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Studies on the Metabolism of Unsaturated Fatty Acids. II.[1] Separation and General Properties of Reduced Nicotinamide Adenine Dinucleotide Phosphate dependent *cis*-2-Enoyl-Coenzyme A Reductase from *Escherichia coli* K-12

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An enzyme fraction which catalyzes the reduction of *cis*-2-alkenoyl-Coenzyme A (-CoA) to the corresponding saturated acyl-CoA derivatives has been separated from *Escherichia coli* extracts.

The enzyme catalyzes the reduction of *cis*-2-octenoyl-CoA with an apparent Michaelis-Menten constant of 2.0×10^{-5} M in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), but not in the presence of reduced nicotinamide adenine dinucleotide (NADH). The reductase is inactive on *cis*-3- or *trans*-2-isomers. The reductase is stable at 45°, but its activity is lost on heating at 55° for 10 min. Some other properties of this enzyme are also described.

Keywords——*cis*-2-octenoyl-CoA reductase; *Escherichia coli*; *cis*-2-octenoic acid; *trans*-2-octenoic acid; metabolism of unsaturated fatty acids; β-oxidation

Previous investigations in a series on hydroxy fatty acid metabolism by microorganisms have shown that 5-hydroxy-*cis*-2-undecenoic acid is reduced to the corresponding saturated acid, which undergoes further β-oxidation, in *Candida*.[3-5] It was also reported that a preparation from the $105000 \times g$ supernatant fraction of *Candida* extracts catalyzed the reduction of *cis*-2-octenoyl-CoA, an intermediate in the β-oxidation of linoleic acid, in the presence of NADPH as a specific electron donor.

The reduction of 5-hydroxy-*cis*-2-undecenoic acid was first observed with *E. coli* cells.[6] When chemically synthesized 5-hydroxy-*cis*-2-undecenoic acid was added to a culture medium of *E.coli*, it was converted to the corresponding saturated acid. Subsequent degradation did not occur, contrary to the case of *Candida*, because the β-oxidation of long-chain hydroxy acids by *E. coli* was incomplete, leaving certain metabolites with chain lengths of 10 to 12 and having a hydroxyl group at the 4th, 5th, or 6th position.[7]

In order to determine whether the reduction actually contributes to the β-oxidation of unsaturated fatty acids, or whether it merely takes place as an ensuing side reaction in *E. coli* cells due to interruption of the β-oxidation of an unusual substrate, we investigated the metabolism of *cis*-2-octenoyl-CoA.

Materials and Methods

Substrates——*cis*-2-Octenoic acid, *trans*-2-octenoic acid, and their CoA derivatives were prepared as described in the previous paper.[3]

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Biochemicals——Coenzyme A, NADPH, and NADH were purchased from Kyowa Hakko Kogyo Co., Ltd.

Enzyme Preparations——A typical procedure is described below. *E. coli* K-12 was grown in a liquid medium containing 2% beef extract and 0.3% linoleic acid (initial pH 7.2) at 37° with vigorous shaking. The cells were collected at the middle of the logarithmic phase by centrifugation and washed twice with cold water. The washed cells were suspended in 0.05 M potassium phosphate buffer (pH 7.2) containing 5 mM 2-mercaptoethanol and 1 mM EDTA-2K, and sonicated for 10 min with a Tomy UR-200P ultrasonic disruptor at a temperature not exceeding 4°. The resulting suspension was centrifuged at 105000×***g*** for 60 min and the precipitate was discarded. A sufficient amount of 1 M potassium phosphate buffer (pH 7.2) was added to the supernatant solution to give a final concentration of 0.1 M potassium phosphate. Solid ammonium sulfate was added to give 90% saturation, and the resulting precipitate was collected and redissolved in a minimum volume of potassium phosphate buffer (pH 7.0, ionic strength, $\mu=0.1$). An aliquot of the preparation was filtered through a Sephacryl S-200 Superfine (Pharmacia Fine Chemicals Inc.) column (2.6 cm×51 cm), eluting with potassium phosphate buffer (pH 7.0, $\mu=0.1$). Fractions of 3.0 ml were collected and assayed for the reductase activity. The fractions containing high activity were combined, and the proteins were precipitated by adding ammonium sulfate to 90% saturation. The resulting precipitate was collected as usual and stored at −20°. The stored protein was dialyzed against potassium phosphate buffer (pH 7.0, $\mu=0.1$) before use.

Assay for Enoyl-CoA Reductase——Since the reduction of 2-alkenoyl-CoA derivatives is NADPH-dependent (Fig. 1), the decrease in absorbance at 340 nm provided a basis for enzymatic assay with *cis*-2-octenoyl-CoA or its *trans* isomer. A Gilford 250 recording spectrophotometer was used for these measurements. A typical reaction mixture contained 20 nmol of substrate, 125 nmol of NADPH, 40 μmol of potassium phosphate (pH 7.2), and water to give a final volume of 0.8 ml. The reaction was initiated by adding a suitable amount of the enzyme preparation. In the experiments on heat denaturation, the protein precipitated after heating was removed by centrifugation, and the supernatant solution was used for the assay.

Results and Discussion

Cofactor Requirement and Substrate Specificity

Experiments with cell-free extracts of *E. coli* obtained by sonication showed that the reduction of *cis*-2-octenoyl-CoA proceeded in the presence of NADPH, the oxidation of which was followed in terms of the decrease in absorbance at 340 nm (Fig. 1).

Filtration of the 105000×***g*** supernatant solution on a Sephacryl S-200 column gave two enzymatically active fractions (I and II), with *cis*-2-octenoyl-CoA as a substrate, as shown in Fig. 2-A. The bulk of the activity was recovered in Fraction II.

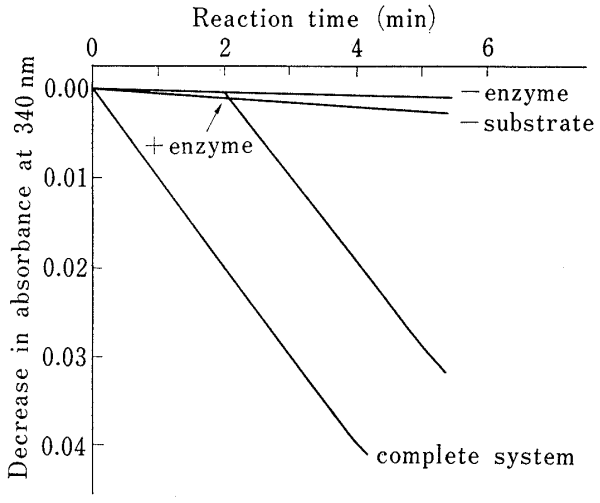

Fig. 1. Requirements for Assay of the Reductase

The complete reaction mixture contained 20 nmol of *cis*-2-octenoyl-CoA, 125 nmol of NADPH, 40 μmol of potassium phosphate (pH 7.2), and the enzyme fraction in a final volume of 0.8 ml.

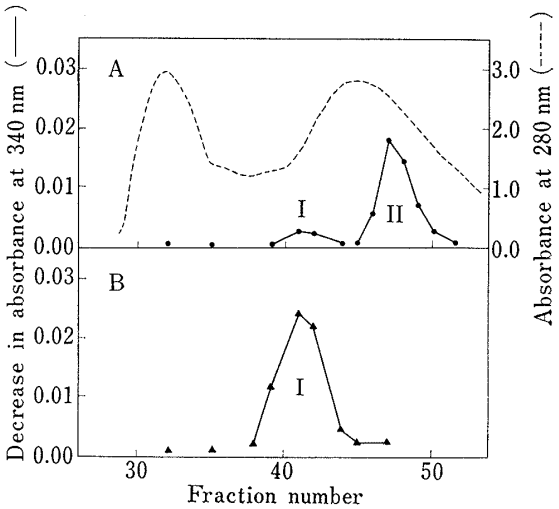

Fig. 2. Gel Filtration of *E. coli* Extracts on a Sephacryl S-200 Superfine Column

A: reductase activity on *cis*-2-octenoyl-CoA (●—●) and protein contents of the fractions (———). B: reductase activity on *trans*-2-octenoyl-CoA (▲—▲).

It was possible that there were two enzymes with different molecular sizes capable of catalyzing the same reaction, or that there was a single enzyme and the two peaks were due to either polymeric forms or its association with other proteins. However, the following evidence suggestsed that there were two enzymes with different functions. Firstly, when *trans*-2-octenoyl-CoA was employed, the oxidation of NADPH was observed exclusively in Fraction I (Fig. 2-B). Secondly, the presence of octanoic acid, the reduction product, was confirmed by gas chromatography after incubation of *cis*-2-octenoyl-CoA and Fraction II in the presence of NADPH, followed by mild saponification and esterification with diazomethane. Incubation of *trans*-2-octenoyl-CoA with Fraction II and NADPH did not give the saturated acid. Gas chromatography revealed that most preparations of the *cis*-2-alkenoyl-CoA contained about 20% of the *trans*-isomer, which was formed during acylation of CoA with the mixed anhydride.

When NADH was added to the $105000 \times g$ supernatant solution, a rapid decrease in the absorbance at 340 nm was observed due to endogenous consumption even without addition of the *cis*-2-alkenoyl-CoA, so that it was difficult to determine whether NADH was the electron donor or not. However, consumption of NADH was not observed when *cis*-2-octenoyl-CoA or its *trans*-isomer was incubated in the presence of NADH and Fraction II.

The enzyme preparation was active on *cis*-2-alkenoyl-CoA derivatives of other chain lengths (C_{10} and C_{12}) tested, but was essentially inactive on *cis*-3-octenoyl-CoA.

Based on these results, we concluded that the reductase contained in Fraction II is specific for *cis*-2-alkenoyl-CoA and requires NADPH as an electron donor. Therefore, the enzyme can be referred to as NADPH-dependent *cis*-2-enoyl-CoA reductase.

The initial rates of reduction of *cis*-2-octenoyl-CoA were studied at various concentrations (Fig. 3). The apparent Michaelis-Menten constant for this substrate was calculated from double reciprocal plots of the same data and was found to be 2.0×10^{-5} M (Fig. 3, inset).

Effect of pH on the Reductase Activity

When the rate of reduction of *cis*-2-octenoyl-CoA was tested in the range of pH from 6.1 to 9.0 with potassium phosphate buffer ($\mu=0.1$) and Tris-hydrochloride buffer ($\mu=0.1$), the enzyme preparation showed a rather broad pH optimum between 7.0 and 8.0.

Heat Denaturation

The enzyme preparation was treated at the temperatures indicated in Fig. 4. The denatured protein was separated by centrifugation, then the supernatant fluid was used

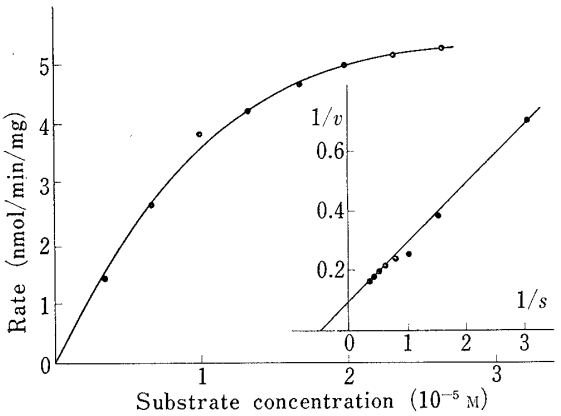

Fig. 3. Rate of Reduction of *cis*-2-Octenoyl-CoA as a Function of Substrate Concentration

Inset, Lineweaver-Burk plot of the same data.

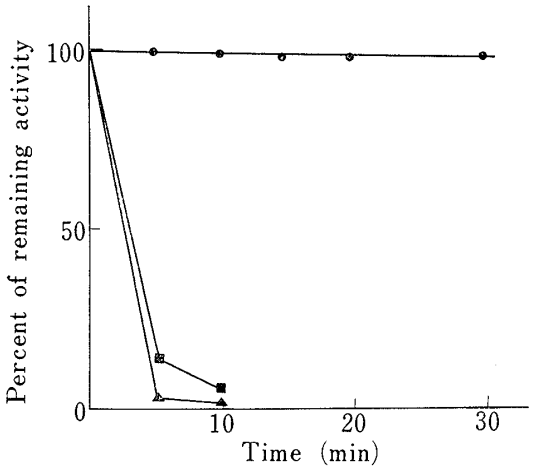

Fig. 4. Remaining Activity of Enoyl-CoA Reductase after Heat Treatment

After heating at 45° (●—●), 55° (■—■), or 65° (▲—▲), the precipitated protein was removed by centrifugation and the supernatant solution was used for assay of the reductase activity using *cis*-2-octenoyl-CoA as a substrate.

Table. Effect of Thiol Inhibitors on Enoyl-CoA Reductase Activity

Inhibitor	Concentration (M)	Inhibition (%)
p-Hydroxymercuribenzoic acid	10^{-5}	7
	10^{-4}	32
	10^{-3}	77
Iodoacetic acid	10^{-5}	9
	10^{-4}	29
	10^{-3}	34

for the assay. Whereas the activity after treatment at 45° for 30 min was about the same as the original, most of the activity disappeared after treatment at 55° for 10 min.

Effect of Thiol Inhibitors

Thiol alkylating reagents, *p*-hydroxymercuribenzoic acid and iodoacetic acid, were found to inhibit the reductase activity considerably, as shown in the table. *p*-Hydroxymercuribenzoic acid at concentrations of 10^{-4} M and 10^{-3} M inhibited the activity by 32% and 77%, respectively, and iodoacetic acid at a concentration of 10^{-3} M inhibited the activity by 34%.

Although the reductase is considered to be a sulfhydryl enzyme, no thiol compound was added to the assay system to avoid addition of the thiol group across the double bond of the substarte.

During the β-oxidation of naturally occurring unsaturated fatty acids, intermediates are formed with a double bond in the 3- or 2-position, depending on the position of the double bond in the parent acid. For example, *cis*-3-dodecenoyl-CoA is formed from oleyl-CoA, and *cis*-2-octenoyl-CoA from linoleyl-CoA. Stoffel *et al.* have reported that *cis*-2-octenoyl-CoA is first converted by enoyl-CoA hydratase to 3-D(−)-hydroxyoctanoyl-CoA, which is then epimerized to the 3-L(+)-hydroxyl isomer by an epimerase.[8] On the other hand, the results presented in this paper, as well as previous evidence described in the introduction, support the existence of an alternative pathway for the metabolism of *cis*-2-alkenoyl-CoA intermediates, which are reduced to the corresponding saturated compounds prior to further degradation in the β-oxidation cycle.

Further experiments to determine the contribution of NADPH-dependent *cis*-2-enoyl-CoA reductase to the β-oxidation of unsaturated fatty acids are in progress.

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