Tyrosine 115 Participates Both in Chemical and Physical Steps of the Catalytic Mechanism of a Glutathione S-Transferase*

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The participation of the hydroxyl group of tyrosine 115 in the catalytic mechanism of isoenzyme 3–3 of rat glutathione (GSH) S-transferase is implicated by x-ray crystallographic analysis of a product complex and confirmed by comparison of the catalytic properties of the native enzyme and the Y115F mutant. Tyrosine 115 is located in domain II of the protein (the xenobiotic substrate binding domain) and is the first residue in this domain to be shown to play a direct role in catalysis. The 1.8-Å structure of isoenzyme 3–3 in complex with (9S,10S)-9-(S-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene, one of the diastereomeric products of the reaction of GSH with phenanthrene 9,10-oxide, indicates that the hydroxyl group of Tyr115 is within hydrogen-bonding distance of the 10-hydroxyl group of the bound product and, by implication, is proximal to the oxirane oxygen of the substrate (Ji et al., 1992). It is reasonable to anticipate that other residues, perhaps contributed by the xenobiotic substrate binding domain, might participate directly in catalysis by helping to stabilize particular transition states such as the oxiranion in epoxide ring openings (Scheme I), the enol (enolate) in Michael additions, or the Meisenheimer complex in nucleophilic aromatic substitutions (Scheme I). For example, the enzyme might provide electrophilic assistance in epoxide ring openings by donating a hydrogen bond to the developing charge on the oxirane oxygen (Cobb et al., 1991). The effectiveness of this participation in any given reaction type, which helps define the catalytic diversity of the various isoenzymes, depends on the electronic demands of the transition state and the nature of the rate-limiting step. Although the construction of chimeric isoenzymes has implicated residues in the C-terminal tail as participating in catalysis (Zhang and Armstrong, 1990; Zhang et al., 1992), no evidence has been forthcoming defining the direct participation of any individual residue from domain II in the catalytic mechanism.

In this paper we present evidence that the hydroxyl group of tyrosine 115 (Tyr115) from the xenobiotic substrate binding domain participates in both chemical and physical steps of the catalytic mechanism of isoenzyme 3–3 and that the net effect of that participation is highly dependent on the nature of the transition state and the rate-limiting step for the reaction in question. Katusz and Colman (1991) provided the first evidence that Tyr115 is located near the active site of mu class isoenzymes by showing that this residue in isoenzyme 4–4 was modified by the active site-directed reagent 1-(S-glutathionyl)-2,3-dioxo-4-bromobutane. Subsequent crystallographic studies of the isoenzyme 3–3-GSH complex located Tyr115 in domain II on the face of the a4-helix that forms one wall of the xenobiotic substrate binding site, the hydroxyl oxygen being about 7.5 Å from the sulfur of GSH (Ji et al., 1992). In this paper we provide crystallographic and kinetic evidence that the hydroxyl group of Tyr115 provides electrophilic assistance in the addition of GSH to an arene oxide substrate, a reaction in which the chem-
The crystal structure of isoenzyme 3-3 in complex with (9S,10S)-9-(S-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene, (9S,10R)-9-(S-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene, respectively. The difference appears to be due to a crystal packing effect. These observations are highly suggestive that the side chain of Tyr des 115 may also be quite close to the oxirane oxygen in the Michaelis complex with the substrate, PhenO. If the hydroxyl group of Tyr des 115 were to act as a hydrogen bond donor to the oxirane oxygen of the substrate, then it could provide electrophilic assistance in the transition state for oxirane ring opening.

Kinetic Properties of the Tyr 115F Mutant—The role postulated for Tyr des 115 from the crystal structure can be tested by site-specific mutagenesis. Removal of the hydroxyl group from the Tyr des 115 has a rather dramatic effect on the enzyme-catalyzed addition of GSH to PhenO, reducing kcat and kcat/Kmphen-o by 2 orders of magnitude (Table I). There is little difference in the stereoselectivity of the Tyr des 115 mutant (51% (S,S)-GSPhen) and the native enzyme (43% (S,S)-GSPhen) toward PhenO. Thus, the severely impaired catalytic efficiency of the mutant is consistent with the removal of an interaction crucial for the chemical step of the reaction and not a deleterious change in the conformation of the active site. Given the proximity of the hydroxyl group of Tyr des 115 to the hydroxyl group of the product in the crystal structure, it seems likely that the side chain of Tyr des 115 acts as a hydrogen bond donor to the oxirane oxygen which stabilizes, with roughly equal facility, the two diastereomeric transition states for opening of the oxirane ring. The magnitude of the transition state stabilization provided by the hydroxyl group, evaluated from the differences in kcat and kcat/Km, was quite different consequences for reactions in which the physical step of product release, rather than chemistry, is rate-limiting.

EXPERIMENTAL PROCEDURES

General Materials and Methods—The substrates CDNBl and Phen-O were obtained as previously described (Cobb et al., 1983). The products (S,S)- and (R,R)-GSPhen and GSDNB were synthesized by the method described by Chong et al. (1987). X-ray crystal structure determinations were carried out by the general methods described by Ji et al. (1992) and will be described in detail elsewhere.2 Reaction kinetics were determined as previously described (Liu et al., 1992) at pH 7.0 and 25°C. Kinetic constants were derived by fitting initial velocity data to a hyperbola with the program HYPER (Gland, 1979). DNA sequencing was performed with Sequenase version 2 from U. S. Biochemical Corp. with the enzyme described by Tabor and Richardson (1989).

Preparation of the Tyr 115F Mutant—An expression vector encoding the Tyr des 115F mutant was prepared by using overlap extension for site-directed mutagenesis via the polymerase chain reaction (Higuchi, 1983). The products CDNBl and Phen-O were obtained as previously described (Cobb et al., 1983). The products (S,S)- and (R,R)-GSPhen and GSDNB were synthesized by the method described by Chong et al. (1987). X-ray crystal structure determinations were carried out by the general methods described by Ji et al. (1992) and will be described in detail elsewhere.2 Reaction kinetics were determined as previously described (Liu et al., 1992) at pH 7.0 and 25°C. Kinetic constants were derived by fitting initial velocity data to a hyperbola with the program HYPER (Gland, 1979). DNA sequencing was performed with Sequenase version 2 from U. S. Biochemical Corp. with the enzyme described by Tabor and Richardson (1989).

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Kinetic constants for the enzyme-catalyzed additions of GSH to PhenO and CDNB and rate constants for dissociation of the corresponding products

| Substrate or product | Enzyme | $k_{cat}$ | $k_{cat}/K_{m}$ | $k_{cat}^\theta$ |
|----------------------|--------|----------|----------------|-----------------|
| PhenO                | Native | 0.39 ± 0.05 | (1.2 ± 0.1) $\times$ 10^4 | 2.6 ± 0.4 |
| (R,R)-GSPhen         | Y115F  | 0.0044 ± 0.0001 | (5.4 ± 0.3) $\times$ 10^4 | 6.6 ± 0.9 |
| (S,S)-GSPhen         | Y115F  | 20.0 ± 2 | (8.4 ± 0.5) $\times$ 10^4 | 6.3 ± 1.5 |
| GSDNB                | Native | 72.0 ± 2 | (1.4 ± 0.04) $\times$ 10^4 | 17. ± 1 |
| CDNB                 | Y115F  | 20.0 ± 2 | (8.4 ± 0.5) $\times$ 10^4 | 3.2 ± 1.5 |

$^\theta$ Measured by stopped flow fluorescence.

TABLE I

FIG. 2. Dependence of the reciprocal of the relative turnover numbers ($k_{cat}/k_{cat,\theta}$) on the relative viscosity ($\eta/\eta^\circ$) for the substrate CDNB with the native enzyme (●) and Y115F mutant (△) and for PhenO with the native enzyme (●) and Y115F mutant (○). Slopes of the dependences derived from least squares fit of the experimental data are 1.05 ± 0.08 and 1.05 ± 0.24 for the native enzyme- and Y115F-catalyzed additions to CDNB, respectively, and approximately zero for the additions to PhenO. Error bars represent propagated errors in the ratio from individual errors in $k_{cat,\theta}$ obtained from the program HYPER (Cleland, 1979). The two lines represent the expected effect of the relative viscosity where the rate-limiting step is a diffusional process (slope = 1.0) and where diffusion is not rate-limiting (slope = 0).

Expected that $k_{cat}$ will decrease as $\eta$ increases. Thus, $k_{cat}^\theta$ observed at a reference viscosity $\eta^\circ$ will be related to $k_{cat}$ observed at some higher viscosity, $\eta$, in the presence of a viscogen by $k_{cat}^\theta/k_{cat} = \eta/\eta^\circ$. A plot of the inverse relative rate constant versus the relative viscosity ($\eta/\eta^\circ$) should be linear with a unit slope when the release of product is limited by a strictly diffusional barrier or should have a slope approaching zero if chemistry or another non-diffusional barrier is rate-limiting. The inverse relative rate constant for the enzyme-catalyzed reaction with CDNB shows the expected linear dependence on the relative viscosity with a slope (1.0 ± 0.08) very close to unity as illustrated in Fig. 2. In contrast, the reaction with PhenO shows no detectable dependence on viscosity. Moreover, the viscosity dependences of the Y115F-catalyzed reactions are the same as those catalyzed by the native enzyme, indicating that the mutation does not change the identity of the rate-limiting step in either reaction. Both sets of results support the conclusions that a chemical barrier limits the turnover of PhenO whereas the turnover of CDNB is limited by a diffusional barrier.

**Conclusions**—Tyrosine 115 from the xenobiotic substrate binding domain of isoenzyme 3--3 of GSH transferase has been

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1. Kinetics of the enzymatic reaction with PhenO and CDNB, showing the dependence of the turnover numbers on the relative viscosity.

2. Table listing the kinetic constants for the enzyme-catalyzed additions of GSH to PhenO and CDNB.

3. The frequency of diffusion encounter (or its microscopic reverse, separation) of two molecules is inversely proportional to the viscosity, $\eta$, of the medium as anticipated by the Stokes-Einstein relationship (Kramers, 1940). If the turnover number of an enzyme is limited by the rate of product release, then it is

4. Intermediate values of the slope (0 < slope < 1) could be indicative of a viscosity-dependent segmental motion or conformational isomerization of the protein in which the solvent viscosity effect is damped by the "internal friction" of the protein motion (Anasser et al., 1992; Sampson and Knowles, 1992) or to a nonspecific effect of the viscogen on the reaction.
and the Mechanism of Glutathione Transferase

identified by x-ray crystallography and by comparison of the kinetic properties of the Y115F mutant to the native enzyme, as a residue that directly participates in catalysis. Two substrates, which have distinctly different requirements for transition state stabilization and different rate-limiting steps, were used to dissect the contribution of the hydroxyl group of Tyr115 to both chemical and physical (product release) steps in catalysis. Although the hydroxyl group appears to provide substantial electrophilic assistance in the addition of GSH to epoxides, accelerating the formation of product, it also seems to slow the egress of products from the active site. The net result of the Y115F mutation is to increase the substrate selectivity ratio ($k_{cat}/K_m^{CDNB}/k_{cat}/K_m^{PhenO}$) from about 70 in the native enzyme to close to 26,000 in the mutant.

REFERENCES

Ansari, A., Jones, C. M., Hensery, E. R., Hofrichter, J., and Eaton, W. A. (1992) Science 256, 1796-1798
Armstrong, R. N. (1991) Chem. Res. Toxicol. 4, 331-140
Chung, H., Harvey, R. G., Armstrong, R. N., and Jarabak, J. (1987) J. Biol. Chem. 262, 12445-12451
Cleland, W. W. (1979) Methods Enzymol. 63, 103-138
Cobb, D., Boehlert, C., Lewis, D., and Armstrong, R. N. (1983) Biochemistry 22, 805-812

FIG. 3. Stereo view of the hydrogen bond interaction (dashed line) between Tyr$^{115}$ located on the α4-helix and the side chain of Ser$^{200}$ located in the C-terminal tail. The α4/α5 helix-turn-helix and the C-terminal tail (both shown in the boldface line) appear to be held together by this interaction. GSH is shown in the boldface line bound at the bottom of the channel leading to the active site. The hydrogen bond to tyrosine 6 is also illustrated as a dashed line.

Higuchi, R. (1991) in PCR Technology (Erlich, H. A., ed) pp. 61-70, W. H. Freeman and Co., New York
Jakobson, L., Askarof, P., Warholm, M., and Mannervik, B. (1977) Eur. J. Biochem. 77, 253-262
Jakoby, W. B. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. 46, 383-414
Ji, X., Zhang, P., Armstrong, R. N., and Gilliland, G. L. (1992) Biochemistry 31, 10169-10184
Johnson, K. A. (1986) Methods Enzymol. 134, 977-703
Katuta, R. M., and Colman, R. F. (1991) Biochemistry 30, 11230-11238
Kolm, B. H., Suga, G. E., and Mannervik, B. (1992) Biochem. J. 285, 537-540
Liu, S., Zhang, P., Ji, X., Johnson, W. W., Gilliland, G. L., and Armstrong, R. N. (1992) J. Biol. Chem. 267, 4296-4299
Kramers, H. A. (1940) Physica (Utrecht) 7, 284-304
Mannervik, B. (1983) Adv. Enzymol. Relat. Areas Mol. Biol. 57, 357-417
Mannervik, B., and Danielson, U. H. (1988) CRC Rev. Biochem. 23, 283-337
Pickett, C. B., and Lu, A. Y. H. (1989) Annu. Rev. Biochem. 58, 743-764
Reineser, P., Dier, H. W., Ladesreine, R., Schaifer, J., Gallay, O., and Huber, R. (1991) EMBO J. 10, 1997-2005
Sampson, N. S., and Knowles, J. R. (1992) Biochemistry 31, 8499-8494
Tabor, S., and Richardsen, C. C. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4076-4080
Widersten, M., Kolm, R. H., Bjornestedt, R., and Mannervik, B. (1992) Biochem. J. 286, 377-381
Zhang, P., and Armstrong, R. N. (1990) Biopolymers 29, 159-169
Zhang, P., Graminski, G. F., and Armstrong, R. N. (1991) J. Biol. Chem. 266, 19475-19479
Zhang, P., Liu, S., Shan, S., Ji, X., Gilliland, G. L., and Armstrong, R. N. (1992) Biochemistry 31, 10185-10193