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STRUCTURE DEPENDENT REACTIVITY IN THE OXYGENATION OF THIANE ANALOGS BY A CYTOCHROME P-450 RECONSTITUTED ENZYME SYSTEM

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Thiane analogs were oxidized to the corresponding sulfoxides by the reconstituted system of purified rabbit liver microsomal cytochrome P-450 - NADPH-cytochrome P-450 reductase. The stereochemical results and kinetic behaviors were discussed in comparison with those of the nonenzymatic oxidations of the thiane analogs by $NaIO_4$. The enzymatic oxygenation of thianes was found to be nonstereospecific, however, the rates of the enzymatic oxygenation was found to be effected by the hydrophobicity of the substrate.

During the past decade, the cytochrome P-450 enzyme has attracted extensive attentions as a membrane-bound mixed function oxygenase for hydroxylation of a wide variety of substrates such as steroids, hydrocarbons and aromatic compounds[1]. Since oxygenation of divalent sulfur compounds occurs by direct introduction of oxygen atom to sulfur, there should be less mechanistic complexity in the enzymatic oxygenation, than in the oxygenation of hydrocarbons by the cytochrome P-450 enzyme[2]. Thus we studied the oxygenation of cyclic sulfides by the cytochrome P-450 enzyme system and suggested that the reactivities of the substrates appeared to depend upon both hydrophobicities and molecular geometories of substrates[3]. To extend our study along this line, we now have studied the structure-reactivity relationship of the enzymatic oxygenation of 2- and 2,6-substituted thianes in comparison with the nonenzymatic oxidation by $NaIO_4$, and found that there is no special stereospecificity of the substrate but the rate of the oxygenation depends on the hydrofobicity of the thiane derivative. Thiane, 1, 2-methylthiane, 2, 2,6-dimethylthiane, 3 (the ratio of stereoisomers = 1 : 1.4), 2-ethylthiane, 4, 2-n-octylthiane, 5 and 2-methoxythiane, 6 were synthesized and incubated with the reconstituted system of purified phenobarbital induced rabbit liver microsomal cytochrome P-450 - NADPH-cytochrome P-450 reductase at room temperature.

Each of the substrates (60 μmole) was incubated with a mixture of cytochrome P-450 (4.6 nmole)[4], NADPH-cytochrome P-450 reductase (ca. 0.3 unit/ml)[5], lipid fraction (1.5 mg) and NADPH generating system (10 μmole of $NADP^+$, 100 μmole of glucose-6-phosphate, 50 μmole of $MgCl_2$ and 12 units of glucose-6-phosphate dehydrogenase) for 30 min at 36°C in 0.1 M potassium phosphate buffer (pH 7.25, total volume 10 ml). Extraction with dichloromethane followed by separation with column chromatography (200 mesh alumina,

ethyl acetate/chloroform/2-propanol ; 20:20:1) gave the corresponding sulfoxides which were identified by comparing their IR and MS spectra with those of the authentic samples. The yield of sulfoxide ranged from ca. 10 % to 30 % with a preference for the substrate bearing a large substituent. When a 2-substituted thiane is oxidized to the corresponding sulfoxides, formation of two possible isomers, i.e., cis-and trance-thiane sulfoxides, is expected. Indeed, products obtained from 2, 4, 5 and 6 gave two peaks of the stereoisomeric sulfoxides. Ratios of the stereoisomers of the oxidized products of all the 2-substituted thianes by cytochrome P-450 were found to be very similar to those of the nonenzymatic oxidation by $NaIO_4$ which is known as a mild oxidant of sulfide to sulfoxide[6]. A typical example is shown in Fig. 1. Even the gas chromatogram of the stereoisomeric mixture of the enzymatic oxidation product of 3 is very similar to that obtained from the nonenzymatic oxidation, revealing that there is no special stereoselectivity in the oxygenation of thiane derivatives by the cytochrome P-450 enzyme system.

In order to examine the effect of substituent on the rate of oxygenation of the thiane, the rates of NADPH oxidation were measured by following the decrease of UV absorption at 340 nm[3]. Woolf's plots were found to fall on a straight line in all cases from which the Michaelis constants and the V_{max}-values were computed by the least square method (Table 1). Since the rates of oxygenation of cyclic sulfides are known to parallel to those of the oxidation of NADPH[3] under the same condition, relative reactivities of the substituted thianes can be estimated from the data shown in Table 1. Inspection of the data in Table 1 reveals that there is no structural effect on V_{max} except 5 and 6, however, the K_m-value decreases with the increase of the bulkiness of alkyl substituent on thiane, implying that the oxygenation of the thiane is controlled by the binding ability of the enzyme and depends on

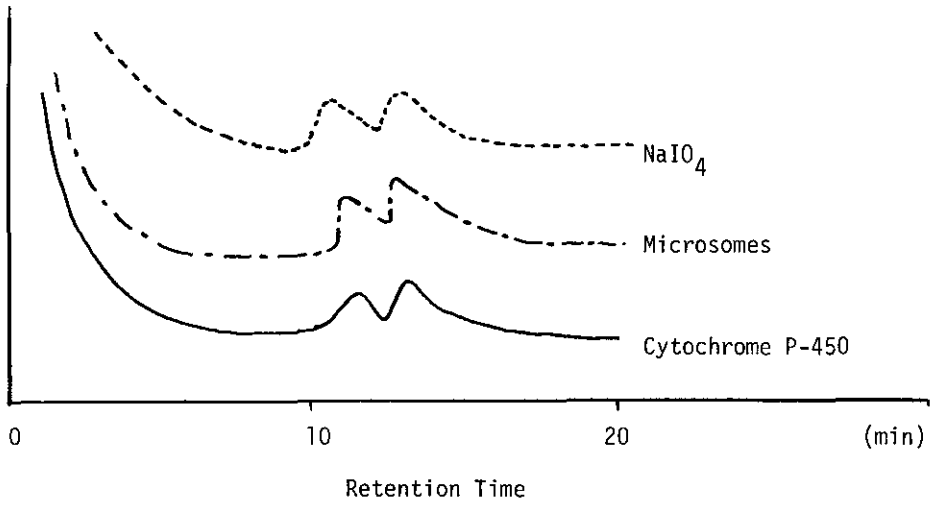
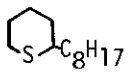
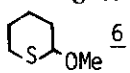


Fig. 1. Gas Chromatogram of the Products of Oxidations of 2-Methylthiane, 2 , by both Biological and Nonbiological Systems

Column : 2m glass column (3 mm) packed with 5 % OV-1 on 60 mesh Celite
Career : 0.8 Kg/cm^2 He
Temperature : programed from 100°C to 200°C of rate 10°C/min

the hydrophobicity of the substrate. In order to support this argument further, the apparent dissociation constants (K_s) between the substrates and cytochrome P-450 in the oxidized state were determined from the dependencies of difference spectra on the concentrations of substrates in the usual manner[3]. An introduction of any substituent on 2- and 2,6-position of thiane was found earlier to change the binding mode of thiane to the enzyme from type II to type I[3]. As was expected, the order of K_s-value is well consistent with that of K_m-values in this case. On the other hand, the Hansch's π-values of alkyl groups may be applied to evaluate the relative hydrophobicities of the substituted thianes[7]. Thus Σ π-values of alkyl

Table 1. Comparison of Kinetics of Oxidation of NADPH in the Reconstituted Pure P-450 Enzyme System with Apparent Dissociation Constants (K_s) between Substrates and Cytochrome P-450 in the Oxidized State, and Hydrophobicities of the Substrates

Substrate	K_m (mM)[a]	V_{max} (mM/min)[a]	K_s (mM)[b]	ΔA_{max}[c]	Binding Type	Hydrophobicity ($\Sigma\pi$)[d]
 1	0.78	0.012	1.17	0.036	II	0
 2	0.33	0.012	0.25	0.019	I	0.5
 3	0.11	0.0083	0.16	0.022	I	1.0
 4	0.11	0.011	0.11	0.038	I	0.97
 5	0.18	0.046	0.097	0.070	I	2
 6	2.11	0.037	0.21	0.015	I	-0.47

a) Kinetic conditions : The reconstituted system (0.55 nmole cytochrome P-450, 0.1 unit/ml of the reductase and 1.5 mg of lipid fraction) was allowed to stand at room temperature for 5 min in UV cavette, and then was diluted by 0.1 M potassium phosphate buffer (pH 7.25) to 2.5 ml. After 10 min standing, 0.36 µl of NADPH and a methanol solution of substrate (1-12 µl) were added into the UV cavette to initiate the reaction. b) Assay condition : Both sample and reference UV cavettes were filled with 0.1 M Tris-HCl buffer, pH 7.4 (2 ml) containing 0.45 µM cytochrome P-450. Then 1-15 µl of 0.2 M methanol solution of substrates was added into the sample cavette. c) Difference of absorbance at $[substrate]_\infty$. d) Hansch's π-values of alkyl substituents of the thiane ring (Ref. 7)

substituents were compared with both K_m-values and K_s-values (Table 1) and the K_m-values are found to be well correlated with the hydrophobicities of the substrates. In contrast to the same stereochemical behavior of the cytochrome P-450 and that with $NaIO_4$ in the oxidation of thianes, the opposite strucural effect has been found in the rates of these reactions, i. e., the order of the rates of nonenzymatic oxidations of thianes by $NaIO_4$ was found to be $\underline{1} > \underline{2} > \underline{3} \approx \underline{4} > \underline{6}$, which is obviously controlled by the steric effect of the substituents. The exceptional behavior of 6 would be due to the electronic effect of oxygen atom of methoxy group.

In summary, the lack of stereoselectivity suggests that the binding of the substrate to the cytochrome P-450 is not sterically specific. However, only the hydrophobic property of alkyl group plays an important role in the binding of the substrate to the active site of the cytochrome P-450 system. Such a preferential hydrophobic interaction increases the value of V_{max}, as has been reported by Imai, Sato and Iyanagi[8]. In the case of 5, the extent of increase of V_{max} is proportional to that of ΔA_{max}. All these observations indicate that the effect of the hydrophobicity of substrate on V_{max} is correlated to both K_s and ΔA_{max}. A more detailed kinetic analysis of the oxygenation of a series of substrates will give a useful information about the transfer of the active oxygen bound to the cytochrome P-450 to the substrate.

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