Unusual reactivity of Tyr-7 of GSH transferase P1-1

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Reaction of human GSH transferase P1-1 (GSTP1-1) with diethylpyrocarbonate (DEPC) at pH 7.0 and 4 °C resulted in covalent modification of an equivalent of one histidine and one tyrosine residue per subunit, with loss of activity. Sequence analysis showed that His-71 and Tyr-7 were modified. Reference to the three-dimensional structure of GSTP1-1 [Reinemer, Dirr, Ladenstein, Huber, Lo Bello, Frederici and Parker (1992) *J. Mol. Biol.* 227, 214–226] shows that the modification of Tyr-7 is most likely to affect enzyme activity. Kinetic analysis of the DEPC modification of Tyr-7 in GSTP1-1 gave a k_2 approx. 150 times

that of a peptide comprising residues 1–11 of GSTP1-1. The reaction of Tyr-7 of GSTP1-1 with DEPC was poorly inhibited by 1 mM GSH (14 %) or 10 μ M *S*-hexylglutathione (18 %). DEPC treatment of the enzyme altered the absorbance at 290 nm in second-derivative spectra, suggesting that a significant amount of tyrosinate ion occurs in the enzyme. GSH, however, did not significantly alter the A_{290} . The data provide the first evidence of unusual chemical reactivity of Tyr-7 and are consistent with its proposed role as a proton acceptor during catalysis.

INTRODUCTION

Studies of the active site of GSH transferases (GSTs) initially involved covalent modification with side-chain-specific reagents which implicated cysteine residues in the activity of rat GST1-1 (Carne et al., 1979) and a histidine residue in the activity of human GSTM1b-1b (Awasthi et al., 1987). The latter study utilized diethylpyrocarbonate (DEPC) as modifier, which similarly caused inactivation of GSTP1-1 concomitant with modification of one histidine residue (C.-L. Xia and H. Chen, unpublished work). More recently, the elucidation of the three-dimensional structure of porcine GSTP1-1 (Reinemer et al., 1991) and human GSTP1-1 (Reinemer et al., 1992) has shown that a histidine residue does not occur in the vicinity of the active site. Furthermore, histidine residues have been found to be non-essential in catalysis by site-directed mutagenesis in the case of rat GST1a-1a (Wang et al., 1991, 1992), human GSTP1-1 (Kong et al., 1991) and human GSTM1-1 (Widersten and Mannervik, 1992), and by n.m.r. spectroscopy in the case of rat GST3-3 (Zhang et al., 1991) and human GSTM1-1 (Pennington and Rule, 1992). Attention has now focused on a tyrosine residue near the N-terminus, found in all four classes of soluble GSTs, as a group which might participate in the catalytic mechanism (Reinemer et al., 1991; Stenberg et al., 1991; Liu et al., 1992; Kolm et al., 1992; Kong et al., 1992; Manoharan et al., 1992). In the present work the effect of DEPC on GSTP1-1 is studied in more detail, providing the first direct evidence that Tyr-7 is unusually reactive and indicating that this residue may function in catalysis as a proton acceptor.

MATERIALS AND METHODS

Purification of GSTP1-1

GSTP1-1 was purified from fresh placenta or *post-mortem* kidney. Steps were carried out at 0–4 °C unless otherwise stated. Tissue was homogenized in a Waring blender in 0.16 M KCl, 2 mM dithiothreitol, 25 μ M phenylmethanesulphonyl fluoride and

10 mM sodium phosphate, pH 7.0 (3 ml/g). The soluble supernatant was obtained by centrifugation at 100 000 *g* for 1.5 h and the GST pool was obtained by affinity chromatography as described by Vander Jagt et al. (1985), except that 5 mM *S*-hexylglutathione was added to the elution buffer. The GST fraction was dialysed for 16 h against 30 vol. of 5 % (v/v) glycerol, 2 mM dithiothreitol and 15 mM sodium phosphate, pH 6.8, and applied to a column of hydroxyapatite prepared in the same buffer. The fraction that was neither retained nor retarded by hydroxyapatite was concentrated by Centriprep, transferred into argon-saturated 10 % (v/v) glycerol, 2 mM dithiothreitol and 20 mM Tris/HCl, pH 7.8, using Sephadex G-25 and purified by anion-exchange f.p.l.c. (Mono Q HR 5/5) in this buffer, eluting with a gradient of NaCl. GSTP1-1 so obtained was > 98 % pure according to reverse-phase h.p.l.c. (Meyer et al., 1989), and activity with 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al., 1974) was in the range 110–150 μ mol/min per mg of protein. Aliquots were snap-frozen and kept at –20 °C until use.

Quantification of histidine residue modification and GSTP1-1 inactivation by DEPC

A solution of 250 mM DEPC in dry ethanol was freshly prepared. Aliquots of GSTP1-1 (10 μ M) in buffer A (2 mM EDTA and 0.1 M sodium phosphate, pH 7.0) were incubated with 0, 0.5, 1, 2 or 5 mM DEPC [in ethanol at 2 % (v/v) in each case] for 45 min at 4 °C. The extent of N-carbethoxylation of histidine residues was determined from measuring the increased absorbance at 242 nm ($\epsilon = 3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Ovádi et al., 1967) compared with control samples lacking only the GST. The activity of treated samples towards CDNB was determined.

Identification of DEPC-modified histidine residue(s)

Samples of GSTP1-1 (75 μ g) in buffer A were treated with 0 or 5 mM DEPC at 4 °C for 1 h as described above, then one-fifth volume of 1.0 M ammonium bicarbonate, pH 8.4, was added

with 3 μ g of chymotrypsin. After incubation at 25 °C for 16 h, portions were analysed by reverse-phase h.p.l.c. using a Brownlee Aquapore RP 300 (30 mm $\times$ 2.1 mm) C8 column. Elution was with a gradient of 2–45% (v/v) acetonitrile in 0.06% (v/v) trifluoroacetic acid at 0.13 ml/min for 1 h, and absorbance was monitored at both 214 and 242 nm. Fractions yielding a relatively high A_{242}/A_{214} , indicative of a carbethoxyhistidine residue, were subjected to automated gas-phase sequencing (Applied Biosystems 470A).

Spectral analysis of tyrosine residue modification by DEPC

To detect modification of tyrosine by DEPC, a sample of GSTP1-1 in buffer A was treated with 5 mM DEPC for 1 h at 4 °C as described above and the u.v. absorption spectrum was obtained using a GSTP1-1 sample treated with ethanol alone as a reference. A baseline was obtained from the spectrum of 5 mM DEPC in buffer A after 1 h at 4 °C using buffer A plus ethanol as reference. Tyrosine residue modification was quantified from the decrease in absorbance in the region of 278 nm ($\epsilon = 1310 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Burstein et al., 1974).

Identification of the DEPC-modified tyrosine residue

Since Tyr-7 was implicated in the active site of GSTP1-1 (Reinemer et al., 1991), its modification by DEPC was sought directly by sequence analysis of the N-terminal region of the enzyme treated as described above with DEPC.

Comparison of DEPC reactivity of tyrosine residues of GSTP1-1 and synthetic peptides

A synthetic peptide corresponding to residues 1–11 of the GSTP1 subunit [GSTP1-(1–11)-peptide] was kindly provided by J. Westwood, Institute of Cancer Research, Sutton, Surrey, U.K., and its sequence verified. Initially, the peptide, in buffer A, was treated with 5 mM DEPC for 1 h at 4 °C and tyrosine modification was analysed by spectral changes and sequencing as described above. To increase the reaction rate of the peptide tyrosine residues, the reaction was subsequently carried out in buffer B (1 mM EDTA and 0.1 M potassium phosphate, pH 7.6) at 20 °C, with DEPC in buffer B as reference. The reaction was monitored by repetitive spectral scanning from 320 to 260 nm using a Perkin-Elmer Lambda 5 spectrophotometer with P1 printer, with a reference lacking peptide. For comparison, GSTP1-1 was similarly treated at 20 °C but in buffer C (1 mM EDTA, 0.1 M potassium phosphate, pH 6.60), to reduce the reaction to a measurable rate. Pseudo-first-order and second-order rate constants for the reaction of tyrosine with DEPC were calculated from the decrease in absorbance in the region of 280 nm.

Derivative spectroscopy

Second-derivative spectra were obtained in real time, scanning at $60 \text{ nm} \cdot \text{min}^{-1}$ with a delta wavelength of 3 nm (spectrophotometer as above).

Effect of GSH or S-hexylglutathione on DEPC modification of GSTP1-1

Aliquots of 4 μ M GSTP1-1 in buffer A were treated with 10 mM DEPC for 1 h at 4 °C in the presence or absence of 1 mM GSH or 10 μ M S-hexylglutathione, and activity towards CDNB was determined. The amounts of carbethoxytyrosine and tyrosine at residue 7 were determined by gas-phase sequencing.

Three-dimensional modelling of structural modification

The effect of carbethoxylation of His-71 and Tyr-7 was studied by computerized modelling using the atomic co-ordinates obtained for porcine GSTP1-1 (Reinemer et al., 1991) and human GSTP1-1 (Reinemer et al., 1992).

RESULTS

Modification of histidine residue

Reaction of GSTP1-1 with increasing concentrations of DEPC resulted in increased absorbance at 242 nm due to the formation of N-carbethoxyhistidine and loss of GST activity towards CDNB (Figure 1). By extrapolation, the equivalent of one histidine residue is modified with complete loss of activity. To ascertain which histidine residue(s) is affected, a sample treated with 5 mM DEPC was digested with chymotrypsin and peptides separated by reverse-phase h.p.l.c. (Figure 2). Fractions which showed relatively high A_{240}/A_{214} values were sequenced. Two peptides were obtained containing His-71 which accounted for the total expected yield of that residue. Histidine appeared unmodified in the sequencer cycle, presumably due to reversal of the modification during sequencing reactions. Other peptides sequenced did not contain the other histidine residue (162).

Modification of tyrosine residue

Spectral analysis of a sample of GSTP1-1 following treatment with 5 mM DEPC at 4 °C (Figure 3) showed that not only histidine but also tyrosine was modified. Similar yields were obtained: 0.80 histidine and 0.81 tyrosine residues modified per subunit. Since Tyr-7 has been implicated in the active site of GSTP1-1 (Reinemer et al., 1991), evidence of its modification by DEPC was sought by N-terminal sequencing. At residue 7, both tyrosine and a novel peak eluting later than the phenylthiohydantoin derivatives of natural amino acids were obtained (Figure 4). The latter peak is presumably due to carbethoxytyrosine. Assuming that the phenylthiohydantoin derivatives of

Figure 1 DEPC-concentration-dependence of inactivation and histidine residue modification of GSTP1-1

GSTP1-1 (10 μ M in buffer A) was treated at 4 °C with various concentrations of DEPC for 45 min. The activity towards CDNB and the extent of histidine residue modification were determined as described in the text.

Figure 2 Identification of the DEPC-reactive histidine residue

A sample of 75 μ g of DEPC-treated GSTP1-1 was digested with 3 μ g of chymotrypsin for 16 h at 25 $^{\circ}$ C and peptides were separated by Microbore reverse-phase h.p.l.c. as described in the text. The eluent was monitored at both 214 nm (continuous trace; 0–1.0 absorbance) and 242 nm (broken trace, 0–0.1 absorbance). Peptides likely to contain a carboxyhistidine residue (arrow-heads) were identified from a relatively high A_{242}/A_{214} value and subjected to automated microsequencing.

Figure 3 Absorption spectrum of DEPC-modified GSTP1-1

Difference spectra were obtained for GSTP1-1 (12 μ M) treated for 1 h at 4 $^{\circ}$ C with 5 mM DEPC in buffer A versus a duplicate lacking only DEPC (continuous line), and DEPC in buffer A after 1 h versus buffer A alone (broken line). Details are given in the text.

tyrosine and carboxytyrosine have similar molar absorption coefficients, the latter comprised 50 % of the total tyrosine. No DEPC modification of Tyr-3 was detected. Treatment of GSTP1-(1–11)-peptide under the same conditions did not result in

Figure 4 Identification of DEPC-reactive tyrosine residue

GSTP1-1 (12 μ M in buffer A) was treated with 10 mM DEPC at 4 $^{\circ}$ C for 1 h and a sample of approx. 150 pmol was subjected directly to automated microsequencing. The h.p.l.c. analysis for residue 7 (continuous trace) is compared with a standard mixture of amino acids (broken trace). The novel peak due to modification of Tyr-7 is arrowed.

modification of tyrosine residue(s) by DEPC according to spectral change or sequencing. The initial sequencing yield was less than expected, presumably due to reaction of DEPC with the N-terminal proline. This effect was more marked in the peptide.

The reaction of 5 mM DEPC with 28 μ M GSTP1-1 at pH 6.60

Figure 5 Rate of reaction of GSTP1-1 tyrosine residue with DEPC

Samples of GSTP1-1 (28 μ M in buffer C, at pH 6.6 and 20 $^{\circ}$ C) were treated simultaneously with either 5 mM DEPC (in ethanol) or an equal volume of ethanol. (a) Ten scans at 0.9 min intervals were made of their difference spectrum at 120 nm $\cdot$ min $^{-1}$, beginning 23 s after mixing. Samples of GSTP1-1-(1-11)-peptide (28 μ M in buffer B; pH 7.6) were similarly treated, with scans at 1.5 min intervals (results not shown). In each case the end point was taken at 20 min. (b) The pseudo-first-order rate constant for tyrosine modification was obtained from a plot of $\log [(A_{280} \text{ at } 20 \text{ min}) - (A_{280} \text{ at } t \text{ min})]$ for GSTP1-1 ($\bullet$) and GSTP1-(1-11)-peptide ($\circ$). The plots showed deviation from first-order kinetics due to the reaction of DEPC with solvent, and an estimate of the rate constant was made using the initial rate of modification (broken lines).

and 20 $^{\circ}$ C (Figure 5) yielded a value for the second-order rate of tyrosine modification (k_2) of approx. 140 M $^{-1}$ $\cdot$ min $^{-1}$. By contrast, reaction of the GSTP1-1-(1-11)-peptide with DEPC at pH 7.60 yielded a k_2 of approx. 9.2 M $^{-1}$ $\cdot$ min $^{-1}$. Thus Tyr-7 in GSTP1-1 is about 150 times as reactive as Tyr-7 in the N-terminal peptide.

Comparison of the second-derivative spectra of GSTP1-1 obtained before and after treatment with DEPC showed reductions in the minima corresponding to peaks of absorbance at 290, 280 and 275 nm (Figure 6a), suggesting that Tyr-7 contributes to

the normal spectrum of GSTP1-1 at these wavelengths. Neither GSH (Figure 6b) nor *S*-hexylglutathione (results not shown) significantly altered the second-derivative spectrum of GSTP1-1 at 290 nm, but both caused a similar but smaller effect to that of DEPC at 280 nm. The major peaks of absorbance of tyrosine residues in the GSTP1-(1-11)-peptide according to minima in the second-derivative spectrum were 284 and 275 nm (Figure 6c).

In order to investigate further the possible interaction of Tyr-7 with the GSH thiol at the active site of GSTP1-1, the effects of GSH, *S*-hexylglutathione and *S*-octylglutathione on the DEPC-dependent inactivation were determined. GSH and *S*-hexylglutathione showed little protective effect against inactivation of GSTP1-1 by 10 mM DEPC at 4 $^{\circ}$ C (Table 1). *S*-Octylglutathione was more effective. In a similar experiment using 5 mM DEPC, the effect of GSH and *S*-hexylglutathione on the degree of inactivation was correlated with the proportion of carbethoxylation of Tyr-7 determined by sequencing (Table 2). The extent of apparent modification was correlated with the degree of inactivation, but was approximately half of the inactivation in each case. It is likely that the relatively low proportion of modified tyrosine is a result of a partial reversal of the modification during the cycles of sequencing.

DISCUSSION

The results clearly show that DEPC treatment of GSTP1-1 results in modification of His-71. No evidence of modification of His-162 was obtained, presumably due to its relative inaccessibility. His-162 is located in the C-terminal region of the helix α F, with the side chain pointing down to the inner region of the molecule. Both N $_{\alpha 2}$ and N $_{\alpha 1}$ of His-162 are within hydrogen bonding distance of Tyr-116 and Leu-156 respectively. His-71 occurs in a region of high sequence conservation among GSTs yet is unique to the Pi class. In classes Alpha, Mu and Theta it is a tyrosine residue. His-71 is situated in helix α C, which is involved in subunit-subunit interaction and is located 21.7 $\text{\AA}$ (2.17 nm) from the G-site (Reinemer et al., 1992). Visual inspection of the structural model shows that His-71 is accessible to small molecules such as DEPC and that carbethoxylation is unlikely to result in significant local or distal conformational changes. Therefore modification of His-71 is unlikely to be involved in the DEPC-dependent inactivation of GSTP1-1. It is likely, therefore, that the major contribution to the inactivation of GSTP1-1 by DEPC is the modification of Tyr-7.

Tyr-7 is conserved in all four families of soluble GSTs (Mannervik and Danielson, 1988; Meyer et al., 1991). According to crystallographic analysis, the phenolic group is within hydrogen bonding distance of the sulphur of bound glutathione sulphonate in the case of porcine GST π (Reinemer et al., 1991) or *S*-hexylglutathione in GSTP1-1 (P. Reinemer, unpublished work). Furthermore, it was suggested that Tyr-7 is activated as a proton acceptor by the neighbouring dipole of helix α A to assist in ionization of the GSH thiol (Reinemer et al., 1991). Through mutational replacement by phenylalanine, Tyr-7 has been shown to be important for the activity of GSTP1-1 (Kolm et al., 1992; Kong et al., 1992; Manoharan et al., 1992); likewise, Tyr-8 for GSTA1-1 (Stenberg et al., 1991) and Tyr-6 for rat GST3-3 (Liu et al., 1992). The reaction of tyrosine residues with DEPC is anomalous, although there is a precedent (Burstein et al., 1974), and demonstrates for the first time that Tyr-7 of GSTP1-1 is activated towards electrophilic attack in comparison with, for example, Tyr-3 in the enzyme itself or tyrosines 3 and 7 in a peptide corresponding to its N-terminal region. If the increased reactivity of Tyr-7 in GSTP1-1 were due solely to increased ionization, the kinetic data obtained would indicate a

Figure 6 Comparison of effects of GSH and DEPC treatment on the second-derivative spectrum of GSTP1-1

Second derivative spectra were obtained, as described in the text, of: (a) GSTP1-1 (8 μ M in buffer C) untreated (continuous trace) or treated with 10 mM DEPC (broken trace); (b) GSTP1-1 (8 μ M in buffer A) in the absence (continuous trace) or presence (broken trace) of 1 mM GSH, and (c) GSTP1-(1-11)-peptide (80 μ M in buffer B) untreated (continuous trace) or treated with 10 mM DEPC (broken trace).

Table 1 Effect of GSH and *S*-hexylglutathione on inactivation of GSTP1-1 by DEPC

GSTP1-1 (3 μ M) in buffer A was treated at 4 $^{\circ}$ C for 1 h with 10 mM DEPC in the presence or absence of 1 mM GSH, 10 μ M *S*-hexylglutathione or 10 μ M *S*-octylglutathione. GST activity towards CDNB was determined in triplicate assays, using parallel incubations lacking DEPC as a reference. Results are means $\pm$ S.D. Significant difference from control was assessed by Student's *t* test. **P* < .001.

Sample	Activity remaining (%)
GSTP1-1 + DEPC	2.3 $\pm$ 0.4
GSTP1-1 + DEPC + GSH	16.8 $\pm$ 0.9*
GSTP1-1 + DEPC + <i>S</i> -hexylglutathione	20.4 $\pm$ 1.7*
GSTP1-1 + DEPC + <i>S</i> -octylglutathione	72.5 $\pm$ 7.8*

Table 2 Comparison of yield of DEPC-modified Tyr-7 with inactivation of GSTP1-1

GSTP1-1 (3.5 μ M) in buffer A at 4 $^{\circ}$ C was treated with 5 mM DEPC for 45 min in the presence or absence of 0.5 mM GSH or 10 μ M *S*-hexylglutathione. GST activity towards CDNB was compared with that of non-DEPC-treated controls (duplicate assays). The samples were subjected to automated gas phase sequencing. The proportions of tyrosine and modified tyrosine were determined from their peak heights, assuming similar absorption coefficients for their phenylthiohydantoin derivatives. nd, not detected (< 0.5%).

Sample	Inhibition of activity (%)	Tyr-3 modified (%)	Tyr-7 modified (%)
GSTP1-1	74.7	nd	38.9
GSTP1-1 + GSH	70.2	nd	37.0
GSTP1-1 + <i>S</i> -hexylglutathione	43.9	nd	20.1

reduction in the pK_a of approx. 2.2. If Tyr-7 interacts with GSH to assist ionization, the reactivity of the tyrosine residue would be expected to be substantially decreased in the presence of GSH, but not greatly affected by *S*-alkylglutathiones. The results presented here of DEPC modification in the presence of GSH, *S*-hexylglutathione and *S*-octylglutathione do not support this proposal. Nor do the spectroscopic data which show that DEPC, but not GSH, reduces the absorption at 290 nm, which is likely to be due to the tyrosinate ion.

Recent pH-activity studies on GSTP1-1 and the mutant form having phenylalanine at position 7 show that at increased pH values (i.e. increased ionization of the tyrosine hydroxy group),

the wild-type but not the mutant enzyme is inactivated (Kolm et al., 1992). The authors conclude that the tyrosinate does not act as a base in assisting the ionization of GSH. Our results are consistent with these conclusions. However, in similar experiments relating pH and activity of wild-type and mutant GSTP1-1, Liu et al. (1992) obtained different results. No inactivation of wild type enzyme occurred at increasing pH and these authors concluded instead that Tyr-7 assisted in dissociation of the GSH thiol proton. It should be noted that in these experiments the mutant enzyme has a pH optimum at pH 9.5, when GSH is largely ionized, but its activity is still less than 10% of that of the wild-type form.

On the basis of the recent determination of the three-dimensional structure of rat GST 3-3, Ji et al. (1992) considered three possible mechanisms whereby the hydroxy group of Tyr-6 (equivalent to Tyr-7 of GSTP1-1) could assist the ionization of GSH: (1) the hydrogen bond Tyr-6-O-H $\cdots$ -S-G assists stabilization of the thiolate anion in the active site; (2) Tyr-6-O $\cdots$ -H-S-G occurs with a very low barrier to proton transfer; and (3) the proton resides in a single well potential, Tyr-6-O $\cdots$ -H $\cdots$ -S-G. The same mechanisms are appropriate for GSTP1-1. The results presented here suggest that the tyrosinate ion exists in GSTP1-1, that it is abolished by DEPC treatment and that it is little affected by the presence of GSH. Our data therefore favour mechanism 2 for GSTP1-1.

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