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STRUCTURAL EFFECTS ON THE REACTIVITY OF SUBSTRATES AND INHIBITORS IN THE EPOXIDATION SYSTEM OF *PSEUDOMONAS OLEOVORANS*

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Summary

The epoxidation reaction catalyzed by an enzyme system of *Pseudomonas oleovorans* exhibits a substrate specificity different from that expected on the basis of chemical reactivity in non-enzymatic epoxidation reactions. Cyclic and internal olefins, aromatic compounds and styrene are not epoxidated. The reactivity of straight chain diolefins is maximal for octadiene and falls off rapidly as the carbon chain is shortened, but decreases only slightly as the chain is lengthened. In contrast, methyl group hydroxylation is less sensitive to decreasing chain length. As a consequence, propylene and 1-butene are hydroxylated but not epoxidated by this enzyme system. With the substrate 1-decene, which is capable of undergoing both epoxidation and hydroxylation, the former reaction predominates. Methyl imidoesters were found to be inhibitors of enzymatic epoxidation, and the potency of a homologous series of imidoester inhibitors was examined. The results parallel the substrate specificity patterns observed, and support the conclusion that the mode of substrate binding severely moderates the inherent chemical reactivity of the activated oxygen in this system. The effect of the bifunctional imidoester, dimethyladipimidate, was also examined and the results compared with those obtained in other investigations.

Introduction

Enzymatic epoxidation reactions are key steps in a variety of biological processes, among which are the metabolism of hydrocarbons [1—3], the bio-

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synthesis of steroids from squalene [4], and the metabolism and interconversion of steroid derivatives [5—7]. In addition, it has been recognized that liver microsomal hydroxylations of aromatic compounds (key steps in the metabolism of amino acids, drugs and vitamins and in the mechanisms of carcinogenicity, mutagenicity and cytotoxicity of many substrates) are probably all actually expoxidation reactions [8,9]. A definition of the molecular basis for and reactivity patterns of such reactions would thus be extremely useful in the design of new drugs and the development of model enzymes systems.

In previous work, we have established that an enzyme system of *Pseudomonas oleovorans* catalyzes the epoxidation of terminal olefins [10—16,25,26] in addition to the previously known methyl group hydroxylation of alkanes and fatty acids [17—24]. We now wish to report that this enzymatic epoxidation reaction exhibits a substrate specificity far different from that expected on the basis of chemical reactivity in non-enzymatic epoxidation reactions. Furthermore, this specificity is reflected in the potency of a homologous series of methyl imidoesters as inhibitors of the epoxidation reaction. When considered together with our recent discovery of the high stereoselectivity exhibited by the enzymatic reaction [15], these findings provide valuable insight into the relative roles of substrate binding and chemical reactivity in this system.

Materials and Methods

Hydrocarbon substrates and organic alcohols were obtained from various sources and were of the highest grade commercially available. Epoxide standards were either purchased or synthesized from *m*-chloroperbenzoic acid and the appropriate olefin [11]. Imidoesters were synthesized from the correspond-

TABLE I

IMIDOESTER STRUCTURES

$CH_3-(CH_2)_n-\underset{\underset{NH}{\|}}{C}-OCH_3$

Imidoester	Value of n	Abbreviation Imidoester	M.P. (°C)
Methyl acetimidate	0	2	83.1—89 (d)
Methyl propanoimidate	1	3	85.9—86.5
Methyl butanoimidate	2	4	79.2—81.5
Methyl pentanoimidate	3	5	76.8—78.0
Methyl hexanoimidate	4	6	99.5—100.2
Methyl octanoimidate	6	8	93.2—93.8
Methyl decanoimidate	8	10	96.5—97.0
Methyl dodecanoimidate	10	12	101.8—102.0
Methyl tridecanoimidate	11	13	95.4—96.0
Methyl tetradecanoimidate	12	14	95.4—96.0
Methyl pentadecanoimidate	13	15	88.9—89.8
Methyl octadecanoimidate	16	18	95.3—96.0
Dimethyl adipimidate	$CH_3O-\underset{\underset{NH}{\|}}{C}-(CH_2)_4-\underset{\underset{NH}{\|}}{C}-OCH_3$	—	219.5—222.5

ing nitriles and methanolic HCl via the Pinner synthesis [27]. Table I lists the structures of the imidoesters used in this study, their melting points, and the abbreviations used in the text. Typical analytical data are as follows. For imidoester 8; calculated: C, 56.02; H, 9.85; N, 7.26. Found: C, 55.56; H, 10.42; N, 7.39. For imidoester 10: calculated: C, 57.85; H, 10.50; N, 6.34. Found: C, 57.93; H, 11.10; N, 6.71.

P. oleovorans TF4-1L was used throughout the experiments. The organism, media, growth conditions, standard resting cell preparation, and epoxide assay have been described [25]. All cultures were grown on *n*-octane.

Preparation of crude enzyme extracts

All procedures were conducted at 4°C in 0.1 M phosphate buffer (pH 7) to which Triton X-100 had been added (0.05%). Following growth, the cell suspension was washed once and the cell pellet was resuspended to an absorbance of 5.2 at 660 nm. 30-ml volumes were subjected to the full output of a Megasonic ultrasonic disintegrator (three bursts, 1.5 min each) and the sonicate was clarified at 48 200 × *g* for 30 min. The turbid supernatant solution represented the crude enzyme extract and contained about 15 mg protein/ml, as determined by the Lowry method. Microscopic examination and spread plates using nutrient agar indicated that the extracts were cell-free.

Samples of rubredoxin and partially purified reductase and ω-hydroxylase were kindly supplied by Drs M.J. Coon and R.F. Boyer.

Epoxidation assays

Reaction conditions for experiments with purified enzyme preparations and our procedures for the extraction, identification and quantitation of epoxide products have been described previously [10,11].

With crude enzyme extracts, the following standard assay procedure was used. To a 16 × 150 ml test tube was added 5 ml enzyme extract, 5 mg NADH and 0.05 ml of 1,7-octadiene. The suspension was mixed on a Vortex mixer and incubated at 30°C with shaking. At the time indicated, 0.5 ml samples were removed and assayed for 7,8-epoxy-1-octene. Control experiments indicated that although the crude extracts already contained significant amounts of NADH, the addition of 1 mg NADH/ml was required for maximal activity.

Imidoester addition

Imidoesters were added to crude enzyme extracts at room temperature. During the addition, the control was also kept at room temperature. All imidoesters were added as the hydrochloride salt to achieve a final concentration of $4.1 \cdot 10^{-5}$ mol/ml. Addition of the imidoesters caused a decrease in pH to approx. 6. The pH was immediately readjusted to 7 with 1 M NaOH. Imidoester addition and pH adjustment was done as rapidly as possible.

In many experiments imidoesters were added to the complete reaction mixture after establishing an initial epoxidation rate. In order to eliminate the possibility that the temporary decrease in pH following imidoester addition caused decomposition of NADH, the order of addition of reagents was reversed, and the extent of inhibition compared. No difference in epoxide production was observed whether NADH and octadiene were added before or after

the imidoester, as long as the pH readjustment was done rapidly.

The effect of imidoesters on whole cells was tested as follows: to standard resting cell preparations [14,25] at room temperature was added either dimethyl adipimidate (5 mg/ml) or methyl hexanoimidate (10 mg/ml) and the pH was adjusted to 7. Immediately after the addition (time-zero) a sample was taken from each tube for titering and epoxidase assay, and the tubes placed in a 30°C shaking waterbath. Titers were estimated on nutrient agar by the spread plate method. Epoxidase activity was assayed after adding 1,7-octadiene (final concentration 1%) to the sample and incubating for 1 h at 30°C. Titering and epoxidase activity assays were repeated after the cells had been exposed to the imidoesters for 1 h and 2 h.

Both 1-butene and a mixture of 1% propylene/99% air were obtained from Matheson. In the case of 1-butene, a cold, stirring solution of 0.05 M Tris buffer (pH 7.5) was saturated with the gas, and an aliquot of this mixture was added to the enzymes in the usual manner. In the case of propylene, the propylene/air mixture was bubbled directly into the solution of enzymes and NADH. In both cases the aqueous reaction mixtures were analyzed directly by gas chromatography on a Porapak Q column (6 ft × 0.25 inch) maintained isothermally at 210°C.

Oxygen stoichiometry experiments were carried out using a YSI Model 53 Biological Oxygen Monitor. The instrument was calibrated before use by measuring the decrease in oxygen tension which accompanied the complete oxidation of a known concentration of protocatechuic acid by protocatechuate-3,4-dioxygenase. The enzyme used for this calibration was kindly supplied by Dr C.T. Hou.

Results

Relative rates of epoxidation and hydroxylation

We have previously reported that on the basis of cofactor oxidation rates, octadiene is more reactive than either octane or octene [10,11]. Since octadiene does not possess a hydroxylatable terminal methyl group, this finding suggests that with straight-chain substrates of moderate length, epoxidation

TABLE II

RELATIVE RATES OF EPOXIDATION AND HYDROXYLATION FOR 1-DECENE

(a) These values are corrected for the endogeneous oxidation of NADH (see refs 11 and 20). (b) Reaction conditions at 25°C as follows: To 1 ml of 0.05 M Tris (pH 7.5) were added 100 μg "hydroxylase", 3.5 μg reductase, and 0.18 mg rubredoxin; 1.06 μmol substrate in 20 μl acetone were added and the reaction was then initiated with 0.2 μmol NADH. (c) Reaction conditions as for (b) except that the reaction mixture contained 500 μg "hydroxylase" and 7 μg reductase. (d) Products were determined by quantitative gas chromatography. Peaks corresponding to aldehydes or other further oxidation products were not observed.

Experiment	Reaction time (s)	NADH oxidized (nMol) (a)	Expoxide formed (nMol)	Alcohol formed (nMol)	Total product (nMol) (d)
A (b)	1450	22	13	7	21
B (c)	470	52	39	12	51

proceeds more rapidily than hydroxylation. However, since it is conceivable that octane, octene and octadiene bind to the enzyme with vastly different affinities, it was of interest to compare the relative rates of epoxidation and hydroxylation with a single substrate capable of undergoing both reactions.

The results obtained when 1-decene was incubated with the ω-hydroxylation system are shown in Table II. Two separate experiments were carried out under different reaction conditions and in both cases substantially more 1,2-epoxydecane than 9-decene-1-ol was formed. In both experiments, an excellent agreement was obtained between the total amount of NADH oxidized and the total amount of product formed. This indicates that under the reaction conditions, no significant enzymatic oxidation of 1,2-epoxydecane or 9-decene-1-ol occurs, and, therefore, the product distribution is a reliable indication of the relative rates of the epoxidation and hydroxylation reactions.

Effect of substrate structure

Table III shows the effect of varying the carbon chain length on the epoxidation of a series of dienes, and the relative rates of cofactor oxidation for an analogous series of alkanes [19]. It is evident that for both epoxidation and hydroxylation maximal activity is observed at a carbon chain length of C_8. In the case of epoxidation, as the chain length is increased, the relative rate of reaction decreases somewhat, but dodecadiene still has 85% of the reactivity of octadiene. On the other hand, when the carbon chain length is decreased by only two carbon atoms, to hexadiene, the relative rate of reaction falls off much more rapidly. In contrast, for the hydroxylation reaction, increasing the carbon chain length has a much more drastic effect on the relative rate of reaction but decreasing the carbon chain to hexane results in only a 25% decrease in reactivity.

If this trend were continued to even shorter substrates, one would expect to reach a point where only the hydroxylation reaction proceeds at a significant rate. As shown in Table IV, this is indeed the case, and both propylene

TABLE III

(a) Reaction conditions as for Table IV, ref. 11. (b) A relative rate of 100 corresponds to the net oxidation of 6.6 nmol of NADH per min. (c) At low concentrations, the peak corresponding to 5,6-epoxy-1-hexene is obscured by the large peak from the hexane used in the extraction procedure (see ref. 11).

Effect of carbon length on epoxidation (a)			Effect of chain length on hydroxylation (data from ref. 19)	
Substrate	Relative rate of NADH oxidation (340 nm) (b)	Product detected by gas chromatography	Substrate	Relative rate of cofactor oxidation
1,5-Hexadiene	40	(c)		
1,6-Heptadiene	67	6,7-Epoxy-1-heptene	Hexane	75
1,7-Octadiene	100	7,8-Epoxy-1-octene	Heptane	87
1,8-Nonadiene	87	8,9-Epoxy-1-nonene	Octane	100
1,9-Decadiene	92	9,10-Epoxy-1-decene	Nonane	99
1,10-Undecadiene	80	10,11-Epoxy-1-undecene	Decane	80
1,11-Dodecadiene	85	11,12-Epoxy-1-dodecene	Dodecane	47
			Hexadecane	3

TABLE IV

SUBSTRATE SPECIFICITY

Standard reaction conditions were used except that gaseous substrates were introduced as described in the Materials and Methods section.

Substrate	Product detected by gas chromatography
Propylene	Allyl alcohol
1-Butene	3-Buten-1-ol
Cyclohexene	2-Cyclohexene-1-ol
	3-Cyclohexene-1-ol
Cyclohexane	Cyclohexanol
cis-5-Decene	*cis*-5-Decen-1-ol
Styrene	—

and 1-butene are hydroxylated to the corresponding unsaturated alcohols, but not epoxidated. Coon and coworkers have reported that whereas internal methylene groups of straight-chain substrates are inert to hydroxylation, cyclohexane stimulates the rate of cofactor oxidation by the ω-hydroxylation system [20]. As shown in Table IV, we find that the internal double bonds of both straight-chain and cyclic compounds are inert to epoxidation. Cyclohexane is converted to both isomeric cyclohexene-ols, which are formed in comparable amounts, but not to epoxycyclohexane. Similarly, neither *cis*-5-decene nor 2-octene are epoxidated. Styrene is not epoxidated at the double bond or the aromatic ring, and Coon and coworkers have reported that several

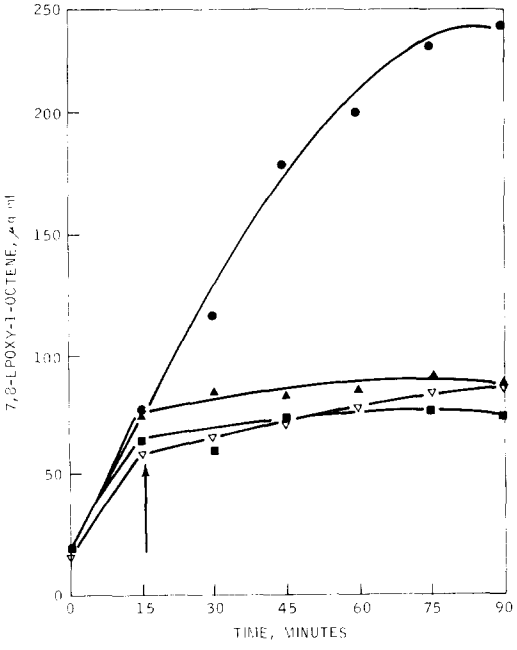

Fig. 1. The effect of imidoester 6, imidoester 8, and imidoester 12 on the epoxidation of 1,7-octadiene by crude enzyme extracts of *P. oleovorans* TF4-IL. Samples were assayed for 7,8-epoxy-1-octene at 15-min intervals. Imidoesters were added after taking the 15-min sample. Control (●); imidoester 6 (▽); imidoester 8 (■); imidoester 12 (▲); imidoester addition (↑).

other aromatic compounds do not stimulate cofactor oxidation [20]. As expected, gas chromatographic analysis revealed that cyclohexanol is formed from cyclohexane.

Inhibition experiments with imidoesters

We have previously suggested that the critical specificity requirement for substrate binding in the *P. oleovorans* enzyme system may be the configuration of the methylene chain of the hydrocarbon [11]. Since it seemed likely that this factor was being reflected in the unusual specificity patterns we observed, it was of interest to us to examine a homologous series of inhibitors in order to determine whether such factors also affect inhibitor binding. Accordingly, a search was initiated for effective inhibitors of the epoxidation reaction. Crude enzyme extracts were used in these studies, since the very high rate of epoxidation with such extracts allows accurate estimation of the relative potency of inhibitory compounds.

Preliminary studies indicated that addition of imidoester 6 at a concentration of $4.1 \cdot 10^{-5}$ M to the standard epoxidation assay mixture (Experimental section) reduced the amount of 7,8-epoxy-1-octene formed after 30 min from 222 mg/ml to 13 mg/ml. Accordingly, a homologous series of imidoester inhibitors were synthesized and their effect on the epoxidation reaction measured. The results of typical inhibition experiments with imidoester 6, imidoester 8 and imidoester 12 are shown in Fig. 1, and it is apparent that rapid inhibition is observed after addition of the imidoester. Table V lists results obtained in similar experiments with imidoesters of varying carbon chain length. The extent of inhibition observed decreases markedly with imidoesters having carbon chains shorter than 6 or longer than 14.

Since it was recognized that imidoesters decompose in neutral solution, methyl hexanoate, the decomposition product of imidoester 6, was tested as an epoxidation inhibitor, and found to inhibit epoxide formation by 36% relative to the control under standard assay conditions. This compares with 85% inhi-

TABLE V

IMIDOESTER INHIBITION OF EPOXIDATION BY CRUDE ENZYME EXTRACT

Percent inhibition is defined as $((C - I)/C) \times 100$, where C and I are the total amount of epoxide synthesized after 60 min in the absence and presence of imidoester, respectively. The standard assay conditions were used as described in the experimental section.

Imidoester	(%) Inhibition
Imidoester 2	3
Imidoester 3	7
Imidoester 4	47
Imidoester 5	47
Imidoester 6	85
Imidoester 8	94
Imidoester 10	83
Imidoester 12	91
Imidoester 13	89
Imidoester 14	93
Imidoester 15	36
Imidoester 18	22

TABLE VI

THE EFFECT OF DIMETHYL ADIPAMATE AND IMIDOESTER 6 ON VIABILITY AND EPOXIDATION ACTIVITY OF *P. OLEOVORANS* STANDARD RESTING CELL PREPARATIONS

Sampling Time (h)	Control [a]		Dimethyl adipamate		Imidoester 6	
	Titer [b]	Epoxide [c]	Titer	Epoxide	Titer	Epoxide
0	$3.3 \cdot 10^9$	388	$3.0 \cdot 10^9$	406	$<10^3$	0
1	$3.0 \cdot 10^9$	393	$3.6 \cdot 10^9$	328	$<10^3$	0
2	$4.0 \cdot 10^9$	430	$4.1 \cdot 10^9$	354	$<10^3$	0

[a] No imidoester present.
[b] Viable cells/ml.
[c] μg 7,8-epoxy-1-octene/ml produced after 1 h at 30° C.

bition obtained with imidoester 6 (Table V), indicating that the presence of the imidoester functionality is responsible for considerable inhibitory potency. Apparently, this additional inhibition is not simply a reflection of the positive charge on the imidoester functionality, since we find that hexyl trimethylammonium bromide inhibits epoxidation by only 37% under standard assay conditions.

Table VI compares the effect of imidoester 6 and its bifunctional analog, dimethyl adipimate, on the epoxidation activity and cell viability of standard resting cell preparations of *P. oleovorans*. Only the monofunctional imidoester 6 causes a rapid loss in both activity and cell viability. This is in sharp contrast to the effects of these reagents on the protocatechuate-3,4-dioxygenase activity and cell viability in *Acinetobacter calcoaceticus* [28].

Discussion

On the basis of the results reported in this paper, it is clear that the epoxidation reaction catalyzed by the *P. oleovorans* enzyme system has some unusual and intriguing characteristics. The great difference in reactivity between terminal and internal double bonds of straight chain compounds seems surprising, steric factors notwithstanding. Cyclic compounds clearly bind to the enzyme and undergo hydroxylation even though the methylene carbons of straight chain alkanes are inert to hydroxylation. Yet, the internal double bonds of cyclic compounds are just as unreactive toward epoxidation as those of straight chain compounds. Our results with the moderately long straight chain alkene, 1-decene, reveal the expected predominance of epoxidation over hydroxylation, presumably reflecting the greater inherent reactivity of the π electron system of the double bond. Yet, with the short chain substrates, propylene and butylene, only hydroxylated products are detected, and the butylene result cannot be ascribed to enhanced reactivity of an allylic carbon atom. Moreover, our results with a homologous series of straight chain substrates indicate that not only is the reactivity of a terminal double bond toward epoxidation affected by the length of the alkyl chain, but that this effect is different than that observed for the hydroxylation of a series of alkanes.

Taken together, these results surely do not fit into any straightforward

chemical reactivity pattern. In peracid epoxidation reactions, for example, cyclohexene and 2-hexene are more than twenty times as reactive as 1-hexene, reflecting the stimulatory effect of alkyl substitution on electrophilic addition to the double bond. Similarly, no significant effect of carbon chain length on epoxidation of a terminal double bond is observed, and even propylene is 80% as reactive as 1-octene [29,30]. It is obvious that factors other than simple chemical reactivity differences are being reflected in our results with the enzymatic system.

We have previously suggested that the configurations of the methylene chains of hydrocarbon substrates critically affect the mode of binding of such compounds to the active sites of oxygenases [11]. The notion that such factors are indeed among those being reflected in the substrate reactivity patterns we observe is supported by our results with the homologous series of imidoester inhibitors. The pattern of relative potency of inhibitors parallels that of substrate reactivity, as would be expected if changes in the methylene chain primarily affect the binding step in the catalytic process. In view of the simple chemical structures of the substrates and inhibitors, hydrophobic interactions must play a key role in the binding process.

We recognize that once bound, the imidoesters are capable of reacting with amino groups on the protein to form a covalent amidine linkage and thereby irreversibly bind to the active site. The facts that extensive inhibition is observed even in the presence of a large excess of substrate, and that imidoester 6 is a considerably more potent inhibitor than hexyl trimethyl ammonium bromide or methyl hexanoate, suggest that such a chemical modification reaction does occur with the imidoester inhibitors. However, the data in hand are insufficient to establish this with certainty.

There is currently considerable speculation as to the nature of the "activated oxygen" species in reactions catalyzed by mixed-function oxidases. Hamilton first suggested that an "oxenoid" species is generated in these reactions by transfer of two electrons to oxygen prior to, or concurrent with, transfer of an oxygen atom to the substrate [31—34]. This mechanism has found particular favor in accounting for the reactions of P-450-containing oxygenases, particularly the "NIH shift" which occurs during aromatic hydroxylation reactions [35]. A number of organic model systems which undergo oxenoid-type reactions have also been designed and investigated [31—35].

In the case of the *P. oleovorans* epoxidation system, the data in Table II and the high reactivity of octadiene relative to octane or octene are in accord with the expected reactivity of an electrophilic "oxenoid" species. However, from much of the other data reported here we conclude that if such a species is indeed involved in reactions catalyzed by this enzyme system, its reactivity is moderated with unusual severity by factors related to the mode of substrate binding. Our recent discovery of the high stereoselectivity of the epoxidation reaction is in line with this conclusion, since production of optically active epoxides requires binding of the substrate in such a way as to totally preclude oxygen attack from one face of the octadiene molecule [15]. Further experiments designed to probe the mechanism of oxygen addition in epoxidation reactions catalyzed by this enzyme system are currently in progress.

Finally, it is interesting to note that the bifunctional imidoester, dimethyl

adipimate, has no effect on either viability or epoxidation activity in the *P. oleovorans* enzyme system. This is in contrast to previous results with *A. calcoaceticus* where dimethyl adipimate was found to inhibit protocatechuate-3,4-dioxygenase activity and cell viability via a mechanism which apparently involves alteration of the structure of cell wall or membrane [28]. Possibly, this difference reflects the inability of the highly charged dimethyl adipimate to interact with either the hydrophobic binding site of the enzyme system itself or with the sites involved in transfer of hydrophobic substrates into the cell. Very recently, it has been reported that dimethyl adipimate is an effective antisickling agent even under deoxygenated conditions, and this effect may well result from alterations in the membrane structure of the modified cells [36].

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