans-2-cis 8,11,14-20:4.
(B) Tracing, representing the trans-2-enoyl CoA reductase activity, and showing formation of cis 8,11,14-20:3 (retention time -8.3 min).
(C) Same as B, except in the presence of 20 μM $\Delta^2$10≡1 CoA; the tracing shows essentially no reduced product, cis 8,11,14-20:3, as a result of inhibition of the enoyl CoA reductase; the β-hydroxy cis 8,11,14-20:3 peak is attributed to the reverse dehydrase reaction which was not affected by the $\Delta^2$10≡1 CoA.

cis-8,11,14-20:3, similar to the inhibition observed with the 16:1 CoA substrate.

Figure 3 represents a radiogaschromatograph of the synthesized radiolabelled trans-2-cis-8,11,14-20:4. As can be seen in Figure 3A the zero

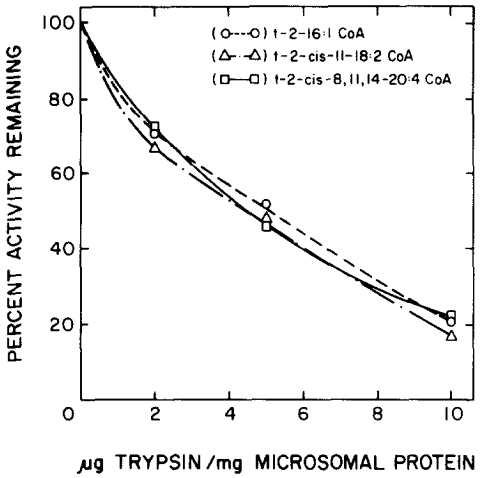

Figure 4. Effect of varying amounts of trypsin on hepatic microsomal trans-2-enoyl CoA reductase activity. The microsomes were treated with trypsin and reductase activity measured spectrophotometrically as described in "Materials and Methods." The concentration of each of the substrates was 5 μM. The values are expressed as percent activity remaining; 100% activity was obtained with microsomes either in the absence of protease or with protease plus protease inhibitor. Specific activities (100%) for 16:1, 18:2 and 20:4 were 18, 24 and 25 nmols/minute/mg microsomal protein, respectively. The data represent a typical experiment.

time incubation of microsomes plus the substrate gives rise to a single radioactive peak demonstrating greater than 90% purity. When the trans-2-enoyl CoA reductase-catalyzed reaction was initiated with NADPH, the substrate was converted to cis-8,11,14-20:3 (Figure 3B). It should be noted that the gas liquid chromatographic tracing (data not shown) of arachidonic acid showed a retention time similar to the cis-8,11,14-20:3 but slightly longer (9 min versus 8.3 min). However, most, if not all, of the first peak (Figure 3B) represents cis-8,11,14-20:3 rather than arachidonic acid since our incubation medium contained 1.0 mM KCN which blocks the Δ^5-desaturase reaction. When 20 μM $\Delta^2$10≡1 CoA was included in the assay medium (Figure 3C) the formation of 20:3 was markedly inhibited but there was no apparent effect on the hydratase which catalyzed the hydration of 20:4 to β-hydroxy-cis-8,11,14-20:3 as evidenced by the large radioactive peak at 22 minutes.

When trans-2-enoyl CoA reductase activity was determined with three different substrates, trans-2-16:1, trans-2-18:2 and trans-2-20:4, in the

presence of increasing amounts of trypsin, the extent of inhibition of reductase activity was the same for all three substrates (Figure 4); for example, 5 μg trypsin caused approximately a 50% decrease in the reductase activity for each of the three substrates. Similar results were obtained with chymotrypsin and subtilisin (data not shown). This is in contrast to the condensation reaction, in which the condensations of palmitoyl CoA, palmitoleoyl CoA and γ-linolenoyl CoA were affected quite differently in the presence of increasing amounts of chymotrypsin (7).

Hence the kinetic data strongly suggest and the inhibition studies are consistent with the idea that multiple condensing enzymes are connected to a single NADPH-dependent elongation pathway.

References

1. Nugteren, D.H. (1965) Biochim. Biophys. Acta 106, 280-290.
2. Seubert, W. and Podack, E.R. (1973) Mol. Cell. Biochem. 1, 29-40.
3. Bernert, J.T. and Sprecher, H. (1977) J. Biol. Chem. 252, 6736-6744.
4. Sprecher, H. (1974) Biochim. Biophys. Acta 360, 113-123.
5. Bourre, J.M., Pollet, S.A., Dubois, G. and Baumann, N.A. (1970) C.R. Acad. Sci. (Paris) 271, 1221-1223.
6. Goldberg, I., Shechter, I. and Bloch, K. (1973) Science 182, 497-499.
7. Prasad, M.R., Nagi, M.N., Ghesquier, D., Cook, L. and Cinti, D.L. (1986) J. Biol. Chem. 261, 8213-8217.
8. Stoffel, W. and Pruss, H.D. (1967) J. Lipid Res. 8, 196-201.
9. Stoffel, W. and Echer, W. (1969) in Methods in Enzymology, (Lowenstein, J.M., ed.) vol 14, pp. 99-105, Academic Press, New York.
10. Fong, J.C. and Schulz, H. (1981) in Methods in Enzymology (Lowenstein, J.M., ed.) vol 71, pp. 390-398, Academic Press, New York.
11. Keyes, S.R. and Cinti, D.L. (1980) J. Biol. Chem. 255, 11357-11364.
12. Nagi, M.N., Prasad, M.R., Cook, L. and Cinti, D.L. (1983) Arch. Biochem. Biophys. 226, 50-64.
13. Nagi, M.N., Cook, L., Ghesquier, D. and Cinti, D.L. J. Biol. Chem. (In Press).