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HYDROXYLATION OF 9-HYDROXYSTEARATE BY A SOLUBLE CYTOCHROME P-450-DEPENDENT FATTY ACID HYDROXYLASE FROM BACILLUS MEGATERIUM

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SUMMARY: A soluble cytochrome P-450 monooxygenase system from Bacillus megaterium ATCC 14581, previously shown to catalyze the monohydroxylation of long-chain unsubstituted fatty acids, has now been found to convert 9-D-hydroxystearate to a mixture of ω-1, ω-2, ω-3 and ω-4 dihydroxystearate isomers. 9-D-Hydroxystearate has a significantly higher affinity than stearate for the enzyme and is a strong competitive inhibitor of palmitate hydroxylation. These results suggest that the enzyme surface has a non-hydrophobic, sterically-permissive binding region between the methyl-group and carboxyl-group binding sites that interacts with polar substituents near the middle of the substrate chain.

Previous publications from this laboratory (1-6) have described a soluble, cytochrome P-450 dependent monooxygenase system from Bacillus megaterium. In the presence of NADPH and oxygen, the enzyme catalyzed the hydroxylation of long-chain unsubstituted fatty acids, amides and alcohols (but not fatty acid methyl esters or alkanes) to a mixture of ω-1, ω-2, and ω-3 monohydroxy isomers. Hydroxylation at the terminal (ω) position was never observed and the relative proportion of the positional isomers formed depended upon both the functional group and the chain length of the substrate. Based on these substrate-affinity studies, we proposed a model for the enzyme-substrate complex that involved the hydrophobic binding and sequestering of the terminal methyl group of the substrate and polar or electrostatic binding of the substrate's functional group at the other terminus (2). The nature of the interaction between the enzyme and the central region of the substrate chain was not investigated but was assumed to be hydrophobic. In the work reported here, however, we present evi-

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dence that a fatty acid such as 9-hydroxystearate with its polar substituent near the middle of the chain has a higher affinity for the enzyme and appears to be a better hydroxylation substrate than the unsubstituted analogs.

MATERIALS AND METHODS

Substrates and cofactors: [U-^{14}C]palmitic acid was purchased from New England Nuclear Corp. and diluted with unlabeled palmitic acid to a specific activity of 4.5 mCi/mmole. Hydroxy fatty acids were obtained from NU-CHEK-PREP, Applied Science Laboratories, Supelco Inc., or P-L Biochemicals at 98 to 99% purity as determined by thin-layer and gas liquid-chromatography. 9-D-Hydroxystearate was the kind gift of Dr. George Schroefer and was found to be greater than 99% purity by TLC and GC/MS analysis. NADPH was obtained from Sigma. All other chemicals were of reagent grade and purchased from commercially available sources.

Enzyme preparation: The growth and harvesting of B. megaterium ATCC 14581 have been described previously (1). The purification procedure for the fatty acid (ω-2) hydroxylase was essentially that of Matson, et al. (5) except that the peak activity fractions from several Sephadex G-200 runs were combined after lyophilization and resuspended in 10 mM phosphate buffer, pH 7.2 to a final protein concentration of 60 mg/ml. The cytochrome P-450 content of this preparation was 0.07-0.09 nmoles/mg protein as determined by the CO-difference spectrum (5); the specific activity was approximately 12.5 nmoles palmitate hydroxylated per min per mg protein. Protein was determined by the Lowry method (7). The enzyme pool was divided into aliquots and stored at -80°C until needed.

Assay for substrate-dependent NADPH oxidation: The rate of NADPH oxidation was determined spectrophotometrically by following the decrease in absorbance at 340 nm utilizing a Beckman model 35 dual-beam recording spectrophotometer. An extinction coefficient of 6.1 mM^{-1} cm^{-1} at 340 nm was used to calculate the number of nmoles of NADPH oxidized. A typical reaction mixture contained: enzyme (100-200 µg protein) NADPH (21.9 nmoles) and substrate (palmitate or hydroxystearate, 0.5-10.0 nmoles) in a final volume of 250 µl buffered at pH 8.0 with 100 mM phosphate buffer. Reactions were started by the addition of the substrate. In these studies as well as subsequent studies utilizing radioactivity, Km's were determined by the Lineweaver-Burke double reciprocal plot and the direct linear plot of Eisenthal and Cornish-Bowden as described by Segal (8). Velocities were corrected for a small, relatively constant substrate-independent NADPH oxidation rate, detectable at a 0.2A full scale deflection.

Assay for palmitate hydroxylation: Incubations were carried out in 15 ml screw-cap culture tubes at 20°C with agitation on a vortex mixer set at a low rpm. The reaction mixture was identical to that described in the previous section, but the order of addition was altered as follows: enzyme was added to the reaction buffer, followed by the addition of substrate and finally NADPH to initiate the reaction. The reactions were terminated after 15 sec by the addition of 10% KOH in water/methanol (1:1 v/v). Isolation and purification of the unreacted substrate and the enzymatically formed hydroxy fatty acids have been described in detail elsewhere (1). Inhibitors, when used, were always added prior to the addition of substrate.

Gas chromatographic/mass-spectral analysis: The enzymatic polar products of 9-D-hydroxystearate were prepared on a large scale using: 3.3 mg enzyme, 500 nmoles hydroxy fatty acid and 600 nmoles NADPH in a final volume of 1.0 ml, buffered with 100 mM phosphate buffer, pH 8.0. The reaction was allowed to

proceed for 1 hr at 20°C with gyrotory agitation (300 rpm). The reaction was terminated by the addition of alcoholic KOH and the polar product methyl esters isolated as described in detail previously (1). For GC/MS analysis, the hydroxy fatty acid methyl esters were converted to trimethylsilyl derivatives by reacting them with TRI-SIL (Pierce Chemical Co., Rockford, Ill.). Analysis was conducted using a Varian Aerograph 1400 GC coupled by a jet glass separator to a Finnigan Gas Chromatograph Peak Identifier (Quadrapole MS). Gas-chromatographic separation was achieved on a 5 ft x 1/8 inch O.D. stainless steel column filled with 3% OV-1 on 60/80 Gas-Chrom Q. Fragmentation patterns and subsequent identifications were conducted on the basis of the interpretations of Kleiman and Spencer (9) for hydroxy fatty acids.

RESULTS

Hydroxylation of 9-D-hydroxystearate: In an effort to extend our present knowledge of the hydroxylase active site model (2), a variety of long-chain monohydroxy fatty acids were screened by the NADPH oxidation assay for activity in the fatty acid (ω-2) hydroxylase system. Although the major products obtained from the enzymatic hydroxylation of saturated long-chain unsubstituted acids (i.e., the ω-1, ω-2, and ω-3 monohydroxy derivatives) did not promote NADPH oxidation, other monohydroxy isomers did stimulate oxidation at rates comparable to those obtained with equivalent concentrations of the unsubstituted analogs. However, because of its established high degree of purity and known optional configuration, the central location of the hydroxy group, and its relatively high NADPH oxidative activity (Fig. 1), 9-D-hydroxystearate was selected for more detailed study. The apparent Km for 9-D-hydroxystearate calculated from the data represented by Fig. 1 was 5.5 μM. Under similar conditions the apparent Km's for palmitate and stearate were 7.0 and 25 μM, respectively. This suggested that, contrary to our expectations, the presence of the 9-hydroxyl group in the 18-carbon chain enhanced substrate binding to the enzyme. It should be noted, however, that the 1:1 stoichiometry between NADPH oxidation and substrate hydroxylation shown for that of palmitate (5) has not yet been rigorously determined for 9-D-hydroxystearate. Thus, a more secure estimation of the relative binding affinity would be to compete 9-D-hydroxystearate against palmitate. As shown in Fig. 2, an increase in the apparent Km for palmitate was demonstrated for [U-^{14}C]palmitate hydroxylation by adding

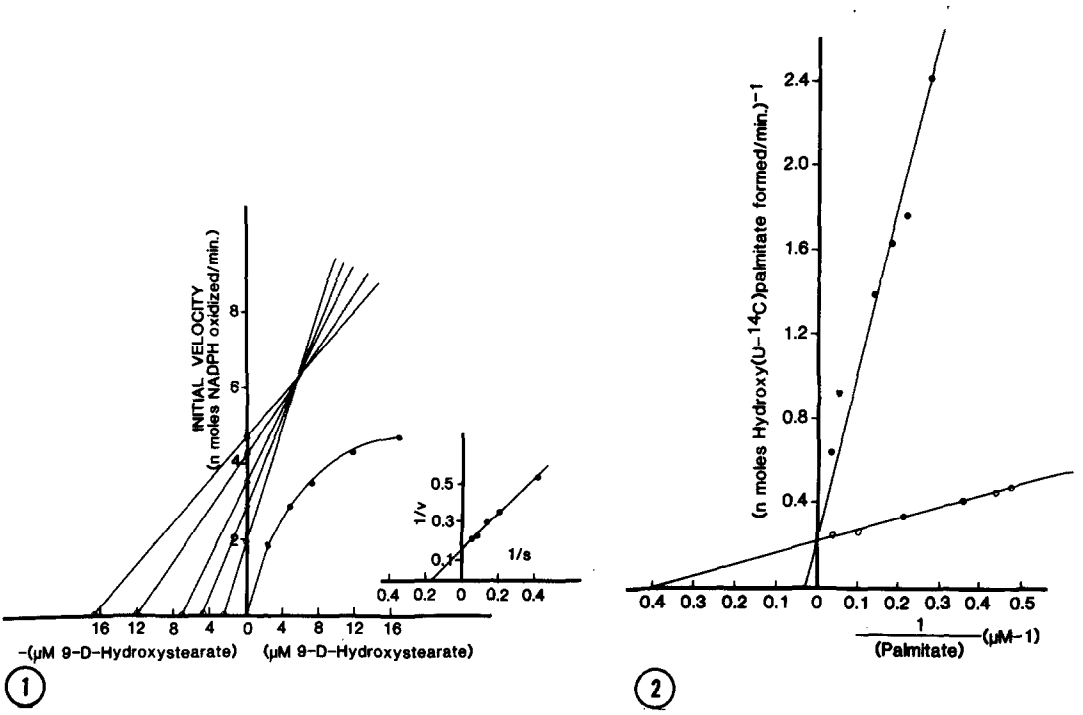

Figure 1: 9-D-Hydroxystearate catalyzed NADPH oxidation: The substrate dependent NADPH oxidation assay conditions and enzyme preparation are described in detail within the Materials and Methods section. Assays were conducted at an enzyme protein concentration of 500 μg/ml. Apparent Km values were determined by the Eisenthal and Cornish-Bowden and Lineweaver-Burke (insert) plots.

Figure 2: Inhibition of palmitate hydroxylation by 9-D-hydroxystearate: The radioactive assay for palmitate hydroxylation as described within the Material and Methods section was utilized. Enzyme protein concentration, 1.0 mg/ml. (●) Inhibitor (9-D-hydroxystearate), 19.2 μM and (o) palmitate concentration, 2.3-28.2 μM.

increasing amounts of 9-D-hydroxystearate. Indeed, hydroxystearate serves as a strong competitive inhibitor of palmitate hydroxylation (Ki 3.1 to 5.4 μM) as measured by this well-established radioactive assay.

Product analysis: Preliminary analysis by thin-layer and gas-liquid chromatographic methods (1,2) of the methyl ester derivatives of the hydroxystearate enzymatic reaction products revealed extensive conversion of the substrate to more polar compounds. Based upon comparison with known standards, the major components corresponded to dihydroxystearates. Subsequent treatment of the product methyl esters with acetic anhydride in pyridine (1) yielded a major

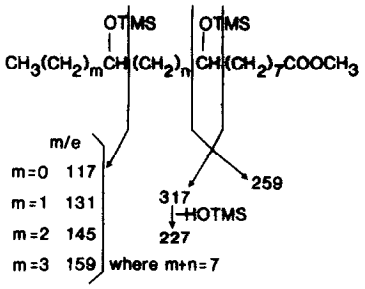

Figure 3: The major mass fragmentation patterns of polar products obtained from enzymatic oxidation of 9-D-hydroxystearate: Arrows indicate points of cleavage for the parent compounds predicted from molecular ion (m/e) fragments that were formed. Details of GC/MS analysis and interpretation are described within the text.

component that behaved like diacetoxystearate methyl ester on both gas-liquid chromatographic columns and thin-layer silica gel plates. GC/MS analysis of the silylated (OTMS) polar product methyl esters are summarized in Fig. 3. The mass fragmentation patterns depicted are characteristic of those found for hydroxy fatty acid derivatives in which primary cleavage points are alpha to the -OTMS position to yield the ions (m/e) observed (9). Thus, m/e's 117, 131, 145, and 159 correspond to ω-1, ω-2, ω-3, and ω-4 dihydroxystearates, respectively. Based upon relative m/e intensities, the ω-3 (m/e 145) product, 9,15-dihydroxystearate occurs in the most abundance. However, determination of the isomer product distribution ratio requires more extensive analysis (2,4) and this observation should be regarded as preliminary.

DISCUSSION

The present study demonstrates that 9-D-hydroxystearate is a strong competitive inhibitor of palmitate hydroxylation, catalyzed by the fatty acid (ω-2) hydroxylase system from B. megaterium. As a substrate, this monohydroxy fatty acid is more active than palmitate or stearate in substrate-dependent NADPH oxidation. The hydroxylation products are similar to those of the unsubstituted long-chain fatty acid substrates in terms of the position for incorporation of the hydroxyl group within the carbon-chain i.e., 9,17 (ω-1)-, 9,16

(ω-2)-, and 9,15(ω-3)-dihydroxystearate. Consistent with earlier observations (1,2,4), hydroxylation at the terminal methyl group was not found, while the major product was 9,15(ω-3)-dihydroxystearate. GC/MS analysis also revealed the presence of the (ω-4)-hydroxylation product, 9,14-dihydroxystearate. Hydroxylations beyond (ω-3) have not previously been reported for the unsubstituted fatty acid substrates, although it is possible that they occur in minor proportion to the ω-1, ω-2, ω-3 isomer products and were not detectable under the conditions employed.

More significant, however, is the high affinity of 9-D-hydroxystearate relative to stearate. The model previously proposed for the fatty acid hydroxylase was based upon the position and relative rates of hydroxylation for long-chain unsubstituted fatty acids and their corresponding amide and alcohol derivatives (2). Important features of this model include two strong substrate-binding areas; a hydrophobic pocket that sequesters the terminal methyl group of the substrate and positions the next three methylene carbons close to the active site and a polar binding site that interacts with the carboxyl, hydroxyl or amide terminus and initiates substrate binding (2,4). The involvement of the substrate's central region in determining effective enzyme-substrate binding was not studied although we assumed that weak hydrophobic interaction occurred. However, the present study shows that the addition of a 9-hydroxy group to stearate actually enhances enzyme-substrate binding and suggests that the enzyme surface has a non-hydrophobic binding region, perhaps involving hydrogen-bond formation, that interacts with polar substituents near the center of the substrate chain. In support of this concept, we have recently tested a variety of 9-substituted stearates (R. Ruettinger and A. Fulco, unpublished) and found that those analogs containing good hydrogen-bonding substituents at this position were much better than stearate as competitive inhibitors of palmitate hydroxylation. On the other hand, 9-D-acetoxystearate was only slightly more effective in this respect while 9-D-bromostearate was about as effective as stearate itself. Although we have not yet investigated the effects

on enzyme-substrate affinity of relatively large substituents at the 9-position of stearate, the present results indicate that the steric requirements in this region are not rigorous.

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