

Separation and Some Characterizations of NADPH-Enoyl CoA Reductase(s) from *Candida albicans*

KOZO ISHIDATE,^{1a)} MICHINAO MIZUGAKI,¹⁾ and MITSURU UCHIYAMA^{1b)}

Pharmaceutical Institute, Tohoku University¹⁾

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Two fractions containing NADPH-oyl CoA reductase activities were isolated from crude extracts of *Candida albicans*. One fraction (Type-I) having larger molecular weight utilizes 5-hydroxyundec-*cis*-2-enoyl CoA, oct-*cis*-2-enoyl CoA and oct-*trans*-2-enoyl CoA as substrates with K_m values of $2.5 \times 10^{-6}M$, $1.1 \times 10^{-6}M$ and $5.0 \times 10^{-7}M$, respectively, while another fraction (Type-II) having smaller molecular weight utilizes these substrates with K_m values of $3.0 \times 10^{-6}M$, $5.3 \times 10^{-6}M$ and $1.0 \times 10^{-6}M$, respectively. This indicates that there exist comparable differences between these two in their affinities especially for the latter two substrates.

Some characterizations, such as, effects of pH and of heat treatment on the activities of these reductase preparations were also investigated.

Introduction

It has been reported that preparations of cell-free extracts of *Candida* catalyzed the reduction of 5-hydroxyundec-*cis*-2-enoyl CoA to its corresponding saturated one in the presence of NADPH as a specific electron donor, and that this process was actually involved in the β -oxidation of homoricinoleic (13-hydroxynonadec-*cis*-10-enoic) acid in *Candida*.²⁻⁴⁾ The NADPH-oyl CoA reductase also catalyzed the reduction of oct-*cis*-2-enoyl CoA which was a common intermediate in the β -oxidation pathway of unsaturated fatty acids, and produced octanoate.

These findings suggest that the reductase may play an essential role in the process of β -oxidation of naturally occurring unsaturated fatty acids and may support the presence of another pathway different from existing theories on the mechanisms of this process, ...e.g. 1) involving isomerase and/or epimerase^{5,6)} and 2) splitting off of propionyl CoA from 3-enoate.^{7,8)}

The present report is to demonstrate two reductase fractions of different molecular weights and of differences in their substrate specificity and in some other behaviors which were found in the process of purification.

Materials and Methods

Substrates—Preparations of 5-hydroxyundec-*cis*-2-enoyl CoA, oct-*cis*-2-enoyl CoA and oct-*trans*-2-enoyl CoA have been described in the preceding paper.^{9,10)} Dodec-*cis*-3-enoyl CoA and dodec-*trans*-3-enoyl CoA were prepared by the methods of Stoffel¹¹⁾ and Boxer,¹²⁾ respectively.

- 1) Location: Aobayama, Sendai; a) Present address: Department of Chemical Toxicology, Medical Research Institute, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo; b) National Institute of Hygienic Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo.
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Assay of NADPH-Enoyl CoA Reductase—Since the reduction of 2-enoyl CoA (or 3-enoyl CoA) is absolutely NADPH-specific,²⁾ the decrease in absorbance at 340 nm provided a basis for the enzymic assay with various enoyl CoA derivatives. A Shimadzu double 40 recording spectrophotometer was used to monitor continuously the decrease in absorbance. All reaction mixtures were assayed at 30°. A typical reaction mixture contained 40 nmoles of substrate, 125 nmoles of NADPH, 40 μ moles of potassium phosphate, pH 7.5, and water added to a final volume of 0.8 ml. The concentration of each substrate and the pH of the phosphate were varied in the experiments of substrate specificity and pH dependency, respectively.

In the experiment of heat denaturation, the denatured protein in each reductase preparation after heating at pH 7.5, was precipitated by centrifugation (10000 $\times g$, for 10 min) and the supernatant was used for the assay of reductase activity in a typical reaction medium.

Enzyme Preparation—The cell-free extracts from *Candida albicans* were prepared as previously described.²⁾

The protein precipitated by 90% ammonium sulfate saturation was dialyzed against 0.01M potassium phosphate buffer, pH 7.2, containing 5 mM 2-mercaptoethanol and 1 mM EDTA-2Na and passed through a Sephadex G-100 column (2.5 \times 90 cm) with 0.01M phosphate (pH 7.2), 5 mM 2-mercaptoethanol and 1 mM EDTA as the eluting solvent. Fractions of 8.0 ml each were collected and NADPH-enoyl CoA reductase activities were assayed with 5-hydroxyundec-*cis*-2-enoyl CoA as a substrate. The tubes containing high enzymatic activities were combined and adsorbed directly on DEAE-cellulose column (2.5 \times 30 cm) which had previously been equilibrated with 0.01M potassium phosphate (pH 7.2), 5 mM 2-mercaptoethanol and 1 mM EDTA. The column was washed with the same solvent until no ultraviolet-absorbing components were eluted. At this point, the eluting solvent was changed to 0.01M phosphate, pH 7.2, 0.1M sodium chloride, 5 mM 2-mercaptoethanol and 1 mM EDTA.

Next, the concentration of sodium chloride in the eluting solvent was stepwisely changed to 0.3M, then to 0.5M. All ultraviolet-absorbing materials of each fraction were pooled and the phosphate buffer concentration was adjusted to 0.1M with 1M phosphate, pH 7.2. Ammonium sulfate was added to make 90% saturation, then the precipitated protein was separated by centrifugation and the pellets were stored at -20° .

The stored protein of each fraction was dialyzed against 0.05M phosphate, pH 7.2, 5 mM 2-mercaptoethanol and 1 mM EDTA before use.

Results

Separation of Type-I and Type-II Reductase(s) on Sephadex G-100 Column

When the protein of crude extracts precipitated by 90% ammonium sulfate saturation was dialyzed and passed through a Sephadex G-100 column (2.5 \times 90 cm), two fractions appeared containing high reductase activities with 5-hydroxyundec-*cis*-2-enoyl CoA as a substrate as shown in Fig. 1. The first reductase fraction, Type-I, had larger molecular weight of about 180000 roughly calculated from its elution volume, and the second reductase fraction, Type-II, had smaller molecular weight of about 85000.

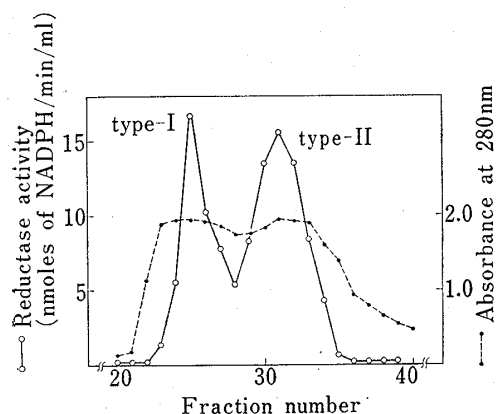


Fig. 1. Elution Pattern of NADPH-Dependent Enoyl CoA Reductase(s) on Sephadex G-100 Column

The size of the column used was 2.5 \times 90 cm. Fractions of 8.0 ml each were collected. Other procedures for the chromatography are described in Methods. The dotted line represents protein contents of the fractions and the solid line does the NADPH-enoyl CoA reductase activity with 5-hydroxyundec-*cis*-2-enoyl CoA as a substrate.

In contrast, Type-II reductase activity (tube number 29—34 after fractionation on Sephadex G-100 column), in the same procedure, was eluted by the eluting buffer containing

Successive Fractionation of Type-I and Type-II Preparations on DEAE-Cellulose Column Chromatography

As shown in Fig. 2a, Type-I reductase fraction (tube number 24—28) was adsorbed directly on a DEAE-cellulose column (2.5 \times 30 cm) and after washing with 0.01M potassium phosphate, pH 7.2, 5 mM 2-mercaptoethanol and 1 mM EDTA, the stepwise elution was carried out by changing the concentration of sodium chloride. Type-I reductase activity was eluted by the eluting agents containing 0.3M sodium chloride. No reductase activity was detected in any other fractions.

0.1M sodium chloride and no NADPH-enoyl CoA reductase activity was detected in any other fractions as shown in Fig. 2b.

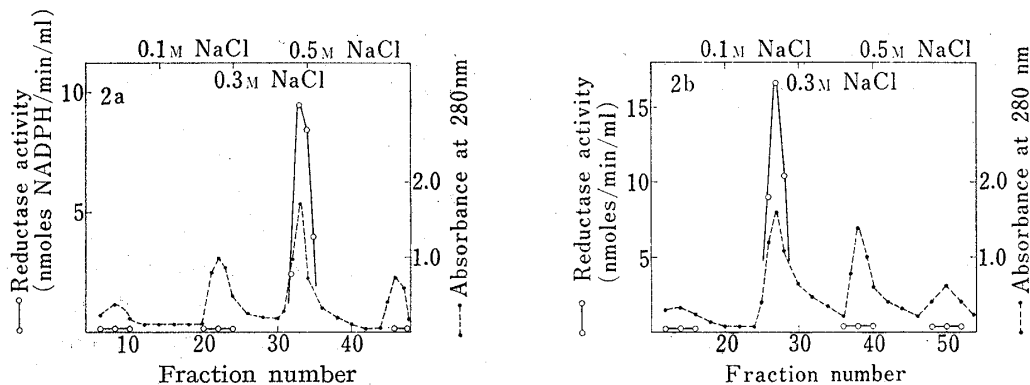


Fig. 2a and 2b. The Stepwise Fractionation of Type-I (2a) and Type-II (2b) Reductase(s) on DEAE-Cellulose Column

The size of the column used was 2.5×30 cm. Fractions of 8.0 ml each were collected and assayed for NADPH-enoyl CoA reductase activity with 5-hydroxyundec-*cis*-2-enoyl CoA as a substrate. Other procedures for the chromatography are described in Methods.

During these procedures of fractionation through both Sephadex G-100 and DEAE-cellulose column, the specific activity of the two fractions appeared about 10 folds higher than that of crude extracts.

Behavior of NADPH-Dependent Enoyl CoA Reductase(s) to Various 2-Enoyl CoA Derivatives

Table I shows the behavior of Type-I and Type-II reductase preparations to various 2-enoates during the process of separation by DEAE-cellulose column chromatography. The higher reaction rates for both of oct-*cis*-2-enoyl CoA and oct-*trans*-2-enoyl CoA were obtained in Type-I preparation than in Type-II preparation. On the other hand, 5-hydroxyundec-*cis*-2-enoyl CoA was more rapidly reduced by Type-II preparation.

The recovered activities during the procedure of separation through DEAE-cellulose column were 60–70% for either 2-enoates as substrates. In order to minimize the loss of total activity, separation by DEAE-cellulose column chromatography was frequently employed.

TABLE I. Behavior of NADPH-Enoyl CoA Reductase Preparations (Type-I and Type-II) to Various 2-Enoyl CoA Derivatives

Each incubation mixture contained 40 μ moles of potassium phosphate, pH 7.5, 40 nmoles of each substrate, 125 nmoles of NADPH and water added to a final volume of 0.8 ml. The reaction was initiated by the addition of sufficient amount of each enzyme preparation to give an absorbance change of 0.01 to 0.02 per min. Type-I and Type-II reductases used here were separated by the stepwise fractionation on DEAE-cellulose column chromatography.

	Specific activity ^{a)}			
	Crude ext.	90% $(\text{NH}_4)_2\text{SO}_4$	Type-I	Type-II
5-Hydroxyundec- <i>cis</i> -2-enoyl CoA	1.39(2060) ^{b)}	4.13	2.90(510)	4.55(743)
Oct- <i>cis</i> -2-enoyl CoA	1.04(1541)	3.10	4.84(852)	1.90(310)
Oct- <i>trans</i> -2-enoyl CoA	1.14(1690)	3.44	4.68(824)	1.89(308)

a) specific activity: nmoles of NADPH oxidized per min per mg protein

b) total activity: nmoles of NADPH per min

Substrate Specificity of Type-I and Type-II Enoyl CoA Reductase Preparations

Figure 3 shows the initial rates of reduction exhibited by the reaction mixtures of either Type-I (a) or Type-II (b) preparation and of various concentrations of oct-*cis*-2-enoyl CoA. Typical relationships between substrate concentrations and enzyme activities were observed, so that the apparent K_m values of Type-I and Type-II preparations with oct-*cis*-2-enoyl CoA were determined to be about $1.1 \times 10^{-6} \text{ M}$ and $5.3 \times 10^{-5} \text{ M}$, respectively, by calculation from double reciprocal plots of the data (Fig. 3, insets).

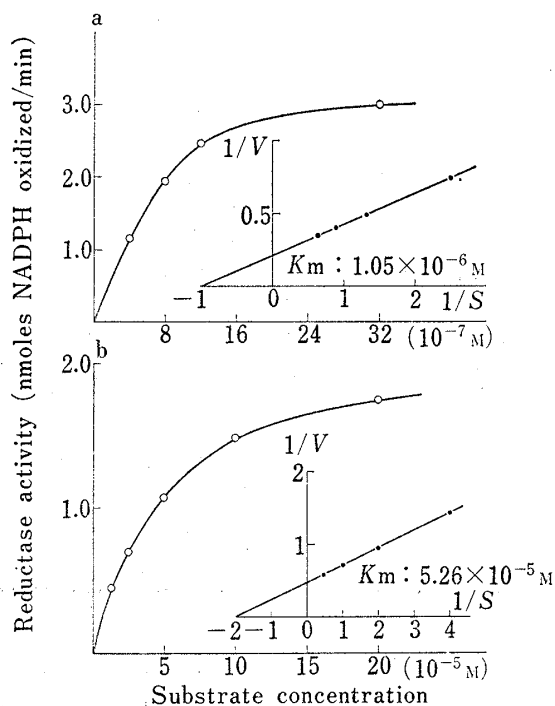


Fig. 3. Effect of Substrate Concentrations on Type-I (a) and Type-II (b) Reductase Activities with Oct-*cis*-2-enoyl CoA as a Substrate

Insets, Lineweaver-Burk plots of the same data.

The K_m values with 5-hydroxyundec-*cis*-2-enoyl CoA, oct-*cis*-2-enoyl CoA and oct-*trans*-2-enoyl CoA using crude extracts as an enzyme source were very similar with each other and showed about $4.0 \times 10^{-6} \text{ M}$, $4.0 \times 10^{-6} \text{ M}$ and $2.5 \times 10^{-6} \text{ M}$, respectively (Table II). It is interesting that Type-I reductase preparation had a higher affinity for any 2-enoyl CoAs than Type-II reductase preparation had, and that between both types of reductase(s), the values with 5-hydroxyundec-*cis*-2-enoyl CoA were of similar level, while those with oct-*cis*-2-enoyl CoA and its *trans* isomer were markedly different. The values observed in crude extracts with either 2-enoyl CoAs would show the average ones between those observed in Type-I and Type-II reductase preparations.

The data suggest that these values would not be in contradiction to the physiological range and that, in addition, the different specificity between two types of reductase preparations may have a possible physiological significance in intact *Candida* cells.

Effect of pH on the Activities of NADPH-Enoyl CoA Reductase Preparations

In order to characterize each type of reductase preparation, effect of pH on the reductase activities was investigated. As shown in Table III, Type-I enzyme preparation had similar activities in the pH range from 6.5 to 8.5 with either 2-enoyl CoAs as substrates. In contrast, Type-II reductase preparation displayed an acidic range of optimal pH in catalyzing the reduc-

TABLE II. Kinetic Data of NADPH-Enoyl CoA Reductase(s) (Type-I and Type-II) for Various 2-Enoyl CoA Derivatives

The K_m value of each reductase preparation was calculated from double reciprocal plots obtained for various 2-enoyl CoA derivatives, respectively.

	K_m (M)		
	Crude ext.	Type-I	Type-II
5-Hydroxyundec- <i>cis</i> -2-enoyl CoA	4.0×10^{-6}	2.5×10^{-6}	3.0×10^{-6}
Oct- <i>cis</i> -2-enoyl CoA	4.0×10^{-6}	1.1×10^{-6}	5.3×10^{-5}
Oct- <i>trans</i> -2-enoyl CoA	2.5×10^{-6}	5.0×10^{-7}	1.0×10^{-5}

tion of oct-*cis*-2-enoyl CoA and its *trans* isomer, but a rather broad range of optimal pH from 6.5 to 8.5 with 5-hydroxyundec-*cis*-2-enoyl CoA.

TABLE III. Effect of pH on the Activities of NADPH-Enoyl CoA Reductase(s) (Type-I and Type-II)

The reaction mixture contained the same components as in Table I, except that the pH of the phosphate was varied as indicated.

	Type-I			Type-II		
	pH 6.5	pH 7.5	pH 8.5	pH 6.5	pH 7.5	pH 8.5
5-Hydroxyundec- <i>cis</i> -2-enoyl CoA	2.58 ^a)	2.86	3.58	5.50	5.39	5.27
Oct- <i>cis</i> -2-enoyl CoA	3.15	3.15	3.25	1.57	1.12	1.12
Oct- <i>trans</i> -2-enoyl CoA	3.06	2.77	2.96	1.68	1.23	1.12

a) specific activity: nmoles NADPH oxidized per min per mg protein

Heat Denaturation

Each type of reductase preparations was treated in a centrifuge tube at a temperature indicated in Table IV, the mixture was then rapidly cooled to 0° in an ice bath and centrifuged at 10000 × *g* for 10 min, and the supernatant fluid was used for the assay of reductase activity. As shown in Table IV, the reaction rates with Type-I preparation on 5-hydroxyundec-*cis*-2-enoyl CoA, oct-*cis*-2-enoyl CoA and oct-*trans*-2-enoyl CoA were equally decreased to 50% after heat treatment at 50° for 30 min, and to 20% after heating at 60° for 3 min. On the other hand, the remaining activity of Type-II preparation on 5-hydroxyundec-*cis*-2-enoyl CoA was not detected after heat treatment at 50° for 30 min, but the activity on the *cis*- and *trans*-isomers of oct-2-enoyl CoA were observed such extents as seen with Type-I reductase preparation on these substrates.

TABLE IV. Percent of Remaining Activities of NADPH-Enoyl CoA Reductase(s) (Type-I and Type-II) for Various 2-Enoates after Heat Treatment

The denatured protein of each enzyme preparation after heating at pH 7.5, was precipitated by centrifugation and the supernatant was used for the assay of reductase activity. The assay mixture contained the same components as in Table I.

Treatment	Type-I			Type-II		
	5-OH C _{11:1}	2- <i>cis</i> C _{8:1}	2- <i>trans</i> C _{8:1}	5-OH C _{8:1}	2- <i>cis</i> C _{8:1}	2- <i>trans</i> C _{8:1}
None	100	100	100	100	100	100
50°, 10 min	69	65	65	8	71	79
50°, 30 min	50	46	47	0	43	50
60°, 3 min	21	19	19			

Discussion

The pathway of the β -oxidation, the major one of fat oxidation, of saturated fatty acids has been well established,¹³⁾ and in which *trans*-2 unsaturated acyl CoA derivatives are formed as metabolic intermediates.

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On the other hand, β -oxidation of naturally occurring unsaturated fatty acids would give rise to fatty acids having *cis*-unsaturation in the 2- or 3-position, after removal of several acetyl units. Such *cis*-unsaturated fatty acids, however, do not impair fatty acid oxidation.¹⁴⁻¹⁸⁾

It is generally accepted that *cis*-2-enoates are converted by an enoyl CoA hydratase [EC 4.2.1.17] to D(-)-3-hydroxy derivatives which are subsequently converted by an epimerase to L(+)-3-hydroxy compounds to ultimately re-enter to the β -oxidation cycle.⁶⁾ With respect to the enoyl CoA hydratase, Stern has reported that this enzyme shows only 1/3 of the activity toward *cis*-2-enoyl CoA as toward the *trans*-2-isomer.¹⁹⁾

While, our previous findings using *Candida*²⁾ proposed the presence of a new pathway in which, different from the existing theory of the hydratase-epimerase system mentioned above, the *cis*-2-enoates are first reduced by the action of NADPH-dependent enoyl CoA reductase and then undergoes normal β -oxidation.

In the present experiments, as shown in Figures 1 and 2, NADPH-enoyl CoA reductase activity was found in two fractions, Type-I and Type-II, in *Candida*, each of which was separated by gel-filtration on Sephadex G-100 and/or by DEAE-cellulose column chromatography. It was possible that there existed two enzymes with different molecular sizes capable of catalyzing the same reaction, and it was also possible that there was a single enzyme and Type-I activity was due to either a polymeric form of the enzyme or its association with other proteins. Type-I reductase preparation showed the higher affinities and reaction rates for both oct-*cis*-2-enoyl CoA and its *trans* isomer than showed for 5-hydroxyundec-*cis*-2-enoyl CoA (Tables I and II).

Differences between Type-I and Type-II reductase preparations were seen by experiments of pH dependency and of heat denaturation (Tables III and IV). Type-I reductase preparation showed similar activities in the pH range from 6.5 to 8.5, while Type-II reductase preparation displayed an acidic range of optimal pH.

It is uncertain whether there might exist more than one reductase in Type-II preparation, or some conformational changes of reductase caused by changing the pH and by heating might decrease the affinity of reductase for 5-hydroxyundec-*cis*-2-enoyl CoA due to its steric hindrance.

It has been observed that acetate incorporation into fatty acids in the mitochondrial elongation system requires both NADH and NADPH²⁰⁻²⁴⁾ and NADPH is required for the reduction of *trans*-2-enoyl CoA.²⁴⁻²⁶⁾

As shown in Tables I and II, the two reductase preparations catalyzed the reduction of oct-*trans*-2-enoyl CoA as well as oct-*cis*-2-enoyl CoA in the presence of NADPH. The relationship between the β -oxidation system and fatty acid chain-elongation system in mitochondria as a function of the reductase should be investigated.

In our preliminary experiments, dodec-*cis*-3-enoyl CoA and dodec-*trans*-3-enoyl CoA were efficiently reduced in the presence of NADPH by the addition of Type-I preparation.

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With respect to *cis*-3-enoates, intermediates during the β -oxidation of unsaturated fatty acids as well as *cis*-2-enoates, Stoffel, *et al.*⁵⁾ extracted an isomerase from rat liver mitochondria converting *cis*-3-enoate to the corresponding *trans*-2 form which re-enter to the β -oxidation cycle. On the other hand, Sinclair⁷⁾ suggested that propionyl CoA would be split off from 3-enoate from the facts of less ketosis in patients eating high fat diets which contained more polyunsaturated fatty acids than saturated ones. In this point of view, there have presented a few supporting articles.^{8,27,28)}

More precise experiments with respect to the possible contribution of NADPH-enoyl CoA reductase to the β -oxidation process of *cis*-3(or *trans*-3)-enoate are now under investigation in this laboratory.

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