Biochemical Pharmacology, Vol. 27, pp. 1435–1439.

0006-2952/78/0515-1435/$02.00/0

METABOLISM *IN VITRO* OF PARA-SUBSTITUTED STYRENES

KINETIC OBSERVATIONS OF SUBSTITUENT EFFECTS

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(*Received* 17 *May* 1977; *accepted* 7 *September* 1977)

Abstract—The metabolism of six para-substituted styrene derivatives (p-$XC_6H_4CH{=}CH_2$; X = CN, Br, Cl, Ph, CH_3, CH_3O) was studied *in vitro* using rat liver microsomes. With p-methyl-, p-methoxy- and p-phenylstyrene it was shown that metabolic attack was completely confined to the vinyl group (as is the case with styrene itself); aromatic hydroxylation, benzylic hydroxylation, and O-demethylation could not be detected. All six styrenes gave a type 1 difference binding spectrum with microsomes *in vitro*, and the role of cytochrome P450 in the metabolism of p-phenylstyrene was further confirmed by its inhibition by CO and stimulation by phenobarbital pretreatment. The spectral dissociation constants (K_s) for the styrenes showed no dependence on the nature of the substituents and were confined to a narrow range of values, each being close to the K_m value observed for the metabolism of that substrate. While the latter varied slightly more than K_s, there was again no regular dependence on the properties of the substituent. Similarly the V_m values obtained for these substrates showed no dependence on the nature of the substituents, which is in contrast to previously observed substituent effects on aromatic hydroxylation by P450 enzymes. These results, as well as related examples from other studies, are discussed in terms of chemical selectivity and mechanistic versatility on the part of a single multi-potential oxygen atom-transfer reagent generated by P450 enzymes.

The importance of substituent effects on the biological activity of chemical agents is widely recognized, and analysis of such effects by Hammett–Hansch methods has become a generally accepted means of probing the chemical mechanisms of biological reactions. As applied to the study of cytochrome P450 drug-metabolizing enzymes in particular, it has been noted that for simple benzenoid substrates, the extent and position of enzymatic ring hydroxylation roughly parallel the reactivity of the substrate to chemical attack by electrophilic reagents [1, 2]. Such observations have given rise to the concept that the enzymatic oxygen atom-transfer reagent generated by cytochrome P450 is an electrophilic species [3, 4]. Styrene is metabolized extensively on the vinyl side chain, with epoxidation by a cytochrome P450 monooxygenase as the initiating step [5, 6]. Since the attack of electrophilic species on styrenes shows pronounced substituent effects characterized by large negative Hammett rho values, we have studied the effects of para substituents on the cytochrome P450 epoxidation of styrenes in an attempt to characterize the chemical nature of the enzymatic oxygen atom-transfer reagent.

EXPERIMENTAL

Synthesis of substrates. The para-substituted styrenes were prepared from the corresponding acetophenones by borohydride reduction followed by dehydration as described previously [7]. Tritiated styrenes (β-^3H) were prepared similarly, except that the acetophenones were first treated with ^3H_2O acidified with PCl_5 in anhydrous tetrahydrofuran for several days [8].

Kinetic studies. V_m and K_m data for the enzymatic epoxidation of the styrenes were obtained as follows. The livers of non-induced male Holtzman rats were homogenized in 0.1 M, pH 7.4, phosphate buffer (2.5 ml/g), and the homogenates were centrifuged at 600 g for 25 min and again at 30,000 g for 25 min, discarding the pellet each time. Final centrifugation at 105,000 g for 2 hr sedimented the microsomes, which were resuspended in 0.1 M, pH 7.4, phosphate buffer to a concentration of 70–80 mg/ml (3–5 mg protein/ml by Lowry assay). Incubations were run in a shaker-incubater under O_2 at 33° in 16 × 100 mm culture tubes containing (in order of addition at 0–3°): (1) 0.5 ml of microsome suspension, (2) 0.1 ml of an NADPH-generating system (contains 7 mg $NADP^+$, 70 mg glucose 6-phosphate, 10 i.u. glucose 6-phosphate dehydrogenase and 100 μmoles $MgCl_2$/ml of 0.09 M, pH 7.4, phosphate buffer) and (3) sufficient substrate with twice its weight of Tween-80 (Sigma) in 25 μl acetonitrile to give initial substrate concentrations of 0.15, 0.45, 0.75, 1.05 or 1.35 mM. Incubations were stopped at 0, 5, 10 or 15 min by addition of KOH (or NaCl for p-cyanostyrene), and loss of substrate was determined by gas–liquid chromatography (g.l.c.) (6 ft × ⅛ in. DC-550 column) after equilibrating 2 ml hexane or heptane containing an internal standard (usually naphthalene or biphenyl) with the incuba-

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tion medium. Epoxidation rates, v, were linear for > 15 min, and were obtained from a plot of [substrate]/[standard] vs time. V_m and K_m were obtained from the slope ($1/V_m$) and intercept (K_m/V_m) of a plot of $[S]/v$ vs $[S]$ by least squares analysis.

Identification of metabolites. Incubations of tritiated 4-methyl- and 4-phenylstyrene (1.41×10^7 and 1.93×10^7 dis./min/μmole respectively) on a 30–50 μg scale were conducted as described above, except that the reactions were stopped after 30 min by acidification with 10% perchloric acid (1 ml) and extraction with ether (4 × 3 ml). The ether extracts were concentrated and analyzed on 0.25-mm Silica gel thin-layer chromatography (t.l.c.) plates eluted with ethyl acetate–hexane (40:60 v/v). Significant radioactivity was found only in the diol band (R_f 0.09 to 0.18) and the olefin band (R_f 0.87 to 0.95). The diols were eluted with ether after scraping the plate, converted to acetonides with anhydrous $CuSO_4$ in acetone [9], and idenitified by g.l.c. comparison to standard samples (5% DC550 at 135–150°) and by their mass spectra. Total recoveries of radioactivity were > 85 per cent in all cases.

Incubations of 4-methoxystyrene were carried out similarly and were analyzed for formaldehyde using Nash's reagent [10]; none was observed, indicating that *O*-demethylation had not occurred.

K_s determinations. Interaction of the styrenes with microsomal cytochrome P450 was studied using a Cary 118 spectrophotometer. Microsomes sedimented between 30,000 and 100,000 *g* as described above were suspended in 0.1 M, pH 7.4, phosphate buffer to a protein concentration of 2–3 mg/ml by Lowry assay. This suspension was placed in both the sample and reference cuvettes. Substrate in acetonitrile (without Tween-80) was added to the sample cuvette in successive 1-μl aliquots, and the contents of the cuvette were stirred gently with a Teflon paddle without otherwise moving the cuvette itself. The reference microsomes received similar aliquots of pure acetonitrile. Between each addition of substrate the spectrum was recorded from 350 to 450 mm. The value of K_s was determined from a plot of the reciprocal absorbance change $(A_{390}-A_{420})^{-1}$, vs the reciprocal of the substrate concentration.

RESULTS AND DISCUSSION

Liver microsomes are known to contain a closely coupled cytochrome P450 mono-oxygenase/epoxide hydrase enzyme system known as the epoxide–diol pathway which catalyzes the conversion of simple olefins as well as some arenes to diols. Epoxide intermediates including styrene oxide may sometimes be trapped or isolated, although only with difficulty [5, 11, 12]. In the present study the role of cytochrome P450 in initiating the metabolism *in vitro* of para-substituted styrenes was investigated using [β-^{3}H]4-phenylstyrene as a representative example (Table 1). It was found that the conversion of this substrate to the α,β-diol depended on the presence of viable microsomes, an oxygen source, and NADPH furnished either as such or via an *in situ* generating system. When NADH was used, the amount of diol formed was decreased by 64 per cent. Incubation under 100% O_2 nearly doubled the amount of diol formed under air, and replacing the nitrogen content of air with CO inhibited diol formation (under ambient lighting) by 40 per cent. Compared to microsomes from normal rats, microsomes from rats pretreated with phenobarbital gave a 2.5-fold greater yield of diol.

Induction by phenobarbital, inhibition by CO, and a requirement for NADPH and oxygen are all characteristics of the cytochrome P450 mono-oxygenase system. Another feature of this system is that the reversible interaction of substrates with the cytochrome perturbs its chromophoric properties and gives rise to a concentration-dependent difference spectrum having a maximum around 385–390 nm and a minimum around 415–420 nm. From a plot of the reciprocal absorbance change vs reciprocal substrate concentration, the spectral dissociation constants listed in Table 2 were ob-

Table 1. Effect of incubation medium components on diol formation from [β-^{3}H]4-phenylstyrene

Incubation conditions*	(μmoles Diol formed/30 min)†
S	1.43 ± 0.11
S‡	3.63 ± 0.20
S, −GS	< 0.05 ± 0.02
S, −GS, + NADPH (1.6 mM)	1.65 ± 0.08
S, −GS, + NADH (1.6 mM)	0.61 ± 0.06
S, $-O_2$, + air	0.72 ± 0.12
S, $-O_2$, + CO—O_2 (80:20)	0.44 ± 0.09

* S, standard conditions. (Each incubation contains 100 mg microsomes corresponding to about 1 g liver, 5.5 μmoles 4-phenylstyrene added with 0.4 mg Tween-80 in 50 μl MeCN, 1 mg $NADP^+$, 10 mg glucose 6-phosphate, 1.2 I.U. glucose 6-phosphate dehydrogenase, and 15 μmoles $MgCl_2$, all in a final volume of 1 ml of 0.1 M, pH 7.4, phosphate buffer; incubations were conducted at 33° under an atmosphere of O_2.) GS, NADPH-generating system ($NADP^+$, glucose 6-phosphate, $MgCl_2$, and glucose 6-phosphate dehydrogenase).

† The reaction is not linear beyond 15 min; N = 3.

‡ Standard conditions except that microsomes from rats given injections of phenobarbital (60 mg/kg, i.p.) once daily for 3 days were used.

Table 2. Substituent effects on the reversible binding and metabolism of *p*-substituted styrene derivatives (p-$XC_6H_4CH=CH_2$) by cytochrome P-450*

X	π^+	σ^+	K_s (mM)	K_m (mM)	V_m (nmoles min^{-1} mg $protein^{-1}$)
CN	−0.57	0.66	‡	0.34 ± 0.22	2.82 ± 0.42
CH_3O	−0.02	−0.27	0.22, 0.25	0.31 ± 0.11	3.96 ± 0.79
CH_3	0.56	−0.17	0.17, 0.23	0.86 ± 0.30	5.33 ± 0.30
Cl	0.71	0.23	0.28, 0.16	0.31, 0.26	3.53, 2.79
Br	0.86	0.23	0.31, 0.27	0.33 ± 0.14	3.32 ± 0.31
C_6H_5	1.96	−0.01	0.26, 0.12	0.17 ± 0.12	2.03 ± 0.62
H§	0	0		0.43	4.77

* K_s, K_m and V_m were determined as described in Experimental; N = 3.

† Values for π and σ are from C. Hansch, A. Leo, S. H. Unger, K. H. Kim, D. Nikaitani and E. J. Lien, *J. med. Chem.* **16**, 1207 (1973).

‡ K_s for *p*-cyanostyrene could not be determined due to interference caused by the u.v. absorption of the substrate itself tending to offset the change in the difference spectrum of the cytochrome.

§ Data from [6].

tained. The fact that these values are very close to the apparent K_m values for the substrates strongly supports the previous conclusion that this metabolism is a P450-dependent process. It also suggests that the kinetics of substrate and product association and dissociation do not limit the overall catalytic process.

Simple benzenoid compounds lacking the vinyl side chain of the styrenes are also oxygenated by cytochrome P450 enzymes. For example, biphenyl [13] is converted to a mixture of 2- and 4-hydroxybiphenyl, toluene [3] is converted mainly to benzyl alcohol but cresols are also formed, and anisole [3] is mainly demethylated but is also ring hydroxylated. In the corresponding para-substituted styrenes these routes of metabolism are not observed; metabolic attack is confined (> 95 per cent) to the vinyl group. The same is true in chemical systems. In general both electrophiles and radicals attack exclusively at the vinyl side chain of simple styrene derivatives. The fact that a vinyl side chain can attract metabolic attack away from groups that are otherwise readily metabolized (e.g. —Ph, —CH_3, —OCH_3) suggests that the vinyl group is also likely to be the sole site of metabolic attack in those styrenes bearing substituent groups which are inert to microsomal drug-metabolizing enzymes (e.g. —CN, —Br and —Cl).

There are two possible explanations for the observed enzymatic selectivity for vinyl attack. One explanation is that incorporation of a para vinyl group onto a simple aromatic such as toluene or anisole sterically blocks the access of the substrate to the active site of the particular P450 species responsible for benzylic hydroxylation or *O*-demethylation, or that the presence of the vinyl group opens a new and facile pathway for metabolism via a vinyl-specific P450 enzyme, thus draining substrate away from the hydroxylation or demethylation enzymes. However, the fact that numerous drugs and other substances containing *p*-tolyl or *p*-anisyl groups attached to large molecular frameworks undergo facile benzylic hydroxylation and *O*-demethylation argues against an adverse steric effect due to the small vinyl group. Furthermore, the overall rate of metabolism of the styrenes is not appreciably different from the metabolism of simple aromatics, so that the selectivity cannot be explained by a vastly greater rate of metabolism by a P450 enzyme specific for vinyl groups.

The second and more likely explanation for the observed enzymatic selectivity is that, like chemical reagents, the enzymatic oxygen atom-transfer reagent generated by P450 enzymes can discriminate between potential sites for attack based on their chemical reactivity. The primary deuterium isotope effect (discrimination agagainst deuterium for hydrogen) so commonly observed [14–16] during P450-dependent hydroxylation and dealkylation reactions is one clear example of enzymatic selectivity based on substrate chemistry. With styrenes the initial attack of both electrophilic and free radical reagents occurs specifically on the β-carbon of the vinyl group because, compared to other types of attack, there is an enormous energetic advantage associated with forming a resonance-stabilized (benzylic) intermediate. Since the function of enzymatic catalysis is to provide a facile, low energy pathway for the reactants to follow, it seems only reasonable to assume that this aspect of substrate chemistry is used to advantage by the enzyme during epoxidation of the styrene side chains. It might even be argued that this is the major reason for the enzyme's selectivity for vinyl attack. Undoubtedly with some substrates, especially those with large rigid structures, it is possible that binding energies may override such that the more chemically reactive sites are sterically less available for enzymatic attack.

The term "electrophilic" has often been used to characterize the attack of cytochrome P450 on aromatic and aliphatic substrates. In fact most of what is known about the properties of the enzymatic oxygen atom-transfer reagent of cytochrome P450 derives from the chemistry of its selectivity toward substrates [1–4]. Thus it has been noted that the extent and position of enzymatic aryl hydroxylation are correlated with the reactivity of the ring to

electrophilic attack. With alkanes there is generally a pronounced preference for hydroxylation at tertiary (CH) > secondary (CH_2) ≫ primary (CH_3) positions. Trifluoroperacetic acid is one chemical reagent that models nearly all of the characteristics of substrate oxygenations by P450 enzymes [3, 4]. It effects aromatic hydroxylation with a concomitant NIH-shift, and it effects aliphatic hydroxylation with retention of configuration and a pronounced preference for tertiary C—H positions. Since trifluoroperacetic acid is an extremely electrophilic reagent, this characteristic is often ascribed to the oxygen atom-transfer reagent generated by cytochome P450, and recently it has been proposed [4] that the latter is a peracid or perimidic acid. However, none of the enzymatic observations modeled by the peracid is inconsistent with the enzymatic reagent having a non-polar or radical-like character. The failure of certain free radical hydroxylating systems to effect an NIH shift [17] simply implies that intermediates other than arene oxides, perhaps aryl radicals, are involved in these particular systems. To the extent that arene oxides are involved in aromatic hydroxylation, the NIH shift and the final position of the phenolic OH are determined by substituent effects on the non-enzymatic rearrangement of the oxide [18], and not on its enzymatic formation. In aliphatic chemistry, free radicals are well known to attack C—H bonds in the order 3° > 2° ≫ 1°, and hydroxylation with retention of configuration is not without precedent [19]. Nor would it be unreasonable to assume that the enzyme could maintain the configuration of a free-radical intermediate if one were to be involved in aliphatic hydroxylation by P450.

The known substituent effects on the reactions of styrene derivatives with a wide variety of chemical reagents provide a clear basis for detecting and quantitating electrophilic character on the part of the attacking species. Attack by electrophiles on the vinyl group of styrenes is greatly accelerated by electron-donating substituents, and the reactions are characterized by substantial negative Hammett rho values. Examples involving the attack of electrophilic oxidants on *p*-substituted styrenes include epoxidation by organic peracids ($\rho = -1.3$) [7], epoxidation by *t*-butylhydroperoxide with a molybdenum catalyst ($\rho = -1.4$) [20], oxidation by CrO_2Cl_2 ($\rho^+ = -1.99$) [21], and bromination by Br_2 in HBr solution ($\rho^+ = -4.3$) [22]. Similarly, the attack of highly electronegative free radicals on styrene, toluene and benzene derivatives is characterized by negative rho values [23, 24]. Although the absolute magnitude of rho is usually somewhat smaller. ($-2 < \rho < -0.8$), it has recently been shown that a given radical will display the same rho value whether adding to styrene or benzene or abstracting a benzylic hydrogen from toluene [25]. On the other hand, radicals such as hydrogen, methyl, phenyl or polystyryl react with rho values close to zero. We anticipated that the oxygen atom-transfer reagent of cytochrome P450 would behave in analogy to these chemical systems. The fact that enzymatic attack occurs only on the vinyl group reinforces the idea that chemical factors dominate the course of these reactions.

In addition to varying the chemical stability of intermediates, and hence the overall rate of an enzymatic reaction, the alteration of substituents on the substrate may also influence the apparent overall rate through changes in the affinity of the enzyme for the substrate. In the case of cytochrome P450 enzymes, substituent effects on substrate binding can be assessed directly by determination of spectral dissociation constants K_s under non-turnover conditions, as well as by the customary determination of apparent K_m values for metabolism of the substrate. The values of K_s and K_m for six *p*-substituted styrenes are given in Table 2; the substituents used were chosen to span a broad range of σ and π values, as well as for their known inertness to metabolic attack (—CN, —Br and —Cl). Inspection of Table 2 shows that, for each substrate, $K_m \approx K_s$. This is expected intuitively, and has been observed in other cases involving P450 enzymes [26]. Perhaps less expected is the narrow range of K_m and K_s values observed for the various substrates. In principle at least, this could be caused by fortuitous cancellation of lipophilic effects on substrate binding by electronic effects on substrate binding. However, comparisons of the σ and π values for the substituents (Table 2) suggest that is not the case here. Although substrate lipophilicity is a requirement for metabolism by P450 enzymes, other investigators have failed to observe a dependence of metabolism parameters *in vitro* on the partitioning behavior of substrates when the substrates were already very lipophilic [27]. Furthermore, the K_s and K_m values for the styrenes are close to those reported for other simple aromatic substrates, for example biphenyl [13] (4-hydroxylation, $K_m = 0.14$ mM, $V_m = 3.5$ nmoles/min/mg of protein) and styrene [6] (epoxidation, $K_m = 0.43$ mM, $V_m = 4.77$ nmoles/min/mg of protein). Since the binding of the styrenes to P450 is apparently not affected by the electronic or lipophilic properties of the substituents, substrate association cannot be rate limiting with respect to turnover of the enzyme. Thus, observation of substituent effects on the enzymatic reaction rates should constitute an uncomplicated probe of chemical nature of the enzymatic oxygen atom-transfer process. Table 2 gives the V_m values obtained for the enzymatic epoxidation of six para-substituted styrenes. Inspection of these values readily reveals that they are independent of the electronic influence of the substituents. The comparison of the cyano- and methoxy-substituted compounds is particularly striking, for while *p*-methoxystyrene is epoxidized by perbenzoic acid sixteen times faster than *p*-cyanostyrene [7], the V_m values for these substrates differ by less than 40 per cent, and their K_m values are almost identical.

One explanation for the lack of substituent effects on the enzymatic epoxidation could be that the enzyme binds the substrate in such a way that the vinyl group is rotated out of conjugation with the aromatic ring. However, this is unlikely as it would also eliminate the mechanistic advantage of resonance stabilization of intermediates, which, as argued above, is probably the reason the enzyme is selective for vinyl attack with these substrates. Furthermore, rotation of the vinyl group would eliminate

only the resonance effects of the substituents and not their inductive effects. In principle, there is also the possibility that the oxygenation of the substrates is not the rate-limiting step in catalytic turnover of the enzyme. However, our observation of secondary deuterium isotope effects in the P450 epoxidation of styrenes implies that substrate oxygenation is, in fact, rate limiting with these substrates [28]. This finding is important here because it emphasizes the validity of using substituent effects as a probe of the oxygen atom-transfer reaction mechanism.

The lack of any significant polar substituent effects on the enzymatic epoxidation of styrenes is not consistent with earlier studies from which it was concluded that the enzymatic oxygen atom-transfer reagent is electrophilic in nature. This discrepancy might be due to the involvement of different species of P450 enzymes as different substrates are studied, e.g. substituted benzenes [1, 2] vs substituted styrenes. On the other hand, it could simply reflect different modes of substrate attack on the part of a single active oxygen species generated by the P450 enzymes. Other evidence suggestive of chemical versatility on the part of the oxygen atom-transfer reagent of P450 includes the site-specific metabolic switching from epoxidation to hydroxylation, apparently dependent only on the presence of saturation or unsaturation in the substrates, at C-11 in steroids [29], on the central C—C bridge in tricyclic antidepressants [30, 31] and related compounds [32, 33], on barbiturate side chains [34], and on simple aromatic hydrocarbons such as styrene [5, 6]/ethylbenzene [35], indene [5]/indane [36] or naphthalene [37]/tetralin [38]. It is clear that further work will be required to resolve the question of mechanistic versatility on the part of the oxygen atom-transfer reagent generated by cytochrome P450, both in terms of its electrophilic vs radical character, and with respect to its ability to attack saturated vs unsaturated vs heteroatom positions in substrates.

Acknowledgement—This work was supported by the National Institutes of Health (GM-21784 and GM-1341) and the University of Kansas General Research Fund.

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