A β-HYDROXYDECANOYL-ACP DEHYDRASE SPECIFIC FOR SATURATED FATTY ACID BIOSYNTHESIS IN E. COLI

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The synthesis of saturated and unsaturated long chain fatty acids in certain bacteria is known to proceed by a common pathway through the production of β-hydroxydecanoyl-ACP.[1] (Goldfine and Bloch, 1961; Lennarz et al., 1962). Bloch and coworkers (Norris et al., 1964; Kass et al., 1967; Brock et al., 1967) have extensively studied an E. coli dehydrase which specifically catalyzes the dehydration of β-hydroxydecanoyl thioesters yielding both trans-2-decenoyl and cis-3-decenoyl thioesters. In the biosynthetic sequence, the former is subsequently reduced and elongated to form primarily palmitate, whereas the cis-3-decenoyl thioester is elongated without reduction of the double bond, giving rise to cis-vaccenate and palmitoleate. Thus, the enzyme could function in the synthesis of saturated and unsaturated fatty acids. Evidence has accumulated suggesting the existence of a thioester dehydrase which produces only trans-2-decenoyl product. Such a dehydrase would function specifically in saturated fatty acid synthesis (Wakil et al., 1964; Pugh et al., 1966; Overath and Stumpf, 1964; Simoni et al., 1967). The existence of this dehydrase is necessarily inferred from the report of the isolation of an E. coli mutant which lacks the dehydrase described by Bloch's group, and is incapable of synthesizing unsaturated fatty acids, but produces normal amounts of saturated acids. The enzymatic defect has been shown to be

[1]Acyl carrier protein.

an absence of the dehydrase described by Bloch's group (Silbert and Vagelos, 1967a, 1967b). Further, Kass and Bloch (1967) have recently shown that the production of unsaturated long chain fatty acids by E. coli synthetase can be specifically inhibited, without impairing saturated fatty acid synthesis, and corrected by addition of their highly purified dehydrase.

The unsaturated fatty acid auxotroph described above has been utilized as a source of a β-hydroxydecanoyl-ACP dehydrase which is shown in this report to have several properties unique among those of thioester dehydrases previously reported. The same activity can be demonstrated in cells which are wild type for fatty acid biosynthesis.

Materials and Methods: An auxotroph of E. coli K-12, Hfr-139, requiring pantothenic acid and thiamine, and an unsaturated fatty acid requiring mutant derived from this strain were used in these studies (Silbert and Vagelos, 1967b).

Crude cell extracts of late log phase cultures were prepared in a French pressure cell at 0°C, buffered with 0.1 M Tris-HCl, pH 7.4, containing 10^{-3} M EDTA.

D-(-)-β-hydroxydecanoic acid was obtained from rhamnolipid derived from Pseudomonas aeruginosa[2] and recrystallized 3 times. The ^{14}C-labelled hydroxy acid was obtained from cultures grown in the presence of ^{14}C-acetate.

D-(-)-β-hydroxydecanoyl-ACP was prepared from E. coli ACP[3] by the mixed anhydride method (Ailhaud et al., 1967), with one modification: the reduced ACP was initially acetylated in 0.1 M phosphate buffer pH 8.0, at 0°C by the addition of a 20-fold molar excess of acetic anhydride. This procedure acetylated all the acylatable groups of ACP. After 15

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[2]Thanks are due Dr. Luis Glaser for generous donation of the rhamnolipid.
[3]The ACP used in these experiments was prepared by Dr. John Elovson.

minutes, excess neutral hydroxylamine was added to cleave the thioester, yielding the ACP-SH. After precipitation and washing with perchloric acid, the ACP preparation was resuspended in 0.1 M Tris-HCl, pH 8.0, and reacted with a mixed anhydride of D-(-)-β-hydroxydecanoate in the usual manner. This procedure yielded β-hydroxydecanoyl-ACP which contained β-hydroxydecanoate only in the thioester position.

D-(-)-β-hydroxybutyryl ACP was synthesized in the same way.

Dehydrase activity was measured spectrophotometrically, observing initial rate of increase in absorbance at 263 mμ, resulting from the formation of α,β unsaturated thioester from D-(-)-β-hydroxydecanoyl-ACP. The standard assay mixture contained 6 mμmoles of β-hydroxydecanoyl-ACP, enzyme preparation, and 2 μmoles of phosphate buffer, pH 7.2, in a volume of 0.2 ml. Enzyme and buffer were contained in the reference cell. Enzyme units are defined as mμmoles of α,β thioester produced per minute per milligram of protein.

Thin layer chromatography of fatty acid methyl esters was carried out in a chloroform - 0.75% ethanol system using silver nitrate impregnated Silica gel G plates (Barrett et al., 1963). Spots were visualized with 2',7'-dichlorofluorescein, 0.2% solution.

Chromatography by gel filtration was performed on a Sephadex G-100 column (3.5 x 75 cm) previously equilibrated with phosphate buffer, 0.01 M, pH 7.2, containing 0.01 M 2-mercaptoethanol. The column was eluted with 275 ml of the same buffer, and 1 ml fractions were collected.

Results and Discussion.-- The crude extract (80 mg) of E. coli Hfr-139, which is wild type with respect to fatty acid synthesis, was chromatographed on Sephadex G-100. As indicated in Figure 1-A, two peaks of α,β dehydrase activity were found when β-hydroxydecanoyl-ACP was used as substrate. Similar chromatography was performed on crude extracts (320 mg) of the mutant incapable of synthesizing unsaturated fatty acids (Figure 1-B). The elution pattern shows that the mutant extract contained only one peak of α,β

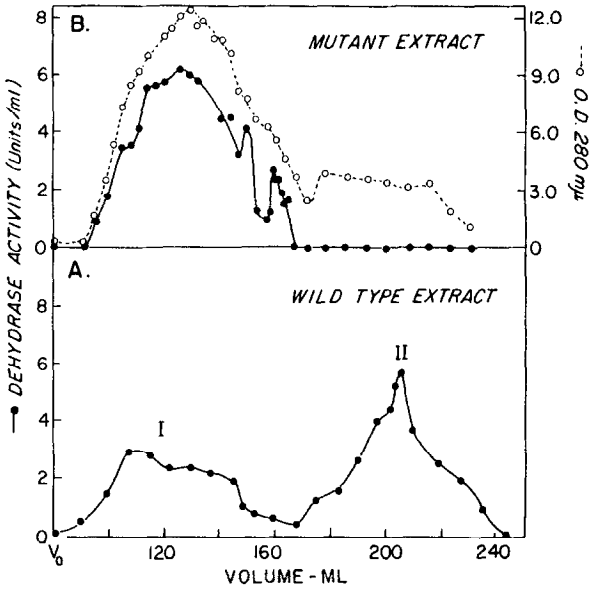

Figure 1.-- Sephadex G-100 chromatography of crude extracts. Procedure described in text.

dehydrase activity. These results suggested that peak II, which is absent in the mutant extract, contains the β-hydroxydecanoyl thioester dehydrase specifically required for unsaturated fatty acid synthesis. The activity of peak I, which is present in both the wild type and mutant extracts, would represent the dehydrase which functions in saturated fatty acid synthesis.

Since Bloch and coworkers have reported that the dehydrase isolated by them is stable at 50°C, heat stability studies were carried out with the enzymes of peaks I and II. With β-hydroxydecanoyl-ACP in the standard assay, the dehydrase activity of peak II was found to be relatively stable at 50° (Figure 2). In contrast, the dehydrase activity of peak I was completely destroyed by heating at 45° for 5 minutes. Similar stability tests with the mutant enzyme indicated complete loss of activity after heating at 50° for 5 minutes.

Majerus et al. (1965) have reported an enzyme in wild type E. coli

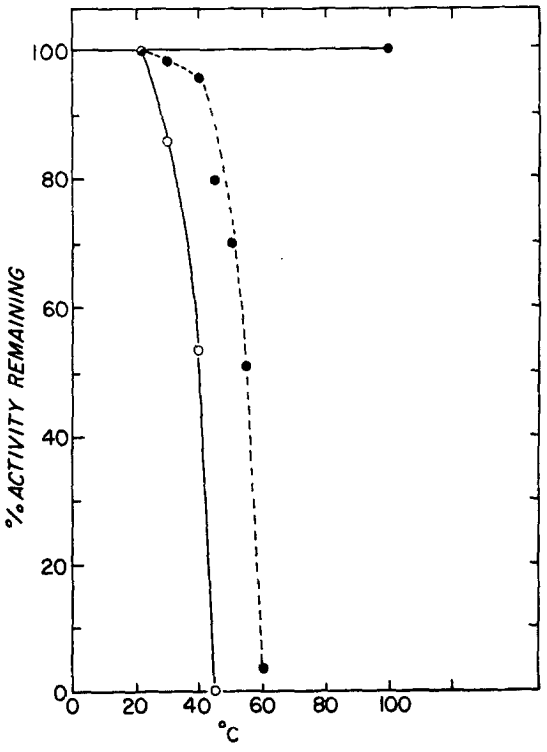

Figure 2.-- Heat stability of dehydrase activities of Peaks I and II (Fig. 1). Procedure described in text. —○— β-hydroxydecanoyl-ACP + Peak I; - - -●- - - β-hydroxydecanoyl-ACP + Peak II; —●— β-hydroxybutyryl-ACP + Peak II.

which catalyzes the dehydration of D-(-)-β-hydroxybutyryl-ACP. This enzyme is not inactivated by heating at 100°. In order to identify this dehydrase activity, both enzyme fractions (peaks I and II) were examined for dehydrase activity using D-(-)-β-hydroxybutyryl-ACP as the substrate in the standard assay. Activity was detected only in peak II, and this activity was not reduced by heating at 100° for 5 minutes (Figure 2). Peak II therefore contains the β-hydroxybutyryl-ACP dehydrase as well as a β-hydroxy decanoyl-ACP dehydrase.

Since the dehydrase activity of peak I appears to contain the enzyme which functions specifically in the pathway of saturated fatty acid synthesis and can be distinguished from enzymes previously described, it has been

partially purified. (Purification procedure to be published). The reaction product formed after incubating the enzyme with ^{14}C-β-hydroxydecanoyl-ACP was hydrolyzed and methylated, and the esters, with appropriate carriers and standards, were chromatographed in the thin-layer system described. Separation of cis and trans monoenoates and hydroxy acid esters is affected by this system. The only product found in this experiment was trans-2-decenoate (20% of recovered counts), along with the ester of unreacted β-hydroxydecanoate.

Kinetic experiments were done with both this enzyme and the dehydrase of peak II which was highly purified according to the method of Kass et al., (1967). Figure 3 demonstrates the substrate concentration curves and the

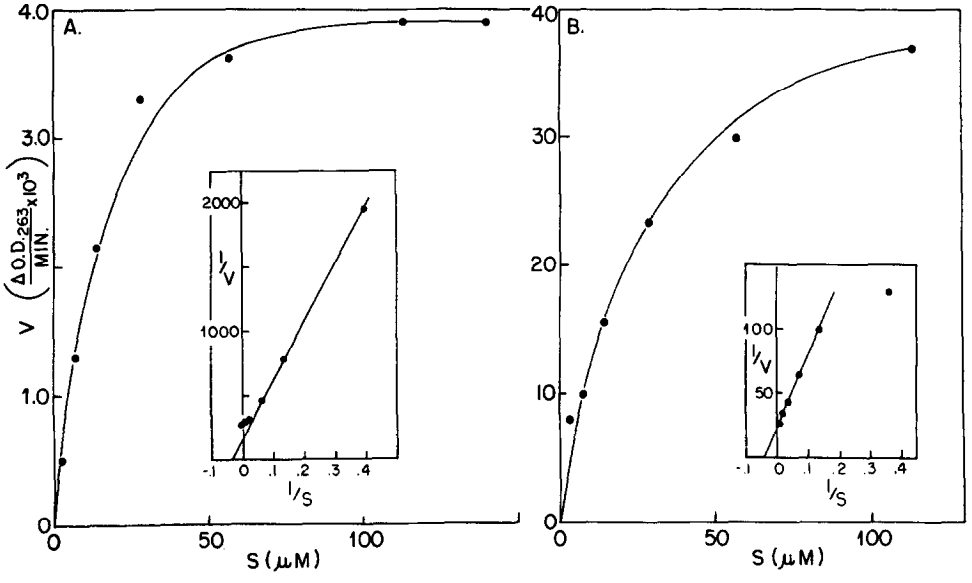

Figure 3.-- Initial velocities of α,β-dehydrase activities of new (cf. Peak I) and previously described (cf. Peak II) enzymes vs. β-hydroxydecanoyl-ACP concentration.

A. New dehydrase, 60 μg of enzyme preparation (described in text), with specified concentrations of β-hydroxydecanoyl-ACP in 0.01 M phosphate buffer, pH 7.2, volume 0.2 ml.

B. Previously described dehydrase, 1.56 μg of enzyme preparation (described in text), other conditions as in A.

Lineweaver-Burke plots of the data for the two enzymes. The K_m of the peak I enzyme for β-hydroxydecanoyl-ACP is 2.8×10^{-5} M (Figure 3-A), and the K_m of the peak II enzyme for this substrate is 2.5×10^{-5} M (Figure 3-B). Thus both of these enzymes have similar affinities which are reasonably high for the ACP substrate.

It is of particular interest that both kinetic and equilibrium studies of the enzyme identified by Bloch's group indicate that it favors the synthesis of the trans-2-decenoyl thioester (Brock et al., 1967), although its functional significance appears to lie in its ability to form a precursor to unsaturated fatty acids. The presence of the dehydrase described in this report in the fatty acid auxotroph which synthesizes normal amounts of saturated, but no unsaturated fatty acids, indicates that this enzyme functions only in saturated fatty acid biosynthesis. Its functional relationship to the dehydrase which produces both cis-3-decenoyl and trans-2-decenoyl thioesters remains to be elucidated.

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