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Stereo- and Enantioselectivity of the Soluble Epoxide Hydrolase-Catalysed Hydrolysis of (±)-*cis*-Dialkyl Substituted Oxiranes

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Abstract: Both enantiomers of *cis*-(±)-2,3-epoxyheptane (**1a**), *cis*-3,4-epoxyheptane (**1b**), *cis*-3,4-epoxynonane (**1c**) *cis*-3,4-epoxynonane-1-ol (**1d**) and *cis*-1-methoxy-3,4-epoxynonane (**1e**) are transformed into the corresponding *threo*-diols **2a-e** by soluble epoxide hydrolase catalysed hydrolysis. The reaction proceeds with a substrate and product enantioselection, which is highly dependent on the substituents at the oxirane ring. Whereas the hydrolysis of racemic **1a** shows practically no enantioselection, that of its isomer **1b**, gives after complete hydrolysis, in a stereoconvergent way, the corresponding *threo*-diol (3R,4R)-**2b**. On the other hand, the hydrolysis of epoxides **1c-e** progress with a fairly good (**1c** and **1e**) or complete (**1d**) substrate enantioselection to give racemic (**2c**) or, in a stereoconvergent way, optically enriched (**2e**) or pure (**2d**) diols. Significant differences were observed from the previous results of the hydrolysis of the same substrates with microsomal epoxide hydrolase.

Keywords: soluble epoxide hydrolase, enantiomeric excesses

Epoxide hydrolases[1] (EH) are important enzymes involved in the metabolism of a broad variety of epoxides, many of which are mutagenic and/or carcinogenic, often formed *in vivo* from the Cytochrome P-450 catalysed biooxidation of compounds containing olefinic or aromatic functionalities.[2] Two main mammalian EH forms, a microsomal, mEH, and a cytosolic soluble one, sEH (or cEH), both catalysing the *anti* addition of water to the oxirane ring to give vicinal diols and having broad and complementary substrate selectivities, are known. However, similar EH activities have been recently identified in organisms as diverse as bacteria,[3] yeasts,[4] fungi,[5] plants[6] and insects.[7] During the past 20 years the mammalian mEH in particular has received a great deal of attention. Stereochemical investigations have demonstrated that the addition of water promoted by this enzyme often exhibits a remarkable substrate enantioselectivity towards racemic epoxides,[8] or product enantioselectivity with meso epoxides.[9] In the latter case the mEH catalysed oxirane ring opening selectively occurs at an (S) oxirane carbon to give (R,R) diols with high enantiomeric excesses.[9] Furthermore, even if the reaction is often endowed with a very high regioselectivity, the nucleophilic attack occurring at the less substituted, or less hindered oxirane carbon,[10] it has recently been demonstrated that the mEH catalysed biotransformation of racemic *cis*-β-disubstituted epoxides can occur in an enantioconvergent way to give at complete hydrolysis pure (R,R)-diols.[11-13] Finally, single turnover experiments have shown that the mEH catalysed hydrolysis proceeds through the formation of a covalent enzyme-substrate ester intermediate, which is subsequently hydrolyzed to release the diol product and the active enzyme.[14-15]

Much less work has involved the soluble epoxide hydrolase (sEH), though it has been recently demonstrated[16] that the mouse liver sEH catalysed hydrolysis also occurs through a two step mechanism. The attack by Asp-333 initiates enzymatic activity leading once again to the formation of an α-hydroxyacyl-enzyme intermediate. At variance with mEH, however, for which the great deal of work has provided a quite clear

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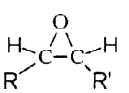
picture of the regio-, stereo-, and enantiospecificity of this enzyme, only few stereochemical data related to sEH, often closely parallel to those for mEH, have been reported.[9a,17,18] It is however noteworthy that, the markedly different regioselectivity observed[19] in the rabbit liver mEH and sEH catalysed hydrolysis of phenyl substituted epoxides strongly suggests that, at least with these substrates, epoxide protonation favouring opening at the benzylic carbon could be more important for sEH than for mEH. The possibility of a push-pull mechanism in the sEH catalysed hydrolysis, consisting of an epoxide oxygen activation by protonation or hydrogen bonding before or during the nucleophilic attack of the carboxylate anion, has also been recently suggested[20] taking into account the electronic effect induced by the *para*-substitution on the phenyl rings of chalcone oxides on the rate of hydroxyalkyl enzyme formation.

In order to further contribute to the understanding of the catalytic mechanism of these two hydrolytic enzymes, we have extended our recent investigation of the mEH-catalysed hydrolysis of *cis*-(±)-2,3-epoxyheptane (**1a**), *cis*-3,4-epoxyheptane (**1b**), *cis*-3,4-epoxynonane (**1c**), *cis*-3,4-epoxynonane-1-ol (**1d**) and *cis*-1-methoxy-3,4-epoxynonane (**1e**) to the soluble enzyme. The stereochemical results are discussed taking into account the proposed mechanisms and the hypothesized topologies of the two active sites.

RESULTS AND DISCUSSION

The rates of biotransformation of the differently disubstituted racemic oxiranes **1a-e**, by the rabbit liver cytosolic preparation,[18] were preliminarily checked and the values compared with those found using the microsomal fraction arising from the same liver preparation. Incubations were carried out at 37 °C and pH 7.4 with 10-20 mM epoxides and 10-20 mg/ml of protein for 10-30 minutes, and stopped by extraction with ethyl acetate. The organic phases were analyzed by GLC, after addition of an appropriate standard.



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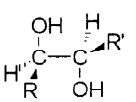

threo-**2**

a: R = CH_3, R' = $(CH_2)_3CH_3$; **b**: R = CH_3CH_2, R' = $(CH_2)_2CH_3$; **c**: R = CH_3CH_2, R' = $(CH_2)_4CH_3$;
d: R = $HOCH_2CH_2$, R' = $(CH_2)_4CH_3$; **e**: R = $CH_3OCH_2CH_2$, R' = $(CH_2)_4CH_3$

In all the cases only the *threo*-diols **2a-e** were found as the sole products arising from the mEH and sEH catalysed hydrolysis. The diol production was always linear with time and with microsomal or cytosolic protein amount, and was independent of the substrate concentration, showing that saturating conditions had been used. The average rates measured using the cytosolic preparations were: **1a**, 1.5; **1b**, 0.45; **1c**, 1.5; **1d**, 3.1; **1e** 0.8 nmol/(mg of protein × min). Similar values were found[13] using the microsomal preparation: **1a**, 3,1; **1b**, 0.5; **1c**, 0.3; **1d**, 5.5; **1e** 4.4 nmol/(mg of protein × min).

In order to determine the substrate and product enantioselection, incubations of racemic epoxides **1a-e** were carried out under saturation conditions (20-30 mM) with the rabbit liver cytosolic preparation, diluted to a protein concentration of 10 mg of protein/ml, at 37 °C and pH 7.4. The reactions were stopped at different times and the residual epoxides and the formed diols were analyzed by GLC using a chiral column. Only in the cases of diols **2d** and **2e** the enantiomeric excesses were determined by HPLC after transformation of these diols into the

corresponding MTPA esters. The results are reported in Table 1, which also includes the absolute configurations of the residual epoxides (**1a-e**) and of the formed diols (**2a-c**), which were determined by comparison of their GLC or HPLC retention times with those of optically active samples.[13]

Table 1. Enantiomeric excesses and absolute configurations of epoxides and diols obtained by partial or complete sEH catalysed hydrolysis of racemic substrates **1a-e**.

Racemic	Hydrolysis %	Enzyme	Unreacted 1 e.e.[a]	Unreacted 1 Abs. Conf.	Formed 2 e.e.[a]	Formed 2 Abs. Conf.
1a	40	sEH	16[b]	(2R,3S)	10[b]	(2R,3R)
	100				0	
1b	50	sEH	4[b]	n.d.	>98[b]	(3R,4R)
	100				>98[b]	(3R,4R)
1c	50	sEH	40[b]	(3S,4R)	6[b]	
	100				4[b]	
1d	50	sEH	>98[b]	(3R,4S)	>98[c]	(3R,4R)
	100		-		>98[c]	(3R,4R)
1e	50	sEH	75[b]	(3S,4R)	70[c]	(3S,4S)
	100		-		70[c]	(3S,4S)

[a]Averages of three determinations: ± 2%. At the same percent of hydrolysis the e.e. values obtained with different rabbit liver cytosolic preparations were reproducible ± 2%. [b]Determined by GLC using a chiral column. [c]Determined by HPLC after transformation into the corresponding MTPA derivatives.

The substrate and product enantioselection was highly dependent on the substituents at the oxirane ring, the relative size of the two alkyl groups and the presence of an hydroxyl group. Whereas the hydrolysis of **1a** shows practically no substrate or product enantioselection, that of isomer **1b**, proceeds without substrate enantioselection to give, in a stereoconvergent way after complete hydrolysis, the corresponding *threo*-diol (3R,4R)-**2b** (>98% e.e.), in agreement with a stereoselective attack at the S oxirane carbon of both enantiomers. On the other hand, the hydrolysis of epoxide **1c** progresses with a fairly good substrate enantioselection to give a racemic diol, **2c**, arising from a non-stereoselective attack at both oxirane carbons of each enantiomer. The introduction of an hydroxyl group on the ethyl substituent completely changes the substrate and product enantioselectivity. The sEH catalysed hydrolysis of **1d** proceeds indeed with a complete substrate enantioselection. Stopping the reaction at 50% hydrolysis it is possible to isolate the practically pure unreacted enantiomer (3R,4S)-**1d**; i.e. an epoxide of opposite configuration with respect to the one obtained at the same conversion from **1c**. Furthermore, with this substrate the oxirane ring opening of both enantiomers occurs in a stereoconvergent way to give, at complete hydrolysis, the corresponding diol (3R,4R)-**2d**. Finally, methylation of the hydroxyl group again modifies the substrate and product enantioselectivity. The enantiomer (3R,4S)-**1e** is preferentially hydrolyzed to give diol (3S,4S)-**2e**, which is isolated with practically the same enantiomeric purity at 50% and 100% hydrolysis, in accord with a selective attack at the R oxirane carbon of both enantiomers.

It is noteworthy[21] that the rabbit liver mEH catalysed hydrolysis of epoxides **1a-e** showed[13] a completely different stereochemical behavior. These substrates were indeed hydrolyzed by the microsomal enzyme to the corresponding diols in an *anti* stereospecific and stereoconvergent way to give, with the exception of the methyl derivative **1a**, almost optically pure *threo*-(R,R) diols at complete hydrolysis. Furthermore, in the case of **1a** and **1e** the reactions were characterized by a high substrate enantioselection; at 50% hydrolysis it was possible to obtain an enantiomerically pure residual epoxide.

Generally, on the basis of the K_M and V_{max} values measured for both enantiomers of some racemic epoxides, and taking into account the proposed topology of the mEH active site,[12] according to which two hydrophobic pockets of different shapes and sizes situated at the right and left back side of the epoxide binding site and able to accomodate two lipophylic syn substituents are present, the frequently observed substrate enantioselection in mEH catalysed hydrolysis has been attributed to an inhibitory effect of one enantiomer on the hydrolysis of the other because of the higher affinity of the former for the mEH active site. However, EH-catalysed water addition to epoxides occurs through a two-step reaction (eq 1) and pre-steady state kinetic measurements have shown[22] that the rates (k_2) and the efficiencies (k_2/K_S) of the alkylation step for both enantiomers may be comparable, and consequently the enantioselectivity may arise almost entirely from the different rate of decomposition of the two substrate-enzyme intermediates.

$$E + S \overset{K_S}{\rightleftharpoons} E\text{-}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}I \xrightarrow{k_3} E + P \qquad (1)$$

Therefore, although it has been hypothesized[20] on the basis of the sEH substrate selectivities that this enzyme can also be viewed as having a hydrophobic binding site on either side of the catalytic site, in light of the two step mechanism, the substituent dependent substrate enantioselectivity observed in the sEH hydrolysis of epoxides **1a-e** may be due not only to the different stability of the enzyme substrate complexes of the two enantiomeric epoxides, but also to the different stability of the corresponding ester intermediates. To fully describe the substrate enantioselectivity in terms of inhibitor mechanism, the rates of binding, acylation, and deacylation should be considered separately. However, independently of the stage at which the inhibitor mechanism occurs, the present results suggest that the steric constraints[20] during the sEH catalytic cycle may determine the stereochemical behaviour, which is probably also affected by hydrogen bonding. Indeed, the opposite substrate enantioselectivity observed in the hydrolysis of **1c** and **1d** may be attributed to some interaction of the hydroxyl group of **1d** with the sEH active site, which can affect the relative stability of the enzyme-substrate complexes of the two enantiomers, and/or, of the two corresponding ester intermediates. Moreover, in agreement with the contribution of a hydrogen bond in the substrate enantioselectivity is also the stereochemical behaviour observed in the hydrolysis of **1e**. This compound, indeed, bearing a methoxy instead of an hydroxyl group is unable to form a hydrogen bond and the related sEH catalysed hydrolysis proceeds with the same substrate enantioselectivity of **1c**; at 50% hydrolysis one recovers the (3S,4R) epoxide enantiomer.

Finally, it is noteworthy that although the reaction of **1c** shows no product enantioselectivity, those of either **1d** and **1e** proceed in a stereoconvergent way to give at complete hydrolysis an optically pure, or highly enriched *threo*-diol. However, while in the case of **1d** the attack occurs at the S oxirane carbon, in the case of **1e** the reaction shows an opposite stereoselectivity giving the corresponding (S,S) diol. For this substrate the product enantioselectivity observed during the sEH catalysed hydrolysis is opposite to that found using the microsomal fraction. Although it is not easy to rationalize these latter results, they are however in agreement with those previously reported on the sEH catalysed hydrolysis of isomeric epoxyeicosatrienoic acids,[23] and show that the stereoselectivity of the nucleophilic attack with the soluble enzyme strongly depends on the substrate structure.

In conclusion, although more data is highly desirable, the present results not only prove that the soluble enzyme presents also an active site able to allow stereo- and regio-selective oxirane ring opening in high

diastereomeric and optical yield in some cases, but also that the two enzymes can be a source of epoxides and diols of opposite configuration.

EXPERIMENTAL SECTION

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. The ^{1}H and ^{13}C NMR spectra were registered in $CDCl_3$ with a Bruker AC 200 instrument using TMS as the internal reference. HPLC analyses were carried out with a Waters 600E instrument equipped with a diode array detector. The e.e.s of the diols **2d** and **2e** were determined, after conversion into the diastereoisomeric MTPA esters, by HPLC on a Nitrile S5 column with hexane/2-propanol (99.5:0.5) as the eluent at a flow rate of 1 ml/min. The yields of recovered epoxides and product diols and the e.e.s of epoxides **1a-e** and of diols **2a-c** were obtained by GLC analysis using a Carlo Erba HRGC 5300 instrument equipped with a 20 m Chiraldex G-TA (ASTEC) column, evaporator and detector 245 °C, helium flow 1 ml/min, at the following temperatures: **1a-c** 80 °C; **1d** 130 °C; **1e** 120 °C. **2a**, as bis(trifluoroacetyl)derivative 80 °C; **2b** 110 °C; **2c** 130°C; **2d**, as trifluoroacetyl derivative 130 °C. *cis*-2,3-Epoxyheptane (**1a**), *cis*-3,4-epoxyheptane (**1b**), *cis*-3,4-epoxynonane (**1c**) *cis*-3,4-epoxynonane-1-ol (**1d**), and *cis*-1-methoxy-3,4-epoxynonane (**1e**) and the corresponding *threo*-diols (**2a-e**) were synthesized as previously reported.[13] Rabbit liver cytosolic preparations were obtained as reported[18] and stored at -40 °C.

Enzymatic Hydrolysis. **A. Rates of sEH catalysed hydrolysis of epoxides 1a-e.** Aliquots (20 μl) of 1-2M ethanolic stock solutions of (±)-**1a-e** were added to 2 ml of diluted cytosolic preparation[13] containing 10 or 20 mg of protein/ml, in a such way as to obtain a 10, 15 and 20 mM final substrate concentration, and the mixtures were incubated with shaking at 37 °C. After 10 and 30 min the reactions were stopped by extraction with ethyl acetate (2 ml) containing a known amount of benzaldehyde as a standard. The extracts were analyzed by GLC for quantification of the unreacted epoxides and the formed diols. The diol formation was linear with time and protein concentration, and was independent of the substrate concentration, indicating substrate saturation. Blank experiments carried out under identical conditions but using boiled cytosolic preparations showed that no spontaneous hydrolysis occurred under the employed conditions. The average saturation rates in nmol/(mg protein x min) obtained for the various substrates with the cytosolic preparation are reported in the text.

B. Determination of Enantiomeric Excesses. Aliquots (20 μl) of 2-3 M ethanolic stock solutions of (±)-**1a-e** were added to 2 ml of rabbit liver cytosolic preparation containing 10 mg of protein/ml and the mixtures were incubated under shaking at 37 °C. At prefixed times the reactions were stopped by extraction with ethyl acetate containing a known amount of benzaldehyde as a standard for the quantification of the residual epoxide, and the extracts were analyzed on the chiral column for the determination of the e.e.s of the residual epoxides and product diols. At least three determinations were made each time. The average results are reported in Table 1. The absolute configurations of the two enantiomers of the unreacted epoxides (**1-e**) and of the formed diols (**2a-c**) were determined directly by comparison of their relative retention time, the elution order on this column being known.[13] The absolute configurations of the diols **2d** and **2e** were established by comparison of the optical rotation values of two sample obtained as reported below with literature data.

C. Diol Isolation. In order to determine the absolute configuration of the excess enantiomer of the formed diols, epoxides **1d** and **1e** as neat liquids (40-60 mg, 0.25-0.4 mmol) were added to 20 ml of the cytosolic preparation, containing 20 mg of protein/ml, and the reaction mixtures were incubated with shaking at 37 °C for the time

necessary to obtain the appropriate conversion (12-14 hours). The incubation mixtures were then stopped by extraction with ethyl acetate (3 x 10 ml). The organic phases were diluted to an exactly known volume (50 ml) by dilution with ethyl acetate and a proper amount of the standard was added to an aliquot (5 ml) of these extracts in order to verify the conversion by GLC. The remaining part of the organic phases were evaporated *in vacuo* and the residues were chromatographed on silica gel columns (70% AcOEt/hexane) to give the corresponding pure diols. 70-75% yield. **2d**, $[\alpha]_D$= + 30 (c = 1, MeOH); e.e. 98%; **2e**, $[\alpha]_D$= -5.9 (c = 1, MeOH), e.e 70%.[24]

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