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Studies on the Metabolism of Unsaturated Fatty Acids. VI.[1] Stereochemical Studies of the Reaction catalyzed by *cis*-2-Enoyl-Coenzyme A Reductase of *Escherichia coli*

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The stereochemical mechanism of the reduction of *cis*-2-octenoyl-coenzyme A (-CoA) catalyzed by *cis*-2-enoyl-CoA reductase from *E. coli* was studied.

cis-2-[7,8-$^2H_5$]Octenoyl-CoA was incubated with partially purified *cis*-2-enoyl-CoA reductase in the presence of 4*R* or 4*S*-[4-$^2H_1$] nicotinamide adenine dinucleotide phosphate (NADPH). The octenoyl-CoA was also incubated with the enzyme in the presence of NADPH in ^2H_2O. Octanoic acids synthesized were isolated and analyzed by gas chromatography–mass spectrometry (GC–MS) to examine the localization of deuterium atoms in the molecule. The octanoic acids isolated from the incubation mixtures were converted to their CoA esters, and then they were dehydrogenated by the action of acyl-CoA oxidase, which had previously been shown to catalyze the *anti*-elimination of the *pro*-2*R* and *pro*-3*R* hydrogens of acyl-CoA. The resulting products, *trans*-2-octenoyl-CoAs, were converted to methyl esters and their deuterium contents at the C-2 or C-3 position were also analyzed by GC–MS.

The results suggested the following stereochemical features of the reaction catalyzed by *cis*-2-enoyl-CoA reductase.

1) *pro*-4*R* Hydrogen of NADPH was incorporated into the C-3 position of octenoyl-CoA.

2) Hydrogen from the medium was introduced into the C-2 position of octenoyl-CoA.

3) The reduction occurred by an *anti*-addition of hydrogen *via* a 2-*Si*, 3-*Re* attack on the *cis*-double bond.

Keywords——*cis*-2-octenoyl-CoA reductase; *Escherichia coli*; *cis*-2-[7,8-$^2H_5$]octenoyl-CoA; stereospecificity; stereochemistry; *anti*-addition of hydrogen; acyl-CoA oxidase

The stereochemical mechanisms of reactions catalyzed by fatty acid synthetases have been investigated by several groups.[2–15] The synthetases catalyze a cyclic series of condensation–β-ketoacyl reduction–dehydration–enoyl reduction reactions. Sedgwick *et al.*[2–4] have reported that the condensation reaction of an acyl group with a malonyl group proceeds with inversion of configuration at C-2 of malonate during the formation of the new C–C bond and that the dehydration reaction involves a *syn*-elimination of the elements of water. We have clarified the stereochemical course of the enoyl reduction catalyzed by various fatty acid synthetases.[6–14] In the course of these investigations, we have reported the following results: (1) the stereospecificity of the enoyl reduction for reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH) is different for synthetases of different origins.[6–11]; (2) the stereochemistry of hydrogen incorporation from NAD(P)H and water is also different among synthetases.[12–14]

Previously we separated a new enzyme, *cis*-2-enoyl-CoA reductase from *E. coli* and *Candida*.[16,17] This enzyme requires NADPH as a specific electron donor and catalyzes the reduction of the double bond of *cis*-2-enoyl-CoA esters. The enzyme is quite different from NADPH-dependent *trans*-2-enoyl-CoA reductase involved in the chain elongation system and is also different from NADPH-dependent *trans*-2-enoyl-acyl carrier protein reductase which is involved in the fatty acid synthetase system. It is assumed that *cis*-2-enoyl-CoA reductase

participates in the β-oxidation of unsaturated fatty acid containing *cis*-double bond(s) on the even-numbered carbon atom(s).

This paper deals with both the stereospecificity of the reaction catalyzed by *cis*-2-enoyl-CoA reductase for NADPH and the stereochemistry of hydrogen incorporation from NADPH and water during the enoyl reduction.

Materials and Methods

Synthesis of cis-2-[7,8-$^2H_5$]Octenoic Acid——This acid was synthesized in order to distinguish the enzymic reaction product from endogenous octanoic acid which is present in the preparation of the enzyme. The synthetic route employed is summarized in Chart 1.

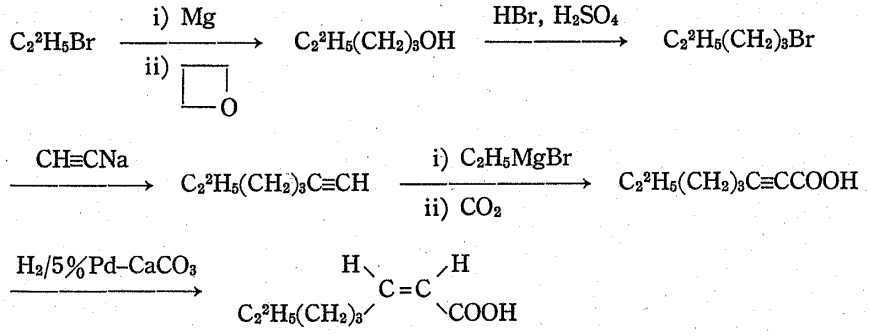

Chart 1

1-[4,5-$^2H_5$]Pentanol (I): Trimethylene oxide (12.82 g, 0.22 mol) in tetrahydrofuran (25 ml) was added dropwise to a solution of a Grignard reagent prepared from [1,2-$^2H_5$]ethyl bromide (20.95 g, 0.18 mol). The mixture was stirred at room temperature overnight and additionally at 45°C for 7 h. Tetrahydrofuran was evaporated off under reduced pressure and ether (60 ml) was added to suspend the residue. After acidification with 2 N H_2SO_4 (25 ml) and removal of insoluble materials by filtration, the ethereal layer was dried with anhydrous K_2CO_3. After removal of ether through a Vigreux column, the residue was distilled to give a colorless liquid (7.50 g, 44%), bp 133—135°C.

1-[4,5-$^2H_5$]Bromopentane (II): The alcohol I (7.50 g, 0.081 mol) was added to a cooled mixture of 47% HBr (12 ml) and conc. H_2SO_4 (5.5 ml), then the mixture was heated with stirring on an oil bath (135—140°C) for 3 h. The product was collected by extraction with ether and a colorless liquid (11.46 g, 91%), bp 125—127°C, was obtained by distillation.

1-[6,7-$^2H_5$]Heptyne (III): Sodium metal (3.40 g, 148 mg·atom) was dissolved in liquid NH_3 (150 ml), and excess CH≡CH gas was introduced with stirring for 3.5 h. Ammonia was evaporated off under an N_2 stream, and the residue was dissolved in anhydrous dimethylformamide (DMF) (45 ml). Then II (11.46 g, 73.5 mmol) in DMF (10 ml) was added dropwise to the stirred solution. The stirring continued at room temperature overnight, then continued at 45°C for 4 h. A mixture of acetic acid (8.9 g) and water (12 ml) was added to the reaction mixture under ice-cooling. The resulting mixture was subjected to steam distillation. The upper layer was separated, dried with anhydrous K_2CO_3, and distilled to yield a colorless liquid (6.03 g, 81%), bp 94—97°C.

2-[7,8-$^2H_5$]Octynoic Acid (IV): An ethereal solution (20 ml) of III (4.52 g, 44.7 mmol) was added dropwise to a solution of a Grignard reagent prepared from ethyl bromide (9.76 g, 89.5 mmol) and Mg (2.28 g, 93.8 mg·atom) in abs. ether (35 ml), and the mixture was refluxed for 3 h. Then dry CO_2 gas was introduced for 6 h and the mixture was left overnight with small pieces of dry ice. The resulting complex was treated with 2 N H_2SO_4 (50 ml) on an ice bath, and the ethereal layer was extracted with 10% NaOH. The extract was acidified with conc.HCl, then extracted with ether, and this extract was dried with anhydrous Na_2SO_4. After removal of ether, the residue was distilled to give a colorless liquid (7.26 g, 92%), bp 80—118°C (7 mmHg).

cis-2-[7,8-$^2H_5$]Octenoic Acid (V): The octynoic acid (2.0 g, 13.8 mmol) in methanol (50 ml) was subjected to catalytic reduction in the presence of 5% Pd-$CaCO_3$ (0.3 g) and quinoline (0.3 g). The reaction was stopped when 300 ml of hydrogen had been absorbed. After removal of the catalyst by filtration, methanol was evaporated off. The residue was purified by silicic acid column chromatography to obtain V (1.06 g, 52.3%).

***cis*-2-[7,8-$^2H_5$]Octenoyl-CoA**——The preparation of mixed anhydride of V with ethyl chlorocarbonate and the conversion to its CoA ester were carried out according to the preceding paper.[1]

Enzyme——*cis*-2-Enoyl-CoA reductase was prepared from *E. coli* K-12 as described previously.[17] Acyl-CoA synthetase and acyl-CoA oxidase from yeast were generous gifts from Toyobo Co., Ltd., Osaka.

Other Materials——Two specimens of stereospecifically deuterium-labeled NADPH were prepared by enzymic reduction of [4-$^2H_1$]NADP$^+$ as described in a previous paper.[6] CoA, NADPH, ATP and FAD were purchased from Kyowa Hakko Kogyo Co., Ltd., and ^2H_2O was from CIBA Ltd., Basle.

Reaction Conditions of cis-2-Enoyl-CoA Reductase——A typical incubation mixture contained potassium phosphate (pH 7.4, 0.3 mmol), NADPH (0.9 μmol), *cis*-2-[7,8-$^2H_5$]octenoyl-CoA (1.2 μmol) and *cis*-2-enoyl-CoA reductase in a total volume of 3.0 ml. The reaction was carried out at 25°C with monitoring of the decrease of absorbance at 340 nm. To terminate the reaction, 0.5 ml of 50% NaOH solution (0.5 ml) was added to the reaction mixtures, which were treated at 50°C for 30 min, and the fatty acids were extracted and subjected to Lemieux-von Rudloff oxidation as described in our previous paper[13] to remove the unreacted *cis*-2-octenoic acid.

Reaction Conditions of Acyl-CoA Oxidase——After the Lemieux-von Rudloff oxidation, the fatty acid fraction containing the deuterium-labeled octanoic acid was subjected to the action of acyl-CoA oxidase. For the acyl-CoA synthetase reaction the incubation mixtures contained potassium phosphate (pH 7.8, 0.1 mmol), 1% Triton X-100 (0.2 ml), $MgCl_2$ (2 μmol), ATP (20 μmol), CoA (2 μmol), appropriate amounts of the octanoic acid, and acyl-CoA synthetase (0.4 unit) in a volume of 1.75 ml. The mixture was incubated at 30°C for 40 min, then a solution (0.25 ml) containing FAD (40 nmol), peroxidase (20 unit) and acyl-CoA oxidase (0.4 unit) was added and the incubation was continued at 30°C for another 2 h. The isolation of products after incubation was carried out according to the method reported previously.[13]

Gas Chromatography-Mass Spectrometry (GC-MS)——Methyl esters of extracted fatty acids were applied to a 2 m × 3 mm glass column containing 1.5% OV-1 on Uniport HP (60—80 mesh) and were chromatographed at 80°C with helium as a carrier gas at a flow rate of 25 ml/min. Mass spectra were taken every 5s with a combined GC–MS instrument (GC–MS 9000s, Shimadzu-LKB, Kyoto), with an ionizing current of 60 μA, an electron accelerating voltage of 70 eV and an ion source temperature of 270°C.

Results

Deuterium Incorporation from Stereospecifically Deuterium-Labeled NADPH and ^2H_2O into Octanoate by the Reaction of *cis*-2-Enoyl-CoA Reductase

The mass spectra of methyl [7,8-$^2H_5$]octanoates which were synthesized from *cis*-2-[7,8-$^2H_5$]octenoyl-CoA with NADPH in the presence of the reductase are shown in Fig. 1. The fragment ion at *m/z* 87 ([$CH_2CH{=}C(OH)OCH_3$]$^+$) represents the oxygen-containing ion produced by the cleavage between C-3 and C-4, and the ion at *m/z* 74 ([$CH_2C(OH)OCH_3$]$^+$), forming the base peak of a methyl ester of a straight chain aliphatic acid, is due to the familiar hydrogen rearrangement and cleavage between C-2 and C-3. The deuterium atom of 4*S*[4-$^2H_1$]NADPH was not incorporated into the octanoate (Fig. 1-C). However, when octanoate was synthesized in the presence of 4*R*[4-$^2H_1$]NADPH, the fragment ions at *m/z* 163 and *m/z* 87 shifted to *m/z* 164 and *m/z* 88, respectively, but the fragment ion at *m/z* 74 remained unchanged (Fig. 1-B). These

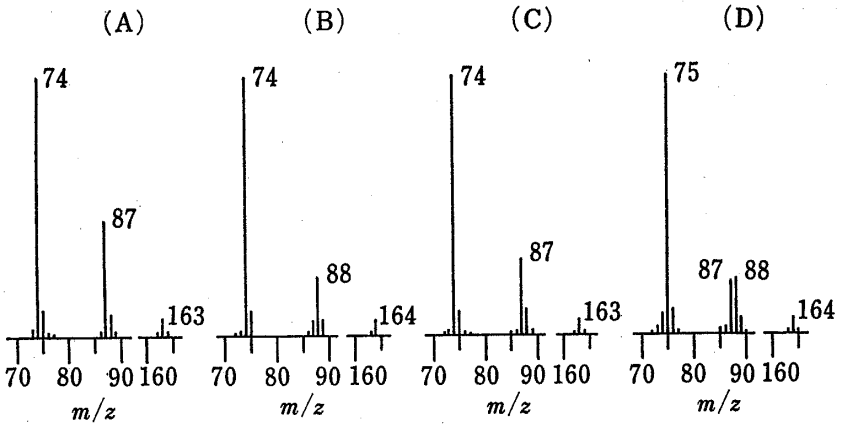

Fig. 1. Mass Spectra of Methyl Octanoates obtained with *cis*-2-Enoyl-CoA Reductase, *cis*-2-[7,8-$^2H_5$]Octenoyl-CoA, and the Following Incubation Mixtures

(A) NADPH and H_2O. (B) 4*R*-[4-$^2H_1$]NADPH and H_2O.
(C) 4*S*-[4-$^2H_1$]NADPH and H_2O. (D) NADPH and ^2H_2O.

observations indicated that *cis*-2-enoyl-CoA reductase directly transferred the *pro*-4*R* hydrogen of NADPH to the C-3 position of *cis*-2-enoyl-CoA substrates as in the case of the reductase from rat liver mitochondria.[18] When octanoate was synthesized with NADPH in ^2H_2O, the fragment ions at *m/z* 163 and *m/z* 74 shifted to *m/z* 164 and *m/z* 75, respectively. A half of the fragment ion at *m/z* 87 shifted to *m/z* 88 ($[CH_2C^2H{=}C(OH)OCH_3]^+$) and the other half of *m/z* 87 remained unchanged (Fig. 1-D). These observations demonstrated that a hydrogen from water was located on the C-2 position of the octanoate.

Stereochemistry of *cis*-2-Octenoyl-CoA Reduction by *cis*-2-Enoyl-CoA Reductase

In this series of experiments, we used acyl-CoA oxidase as a tool to determine the configuration at C-2 and C-3 of octanoic acid synthesized by *cis*-2-enoyl-CoA reductase. This oxidase catalyzes the *anti*-elimination of *pro*-2*R* and *pro*-3*R* hydrogens of acyl-CoA.[19] In order to investigate the stereochemical course of the enoyl reduction, two different kinds of experiments were performed using *cis*-2-[7,8-$^2H_5$]octenoyl-CoA as a substrate: (1) *cis*-2-[7,8-$^2H_5$]octenoyl-CoA and 4*R*-[4-$^2H_1$]NADPH were incubated in H_2O, and (2) *cis*-2-[7,8-$^2H_5$]octenoyl-CoA and NADPH were incubated in ^2H_2O. After isolation of the acids from the incubation mixtures, the samples were subjected to Lemieux-von Rudloff oxidation to remove the unreacted octenoic acid. An aliquot of the resulting acids was treated with acyl-CoA synthetase and then with acyl-CoA oxidase without any separation of octanoic acid from hexanoic acid produced by the oxidation, because the synthetase acts on octanoyl-CoA about 8 times faster than on hexanoyl-CoA.[20]

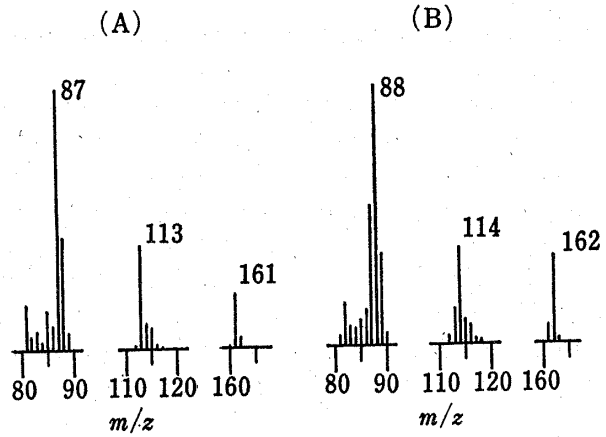

Fig. 2. Mass Spectra of Methyl *trans*-2-Octenoates obtained by the Reaction of Acyl–CoA Oxidase

The substrate was prepared from *cis*-2-[7,8-$^2H_5$]octenoyl-CoA by the catalytic action of *cis*-2-enoyl-CoA reductase in the presence of (A) 4*R*-[4-$^2H_1$]NADPH and H_2O, and (B) NADPH and ^2H_2O.

The mass spectra of methyl *trans*-2-octenoates which were derived from the corresponding deuterium-labeled octanoic acids by the oxidation of acyl-CoA oxidase are shown in Fig. 2. The mass spectrum of methyl *trans*-2-octenoate derived from 4*R*-[4-$^2H_1$]NADPH and H_2O showed the molecular ion at *m/z* 161 (Fig. 2-A), while that of the corresponding methyl octanoate was at *m/z* 164 (Fig. 1-B). Usually methyl 2-alkenoates yield a characteristic peak at *m/z* 113 as a result of cyclization following rupture between C-5 and C-6,[21] as shown in Fig. 2-A. The mass spectrum illustrated in Fig. 2-A was the same as that of authentic methyl *trans*-2-[7,8-$^2H_5$]octenoate (data not shown). These results indicated that the deuterium atom which had been incorporated from 4*R*-[4-$^2H_1$]NADPH into the C-3 position of the octanoate was eliminated by the catalytic action of acyl-CoA oxidase. Since acyl-CoA oxidase-catalyzed dehydrogenation proceeds by *anti*-elinination of the *pro*-2*R* and *pro*-3R hydrogens of an acyl-CoA, it was demonstrated that the reduction catalyzed by *cis*-2-enoyl-CoA reductase occurred by means of *Re*-attack of hydride of NADPH on the C-3 position of *cis*-2-octenoyl-CoA.

On the other hand, methyl *trans*-2-octenoate derived from the incubation mixture in the presence of NADPH and ^2H_2O showed the molecular ion at *m/z* 162 (Fig. 2-B) and the corresponding methyl octanoate had the molecular ion at *m/z* 164 (Fig. 1-D). The mass spectrum illustrated in Fig. 2-B showed that the fragment ions at *m/z* 161, 113, and 87 were shifted to *m/z* 162, 114, and 88, respectively. These results suggested that the deuterium atom which had been incorporated from ^2H_2O into the C-2 position of the octanoate was retained on the molecule after the acyl-CoA oxidase reaction. Therefore, the reaction catalyzed by the *cis*-reductase

proceeded by means of *Si*-attack of a proton of water on the C-2 position of *cis*-2-octenoyl-CoA.

Based on these results, we concluded that the stereochemistry of enoyl reduction catalyzed by *cis*-2-enoyl-CoA reductase of *E. coli* was an *anti*-addition of hydrogens *via* a 2-*Si*, 3-*Re* attack on the *cis*-double bond as shown in chart 2.

Discussion

Kawaguchi *et al.*[12,19] established a method to determine the configuration of deuterium-labeled fatty acids formed by the enzymes in fatty acid synthetase systems. The method involves two enzymic reactions: the first is activation of a fatty acid catalyzed by acyl-CoA synthetase and the second is its dehydrogenation catalyzed by acyl-CoA oxidase. Then the resulting 2-enoyl-CoA is converted to the corresponding methyl ester and analyzed for deuterium content and distribution by GC–MS. Since acyl-CoA oxidase removes exclusively the *pro-R* hydrogens from both C-2 and C-3 of acyl-CoA, information about the deuterium content of the 2-alkenoates enables us to deduce the configuration of the original deuterium-labeled fatty acids. We applied this method to elucidate the stereochemistry of hydrogen incorporation from reduced [4-$^2H_1$]-NADPH or from ^2H_2O during the enzymic reduction of cis-2-octenoyl-CoA catalyzed by *cis*-2-enoyl-CoA reductase. The observed stereoche mistry can best be explained by an *anti*-addition of hydrogen *via* a 2-*Si*, 3-*Re* attack on the *cis* double bond of 2-octenoyl-CoA. The conclusions obtained in this investigation are illustrated in Chart 2.

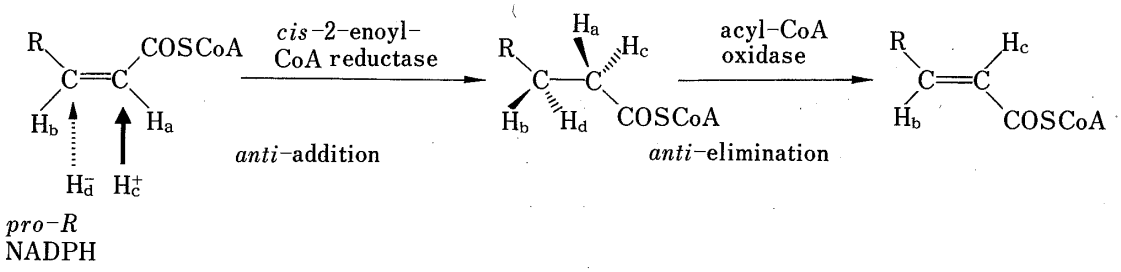

Chart 2

There are no reports on *cis*-2-enoyl reduction other than those of our group. Although we consider that *cis*-2-enoyl-CoA reductase participates in the β-oxidation of unsaturated fatty acids, its role has not been elucidated completely. However, the results mentioned in this paper should help to clarify the nature of the *cis*-2-enoyl-CoA reductase-catalyzed reaction.

Kawaguchi *et al.* have pointed out that the stereochemistry of hydrogen transfer from pyridine nucleotides may be correlated with the stereochemistry of hydrogen incorporation into the C-3 position of the intermediates.[12,13] This is also the case for the *cis*-enoyl reduction, which involves the *Re*-attack of *pro-R* hydrogen of NADPH on the *cis*-2-enoyl-CoA substrate.

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