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HYDROXYSTEARATES AS INHIBITORS OF PALMITATE HYDROXYLATION CATALYZED BY THE CYTOCHROME P-450 MONOOXYGENASE FROM BACILLUS MEGATERIUM

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SUMMARY: The soluble, cytochrome P-450 dependent fatty acid (ω-2) hydroxylase from Bacillus megaterium catalyzes the hydroxylation of both n-saturated and n-monohydroxyfatty acids. Continued hydroxylation of hydroxyfatty acids is dependent upon the position of the hydroxyl group since the ω-1, ω-2 and ω-3 monohydroxy products of the unsubstituted, saturated fatty acid series are not substrates. Utilizing a series of monohydroxystearate positional isomers this study demonstrates that there exists an optimal hydroxy position on the substrate's carbon chain. Competitive inhibition of palmitate hydroxylation by monohydroxystearates indicates that 6-hydroxystearate is a better substrate than palmitate, one of the more active substrates for hydroxylation. This suggests that substrate-binding at the active site is strongly influenced by a "non-hydrophobic" binding region on the enzyme.

Previous publications from this laboratory (1-8) 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). Recently,the involvement of the substrate's central

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region in determining effective enzyme-substrate binding was investigated. A fatty acid such as 9-hydroxystearate with its polar substituent near the middle of the chain was found to have a higher affinity for the enzyme and to be a better hydroxylation substrate than the unsubstituted analogs (7). This suggests that the enzyme active site has a non-hydrophobic binding region which, perhaps through hydrogen-bond formation, interacts with polar substituents near the center of the substrate chain. In the present study, additional monohydroxystearate positional isomers are utilized in an effort to more clearly define the nature of this interaction.

MATERIALS AND METHODS

Substrates and cofactors: [U-^{14}C]palmitic acid was purchased from New England Nuclear Corp. at 99% radiopurity and diluted with unlabeled palmitic acid to a specific activity of 4.5 mCi/mmole. Hydroxyfatty 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 Schroepfer and was found to be greater than 99% pure 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 conditions for the growth and harvesting of B. megaterium ATCC 14581 have been described in detail 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 column purifications were combined after lyophilization and resuspended in 10 mM phosphate buffer, pH 7.2 to yield an enzyme pool adjusted to a final protein concentration of 60 mg/ml. The cytochorme P-450 content of this preparation was approximately 0.1 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 (9). The enzyme pool was divided into aliquots and stored at -80°C until needed for the experiments described.

Assay for palmitate hydroxylation: The hydroxylation of [U-^{14}C]palmitate was determined by a method described in detail elsewhere (1). A typical reaction mixture contained: enzyme (100-200 µg protein), NADPH (21.9 nmoles), substrate (palmitate, 2.3 - 28.2 µM) and inhibitor (monohydroxystearate, 19.2 µM) in a final volume of 250 µl buffered at pH 8.0 with 100 mM phosphate buffer. Incubations were carried out in 15 ml screwcap culture tubes at 20°C with agitation on a vortex mixture set at a low rpm. The order of addition was as follows: enzyme was added to the reaction buffer, followed by the addition of substrate and finally NADPH to initiate the reaction. Inhibitors, when used, were always added prior to the addition of substrate. 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 hydroxyfatty acids have been described elsewhere (1-2). Apparent Km's for palmitate hydroxylation were determined by the ^{V} vs. $^{V}/_{S}$ re-plot of the Lineweaver-Burke double reciprocal plot and subjected to linear regression

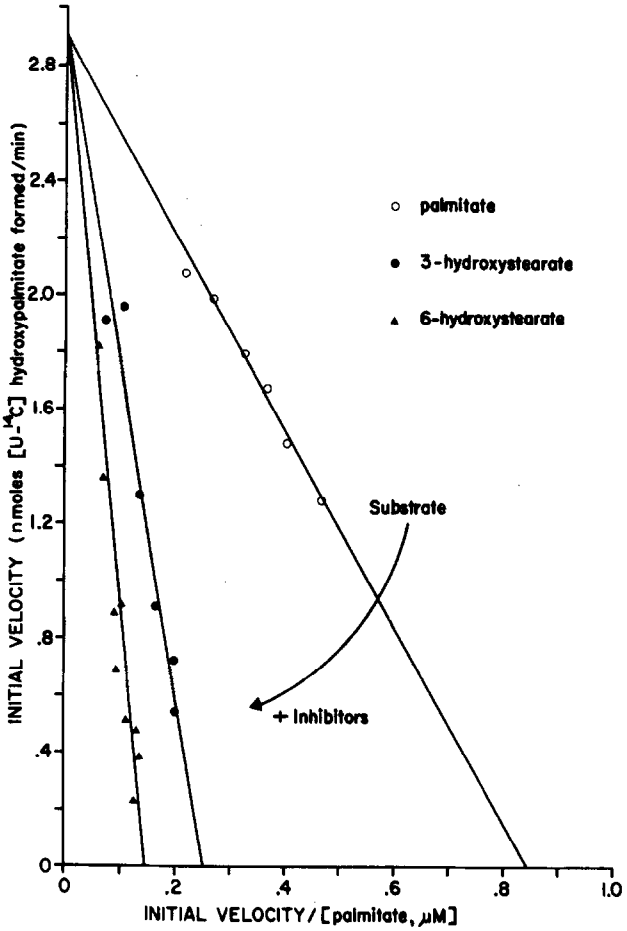

Fig. 1. Monohydroxystearates as inhibitors of palmitate hydroxylation.

analysis. The increase in apparent Km in the presence of an inhibitor was taken as a measure of inhibitor strength for the series of monohydroxystearates studied.

RESULTS AND DISCUSSION

In a previous study (7) it was shown that 9-D-hydroxystearate was a better substrate than stearate in the substrate-catalyzed oxidation of NADPH, as well as being a strong competitive inhibitor of palmitate hydroxylation. Subsequently, a number of additional monohydroxyfatty acid positional isomers were tested in competition with palmitate and the extent of palmitate hydroxylation determined by the well-established radioactive assay (1-7). In Fig. 1, the results of individual competititons for 3- and 6-hydroxystearates with

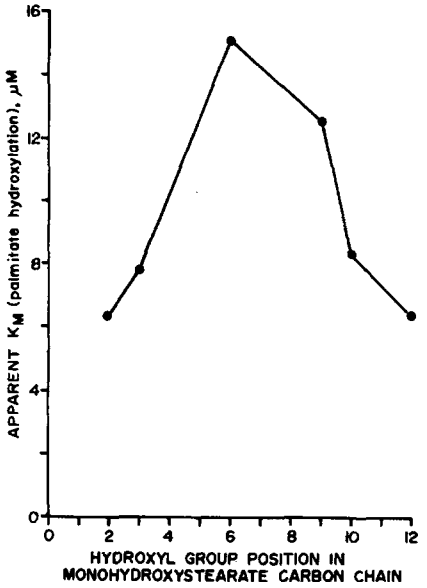

Fig. 2. Change in the apparent Km for palmitate hydroxylation in the presence of hydroxystearate positional isomers.

palmitate are presented as the ^{v} vs $^{v}/s$ re-plot of the Lineweaver-Burke equation. As in the case of 9-hydroxystearate (7), these monohydroxystearates increased the apparent Km for palmitate hydroxylation but did not alter the Vmax and, therefore, can be regarded as competitive (substrate) inhibitors. However, the degree of inhibition appears to be a function of the hydroxyl group position for the monohydroxystearates. As shown in Fig. 2, optimal inhibition, defined in terms of an increased apparent Km for palmitate hydroxylation, is obtained using 6-hydroxystearate. Under the conditions employed, the Km for palmitate in the absence of an inhibitor was approximately 3.5 μM. When unlabeled palmitate (19.2 μM) was utilized as a competitive inhibitor of radiolabeled palmitate hydroxylation (substrate palmitate, 2.3-28.2 μM) the apparent Km for labeled palmitate increased to 12.6 μM. Under identical conditions, the addition of unlabeled stearate increased the Km to 8.6 μM. Thus, 6-hydroxystearate (Km, palmitate = 15.2 μM) would appear to be a better substrate than palmitate or stearate. In the absence of palmitate, 3- and 6-hydroxystearates were hydroxylated predominately to the ω-1, ω-2, ω-3 and ω-4

dihydroxystearate positional isomers as determined by GC/MS analysis (7) of their trimethylsilyl derivatives (R. Matson and R. Stein, unpublished observation). Finally, in agreement with our previous study, 9-hydroxystearate and palmitate have similar substrate reactivities (7). These results suggest that the positioning of a polar functional group, such as the hydroxy moiety, along the fatty acid substrate carbon-chain is an important determinate of effective substrate binding. Furthermore, based upon these inhibitor studies with hydroxystearate positional isomers, there would appear to be an optimal substrate conformation which is strongly influenced by a non-hydrophobic binding region at the enzyme's active site. From our previous study (7) this most likely involves hydrogen-bond formation. However, more detailed studies will be required to delineate the importance and characteristics of these binding sites.

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