Vol. 184, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1992 Pages 194-197

TYROSINE-7 IN HUMAN CLASS Pi GLUTATHIONE *S*-TRANSFERASE IS IMPORTANT FOR LOWERING THE pK_a OF THE THIOL GROUP OF GLUTATHIONE IN THE ENZYME-GLUTATHIONE COMPLEX

Kwang-Hoon Kong, Kazue Takasu, Hideshi Inoue, and Kenji Takahashi[1]

Department of Biophysics and Biochemistry, Faculty of Science,
The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Received February 21, 1992

SUMMARY: Previously, we reported the importance of Tyr7 for the catalytic activity of human class Pi glutathione *S*-transferase [Kong *et al.* (1992) *Biochem. Biophys. Res. Comm.*, 182, 1122]. As an extention of this study, we investigated the pH dependence of kinetic parameters of the wild-type enzyme and the Y7F mutant. The replacement of Tyr7 with phenylalanine was found to alter the pH dependence of V_{max} and V_{max}/K_m^{CDNB} of the enzyme for conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB). The pK_a of the thiol of GSH in the wild-type enzyme-GSH complex was estimated to be about 2.4 pK units lower than that in the Y7F-GSH complex. Tyr7 is thus considered to be important for catalytic activity in lowering the pK_a of the thiol of GSH in the enzyme-GSH complex.

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a family of multifunctional proteins. One of the functions is catalyzing the formation of conjugates between reduced glutathione (GSH) and a wide variety of electrophilic compounds [1-3]. In mammals the cytosolic GST isoenzymes can be grouped into at least three distinct classes, Alpha, Mu and Pi, according to their structures and catalytic properties [4]. They are homo- or hetero-dimers of subunits of about 210-220 amino acid residues. Although there are many investigations on the structures and functions of GSTs, the reaction mechanism is unknown.

Chen *et al.* [5] mentioned that one major contribution of GST to catalysis is to lower the pK_a of the bound nucleophile, GSH. However, the reason for the low pK_a value of the bound GSH is unknown. The participation of a general base in the catalytic mechanism is expected and its pK_a is estimated to be >7.5 in rat GST4-4 [5], and 7.6 in rat GST3-3 [6]. However, the essentiality of a histidine residue(s) as a general base in the catalytic mechanism of GSH-conjugating reaction by human class Pi GST (GSTπ) was rejected by the study using site-directed mutagenesis [7]. Histidine residues were also reported to be unimportant for the catalytic activities of other classes of GSTs [8,9].

[1]To whom correspondence should be addressed.

The abbreviations used are: GST, glutathione S-transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene.

0006-291X/92 $1.50

Stenberg *et al.* [10] reported that Tyr8 in human class Alpha GST A1-1 is essential to the catalytic reaction. We also reported that Tyr7 in GSTπ, the counterpart of Tyr8 in GST A1-1, is essential to the catalytic reaction [11]. Since this tyrosine residue is conserved in all classes of cytosolic GSTs, it is considered to be essential and participate in the enzymatic reaction mechanism of GSTs commonly over the classes. The three-dimensional structure of the glutathione sulfonate-bound class Pi GST from pig lung, reported by Reinemer *et al.* [12], shows that the sulfonate group is located adjacent to the hydroxyl group of Tyr7. Accordingly, the thiol group of GSH in the GST-GSH complex is also expected to lie in the immediate neighborhood of the hydroxyl group of Tyr7. Therefore, Tyr7 seemed to participate in lowering the pK_a of the thiol of bound GSH in the enzyme. In order to clarify whether Tyr7 plays such a role, we examined the effect of mutation of Tyr7 on the pH dependence of the catalytic activity of GSTπ.

MATERIALS AND METHODS

Materials. Wild-type human GSTπ was obtained by expression of a cloned cDNA, gifted by Prof. Muramatsu [13], in *E. coli* as described in the previous paper (7). GSH and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Kohjin Co. and Wako Pure Chem. Ind., respectively.

Preparation of mutant enzymes. The preparation of the mutant Y7F was reported in the previous paper [11].

Enzyme assays. Assay of the enzyme activity was done as described in the previous papers [7,11]. The dependence of V_{max} on pH was determined by using the following buffers (0.1 M) at the indicated pH: Bis-Tris-HCl, from 5.5 to 7.0; Tris-HCl, from 7.5 to 9.0; sodium 3-cyclohexylaminopropanesulfonate, 9.5. Reaction was carried out at saturating GSH (2.5 mM) and variable CDNB concentrations.

RESULTS

The V_{max} and V_{max}/K_m^{CDNB} values of the wild-type GSTπ and Y7F for GSH-CDNB conjugating reaction were determined under the conditions of saturating GSH and at various pH values. The dependence of V_{max} on pH is shown in Fig. 1A. The V_{max} of the wild type increased

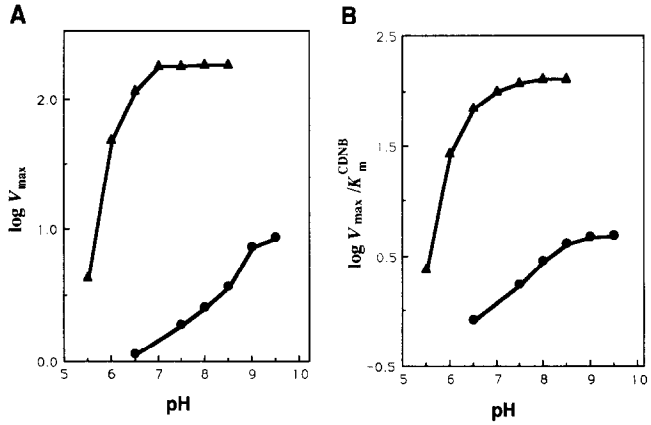
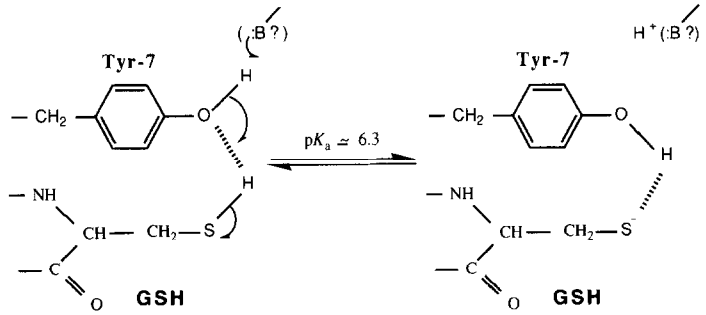


Figure 1. Dependence of V_{max} (A) and V_{max}/K_m^{CDNB} (B) values on pH. The kinetic parameters of the wild type and Y7F for the conjugation of GSH with CDNB were determined under the conditions of saturating GSH (2.5 mM) and variable concentrations of CDNB (0.2 - 1.0 mM). —▲—, wild type; —●—, Y7F.

with raising pH and reached a maximum at pH 7.0. On the other hand, the replacement of Tyr7 with phenylalanine shifted the optimal pH of the enzyme to higher pH. The V_{max} of Y7F increased with raising pH and reached a maximum at pH >9.5 that was at least 2.5 pH units higher than that of the wild type, although the enzymatic reaction rate of GST was not estimated so accurately at alkaline pH, above 8.0, due to the rapid nonenzymatic reaction. The dependence of V_{max}/K_m^{CDNB} values of them on pH was similar to that of V_{max} values (Fig. 1B). From the plots of $\log(V_{max}/K_m^{CDNB})$ against pH, the pK_a values of the thiol of GSH in the wild type-GSH and the Y7F-GSH complexes were estimated to be approximately 6.3 and 8.7, respectively. A similar dependence on pH was observed in potassium phosphate buffer, although the reaction rate was faster than in Tris-HCl or Bis-Tris-HCl buffer (data not shown).

DISCUSSION

One major contribution of GST to catalysis is considered to lower the pK_a of the thiol of GSH bound to the active site [5]. The participation of a general base is expected, but it has been unknown which residue is important for lowering the pK_a. In the previous paper, we reported the importance of Tyr7 for the catalytic activity of GSTπ [11]. However, the way of the participation of Tyr7 in the reaction mechanism was unknown. Therefore, in order to clarify whether the important residue, Tyr7, contributes to lowering the pK_a of the thiol of bound GSH, we examined the pH dependence of the V_{max} and V_{max}/K_m^{CDNB} values of the wild type and Y7F for the conjugation of GSH with CDNB (Fig. 1). The dependence of V_{max} and V_{max}/K_m^{CDNB} on pH is expected to reflect the pK_a of the thiol group in the enzyme-GSH complex [5,14]. From the pH-logV_{max} and the pH-$\log(V_{max}/K_m^{CDNB})$ profiles, the pK_a of the GSH in the wild type-GSH complex was estimated to be about 6.3 that was consistent with the published data [5,6]. On the other hand, from the pH-$\log(V_{max}/K_m^{CDNB})$ profiles, the pK_a of the thiol of GSH in the Y7F-GSH complex was estimated to be around 8.7, which was about 2.4 pK units higher than that of the wild type and close to that (9.1) of GSH in aqueous solution. Therefore, Tyr7 is suggested to be essential for lowering the pK_a and enhancing the nucleophilicity of the thiol of GSH in the active site of GSTπ. Recently, Entsch *et al.* [15] have reported that *para*-hydroxybenzoate hydroxylase shifts the pK_a of the bound substrate from 9.3 to 7.4 by the contribution of a tyrosine residue. A similar mechanism can be presumed also in the case of GST (Scheme 1).



Scheme 1. Putative mechanism. The pK_a of Tyr7 may be reduced by the electrostatic potential of the active site [12] or by interaction with another basic residue (:B).

The pK_a value of the putative base that contributes to reaction mechanism is estimated >7.5 in rat GST4-4 [5] and 7.6 in rat GST3-3 [6]. Since the estimated pK_a is much lower than that of a usual tyrosine residue, about 10, and since the pK_a of Tyr7 is unknown, it is unclear whether the putative base in rat GST4-4 or GST3-3 is tyrosine. However, Reinemer [12] pointed out that the active site may contribute by its electrostatic potential to a reduction in pK_a of both thiol and tyrosine groups, and that the N-termini of the two parallel helices are close to the G-site, where they may generate a positive electrostatic potential. The pK_a value of the Tyr7 in GSTπ is now being investigated by various methods. The contribution of an additional residue as a general base in cooperation with Tyr7 cannot be ruled out yet (:B in Scheme 1). The possibility is also under investigation.

Acknowledgment: We thank Professor Masami Muramatsu (Faculty of Medicine, The University of Tokyo) for providing us with human placental GSTπ cDNA.

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