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Interaction of Glutathione Transferase from Horse Erythrocytes with 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole*

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7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole reacts with two thiol groups of the dimeric horse erythrocyte glutathione transferase at pH 5.0, with strong inactivation reversible on dithiothreitol treatment. The inactivation kinetic follows a biphasic pattern, similar to that caused by other thiol reagents as recently reported. Both *S*-methylglutathione and 1-chloro-2,4-dinitrobenzene protect the enzyme from inactivation. Analysis of the reactive SH group-containing peptide gives the sequence Ala-Ser-Cys-Leu-Tyr, identical with that of the peptide that contains the reactive cysteine 47 of the human placental transferase. In the presence of glutathione, the enzyme is not inactivated by this reagent, but it catalyzes its conjugation to glutathione. At higher pH values, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole reacts with 2 tyrosines/dimer and lysines, as well as with cysteines. Reaction with lysine seems essentially without effect on activity; whether the reactive tyrosines are important for activity could not be determined using this reagent only. However, 2 tyrosines among the 4 that are nitrated by tetranitromethane are important for activity.

Glutathione transferases (EC 2.5.1.18) (GST)[1] are dimeric proteins able to conjugate glutathione (GSH) to a variety of electrophilic compounds (1-3). Multiple isoenzymatic forms have been found in several mammalian tissues and tentatively grouped in three different classes (4). It has been demonstrated that this enzyme has one binding subsite for glutathione (G subsite) and another for the electrophilic substrate (H subsite) both located in each subunit (3), but the structure of these sites and the catalytic mechanism have not been yet defined. Kinetic studies suggest a sequential random mechanism for the reaction by GST (5); a thiol group, guanidino and amino groups, and histidine have been suggested to be present at the active site (6-9). Recently, we confirmed the importance of a thiol group in or near the active site of GST from horse erythrocytes (10). This isoenzyme is a variant of the P_i class transferases and possesses only one reactive thiol group per monomer, whose integrity is essential for the enzymatic activity (10). We also observed a nonequivalence of the two subunits of this protein in their reaction with sulfhydryl reagents, probably due to a nonsymmetrical association of two identical subunits or, alternatively, to heterodimeric structure composed by subunits of very similar charge and size (10). In the present study, we report the interaction of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) with the horse isoenzyme in order to obtain further insights on the topography of the active site. This reagent is a useful chemical probe able to modify selectively cysteine, tyrosine, and lysine residues, depending on the pH of the reaction, giving characteristic absorption spectra in the visible region (11-13). Moreover, this compound becomes highly fluorescent after reaction with cysteinyl and lysyl residues (13-15). Results reported in this paper confirm the presence of two SH groups per dimer that are important for the activity and that can be selectively modified by NBD-Cl at pH 5.0. In the presence of glutathione, NBD-Cl is used as a substrate by this transferase and it is enzymically conjugated to glutathione. At alkaline pH values, NBD-Cl reacts also with 2 tyrosines and 1 lysine. The lysine-modified enzyme has approximately the same activity as the native enzyme, whereas evidence is presented here that the integrity of 2 tyrosines may be important for the enzymatic activity.

EXPERIMENTAL PROCEDURES

Materials

Glutathione transferase was prepared from horse erythrocytes as previously described (10, 16). Specific activity in the standard assay conditions was 24 units/mg at 25 °C. NBD-Cl and tetranitromethane (TNM) were purchased from Aldrich (Germany). *N*-(4-Anilino-1-naphthyl)maleimide (ANM) was obtained from Fluka Chemie AG (Switzerland). TPCK-treated trypsin and *S*-methylglutathione were Sigma products. All other reagents were of reagent grade.

Glutathione transferase-cysteamine mixed disulfide (GST-SS-cysteamine) was prepared as follows. 100 nmol of enzyme were incubated with 10 mM cystamine in 2 ml of 0.1 M potassium phosphate buffer, pH 8.0, at 37 °C for 2 h. The excess of cystamine was removed by a Sephadex G-25 column (1 × 30 cm) equilibrated with 10 mM potassium phosphate buffer, pH 8.0. After this treatment, the enzyme is almost inactive (2.5% of the original activity) and it has no titratable SH groups, as judged by Ellman's procedure (17). The modified enzyme recovers the original activity after a 10-min reaction with 50 mM dithiothreitol (DTT) at pH 8.0.

Methods

Enzyme Assay—GST activity was assayed spectrophotometrically at 340 nm. The standard incubation mixture contained 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 2 mM GSH in 2 ml (final volume) of 0.1 M potassium phosphate buffer, pH 6.5, and 1 mM

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[1] The abbreviations used are: GST, glutathione transferase; GSH, glutathione; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; CDNB, 1-chloro-2,4-dinitrobenzene; TNM, tetranitromethane; GST-SS-cysteamine, glutathione transferase-cysteamine mixed disulfide; DTT, dithiothreitol; ANM, *N*-(4-anilino-1-naphthyl)maleimide; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

EDTA. One enzyme unit is the amount of enzyme that catalyzes the conjugation of 1 μmol of GSH/min at 25 °C. Spectrophotometric measurements were corrected for the spontaneous reaction of GSH with CDNB.

Reaction of GST with NBD-Cl—Reaction of GST with NBD-Cl was carried out at 25 °C in 0.1 M sodium acetate buffer, pH 5.0, 1 mM EDTA, and 1.5 μM enzyme. NBD-Cl was used from a 50 mM stock solution in dimethyl sulfoxide freshly prepared. At fixed times, 10–20-μl aliquots of the mixture were tested for GST activity in the standard assay conditions. Activity values were corrected from both the spontaneous inactivation of GST and the dimethyl sulfoxide-induced inactivation (about 10% after 1 h of incubation at 25 °C). The NBD-modified enzyme at pH 5.0 was prepared by incubating 2 mM NBD-Cl with 1 mg/ml GST in 0.1 M sodium acetate buffer, pH 5.0, and 1 mM EDTA for 3 h. The excess of NBD-Cl was removed by a Sephadex G-25 column (1 × 30 cm) equilibrated with the same buffer. The protein fractions were pooled for spectrophotometric and fluorometric analyses, performed with a double beam Uvikon 860 spectrophotometer (Kontron) and a SFM 25 spectrofluorimeter (Kontron), respectively. The NBD-modified enzyme at pH 8.0 was prepared as above after incubation with NBD-Cl in potassium phosphate buffer, pH 8.0, for 30 min. Molar extinction coefficients of NBD-protein tyrosine and lysine adducts were 11,600 at 380 nm and 26,000 at 475 nm, respectively, in a pH range of 7.5–8.5 (14, 15). The molar extinction coefficient of NBD-protein cysteine is 13,000 at 430 nm at pH 8.0 (13); the same value is obtained in a pH range of 5.0–8.0.[2]

Binding studies on affinity chromatography were performed by glutathione-Sepharose resin prepared, as described by Simons and Vander Jagt (18); about 0.3 mg of native GST or of NBD-modified enzyme were loaded on the affinity column (1 × 5 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, and 1 mM EDTA. Elution of the enzyme was performed as previously reported (10). The elution volume of the native or of the modified enzyme was measured by following the absorbance at 280 nm and/or the enzymatic activity.

Enzymatic Conjugation of NBD-Cl—Glutathione transferase activity with NBD-Cl as substrate was studied by following continuously the increase of absorbance at 430 nm due to the *S*-NBD-glutathione conjugate (ϵ_M = 13,000 M^{-1} cm^{-1}) (13). The standard assay mixture contained 0.5 mM GSH, 0.2 mM NBD-Cl, and 1 mM EDTA in 2 ml of 0.1 M acetate buffer, pH 5.0. The reaction was started by addition of the enzyme. Activity values were corrected for the spontaneous conjugation of GSH with NBD-Cl. Kinetic constants were calculated at pH 5.5 by varying independently the concentration of GSH and NBD-Cl in the ranges of 0.06–1 and 0.004–0.5 mM, respectively.

Inactivation by TNM—Nitration was performed essentially as described by Sokolovsky (19) on 50 nmol of GST-SS-cysteamine in 5 ml of 0.01 M potassium phosphate buffer, pH 8.0, at 37 °C under continuous shaking. The reaction was started by the addition of TNM (130 μM, final concentration) from a freshly prepared 60 mM stock solution in ethanol. The nitration progress was followed spectrophotometrically at 428 nm by using a molar extinction coefficient of 4,100 M^{-1} cm^{-1} for the nitrotyrosine residue (19). At fixed times, 0.1-ml aliquots were incubated with 50 mM DTT for 10 min at 37 °C, both to block the nitration reaction and to restore the protein SH groups (20). A control experiment was done with the same amount of GST mixed-disulfide similarly processed without TNM treatment. Protein concentration was estimated by the procedure of Lowry *et al.* (21).

Labeling of GST by ANM—Glutathione transferase (1 mg) was dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. ANM (1 μmol) was added to the enzyme (1 ml, final volume) and incubated for 30 min at 37 °C. After this treatment, the enzymatic activity was abolished and the reaction was stopped by the addition of 10 mM (final concentration) DTT. Reduction and carboxymethylation of the modified enzyme were performed according to a previously described procedure (23). *S*-Carboxymethylated GST was then digested with TPCK-treated trypsin in 0.1 M ammonium bicarbonate buffer, pH 8.0, at 37 °C for 24 h. Purification of peptides was carried out with the same chromatographic conditions as reported in Ref. 23.

Automated Edman degradation of the fluorescent peptide was performed using an Applied Biosystem model 470A gas-phase sequenator.

[2] G. Del Boccio, A. Pennelli, E. P. Whitehead, M. L. Bello, R. Petruzzelli, G. Federici, and G. Ricci, unpublished observations.

RESULTS

Reaction of GST with NBD-Cl at pH 5.0—GST from horse erythrocytes is inactivated by NBD-Cl at pH 5.0. About 10% of the original activity was recovered after 3 h of incubation with 0.15 mM NBD-Cl without GSH (Fig. 1*A*). A semilog plot of inactivation data revealed a biphasic pattern of inactivation (Fig. 1*B*) that resembles that obtained with other thiol-blocking reagents (10). By extrapolating the lower phase to zero time, it appears that the faster reaction causes a loss of activity of about 50%. Pseudo-first order kinetic constants are 0.086 and 0.0081 min^{-1} for the fast and slow reaction, respectively. Both these constants are linearly proportional to NBD-Cl concentration up to 1.5 mM concentration. The original activity can be completely recovered by treating the inactivated enzyme with 50 mM DTT for 15 min (Fig. 1*A*). NBD-Cl is a thiol-specific reagent below pH 7.0 (13). The interaction product of a protein cysteine-NBD adduct absorbs at 420–430 nm and fluoresces at 545 nm (excitation = 420 nm) (13). As expected, when the NBD-modified enzyme was separated from the unreacted NBD-Cl by Sephadex G-25 chromatography, it exhibits an absorption peak centered at 430 nm (Fig. 2*A*). On the basis of a molar absorption coefficient of 13,000 M^{-1} cm^{-1} (13), the sulfhydryl NBD adduct formed after 180 min of reaction was estimated to involve 2.15 thiol groups/mol of GST. Similarly, it was calculated that 1.2 thiol groups were modified after 30 min of reaction with NBD-Cl (about 50% inactivation). The absorbance at 430 nm almost disappeared upon addition of 50 mM DTT (Fig. 2*A*). Fluorometric analyses performed on the NBD-modified enzyme show a characteristic fluorescence at 545 nm indicative of a cysteine-NBD adduct, which also disappeared upon DTT treatment (Table I).

Localization of Reactive Thiol Group—Isolation and characterization of the peptide containing the NBD-modified cysteine were unsuccessful because the standardized treatment of the enzyme with DTT prior to carboxymethylation and tryptic digestion (22) removes the NBD group from the modified cysteine, as described above; moreover, during the trypsin digestion at pH 8.0, performed without the reduction and carboxymethylation steps, the NBD group migrates from the cysteine residue to a lysine residue as suggested by the shift of the absorption peak from 430 to 475 nm (data not shown). This migration has been reported to occur under alkaline conditions or upon illumination in other NBD-modified proteins (13, 24). Therefore, we modified the reactive cysteine with another specific fluorescent probe (ANM) recently used for the identification of a highly reactive sulfhydryl group of placental GST (23). After the ANM modification performed as described under "Experimental Procedures," the enzyme was completely inactive and unable to react with NBD-Cl as spectrophotometrically determined (data not shown). The high pressure liquid chromatography pattern after TPCK-treated trypsin digestion showed a single fluorescent peptide that elutes after 32 min. The automated Edman degradation gave the following sequence for the first 5 residues: Ala-Ser-Xxx-Leu-Tyr. This sequence is identical with that found for the peptide that contains the reactive cysteine 47 of the placental isoenzyme (23).

Further information on the functional localization of the reactive thiol group is given from protection experiments; the inactivations by NBD-Cl at pH 5.0 were performed in the presence of CDNB (5.0 mM) and *S*-methylglutathione (5.0 mM). At this pH, we found that CDNB does not cause any appreciable loss of activity when incubated with GST for 1 h. Both these compounds, which bind reversibly with the H and G subsites, respectively, protect the enzyme from NBD-Cl

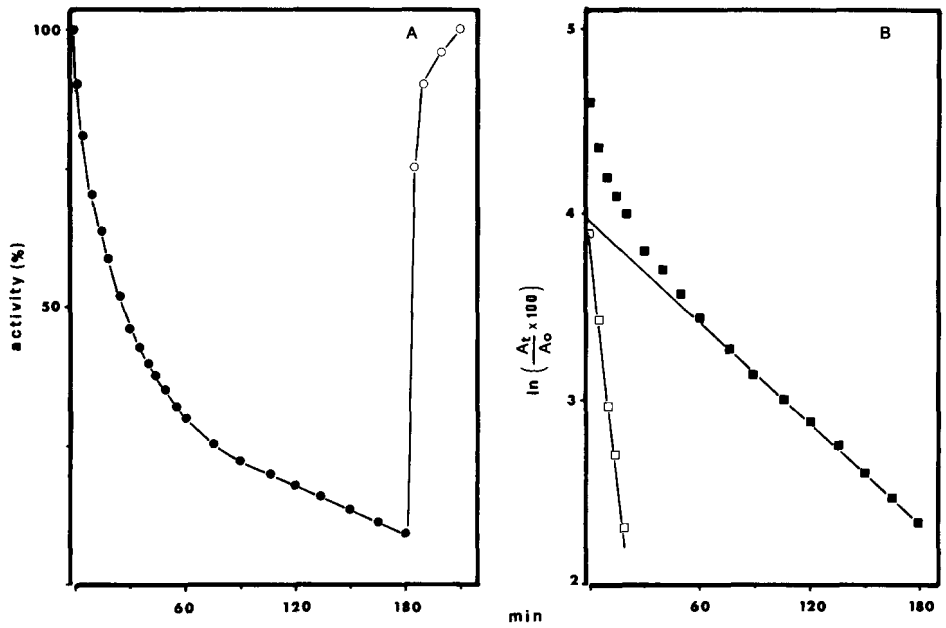

FIG. 1. **Inactivation pattern of GST by NBD-Cl.** *A*, ●, GST (1.5 μM) was incubated with 0.15 mM NBD-Cl in 0.01 M sodium acetate buffer, pH 5.0, and 1 mM EDTA at 25 °C. ○, reactivation after addition of 50 mM DTT. The enzymatic activity was assayed on 20-μl aliquots in the test conditions reported under "Experimental Procedures." *B*, semilog plot from the rate of inactivation from *A*. ■, experimental points; □, points obtained by subtracting the contribution of the slow inactivation from the observed data (25).

inactivation (Table II). Moreover, the inactivated enzyme is not bound or retarded by the glutathione-epoxy-Sepharose column used for affinity chromatography, indicating a structural change of the active site (data not shown).

NBD-Cl as GST Substrate—At pH 5.0, GST is not inactivated by NBD-Cl in the presence of GSH, but it catalyzes an active conjugation of GSH with this reagent. The enzymatic product has the same spectral properties and extinction coefficient as the *S*-NBD-glutathione adduct formed by the nonenzymatic reaction of GSH with NBD-Cl at pH 7.0 (ϵ_M = 13,000 cm^{-1} M^{-1} at 420 nm). The optimum pH value for the enzymatic reaction has been found between 5.0 and 6.0 (Fig. 3), and the K_m value, calculated at saturating GSH concentration, was found to be 30 μM. In the test conditions reported under "Experimental Procedures," GST has a specific activity of 0.8 units/mg with this new substrate, which represents 3.3% of that observed with CDNB and comparable with that obtained with ethacrynic acid (10). These data clearly support the idea that NBD-Cl binds to the H subsite with high affinity; this may be an indirect suggestion that the reactive SH group is located near this subsite.

Reaction of GST with NBD-Cl at pH 8.0—The inactivation of GST by NBD-Cl at pH 8.0 is faster than that observed at pH 5.0; after only 10 min of incubation with 0.15 mM NBD-Cl, the enzyme was almost completely inactivated. Moreover, the kinetic treatment of the inactivation data at pH 8.0, obtained with variable amounts of NBD-Cl, reveal a multiphasic inactivation pattern (data not shown). The inactivated enzyme was passed through a Sephadex G-25 column and then analyzed spectrophotometrically (Fig. 2*B*). The visible spectrum between 350–500 nm is complex and indicative of probable modifications of tyrosines, cysteines, and lysines whose NBD adducts absorb at 380, 430, and 475 nm, respectively (11–13). Also, a fluorometric analysis is consistent with the modification of lysines and cysteines (Table I). Upon DTT treatment, only the 475 nm absorption remains unchanged (Fig. 2*B*); in fact, it has been reported that DTT cannot restore the NBD-modified lysines (15). Kinetics of modification of these amino acids by NBD-Cl is complicated because of the overlapping of the visible and fluorometric spectra. The analysis was simplified by blocking the protein cysteines with cystamine before the NBD-Cl treatment. GST-SS-cysteamine, prepared as described under "Experimental Procedures," was reacted with NBD-Cl at pH 8.0. The appearance of two separated components with absorption maxima at 380 and 475 nm, respectively (Fig. 4) allows a more accurate identification and quantitation of the tyrosine- and lysine-NBD adducts. From the kinetic patterns of the lysine and tyrosine modifications shown in Fig. 5, it appears that 2 tyrosines/dimer react with NBD-Cl within 1 h of incubation, whereas only about 1 lysine is modified in the same period of time. In these conditions, the reaction with tyrosine stops while that with lysine still proceeds; this suggests that there are two particularly reactive tyrosines, whereas the reagent may be reacting indiscriminately with many lysines. DTT treatment allows the restoration of the tyrosines as shown by the disappearance of the 380 nm peak (Fig. 4); on the other hand, the lysine-NBD peak at 475 nm remains unchanged. In this condition, the enzyme recovers about 85% of the original specific activity (Fig. 5). The 15% inactivation does not seem to be due to the lysine modification because this amount of inactivation is obtained after only 5 min of reaction with NBD-Cl when only 0.1 lysine residue/dimer has reacted.

Reaction of GST with TNM—The above reported experiments do not clarify whether the tyrosine modification has any effect on the enzymatic activity, because we have not found conditions giving NBD-Cl reacted tyrosines and unreacted cysteines. Therefore, we treated the enzyme with tetranitromethane, a selective, mild reagent for the nitration of protein tyrosines (19). Since TNM may react also with protein sulfhydryls (19), we performed the nitration experiments on the cystamine-modified enzyme obtained as above. The nitration of the tyrosines was followed spectrophotometrically at 428 nm (19) and, at fixed times, the enzymatic

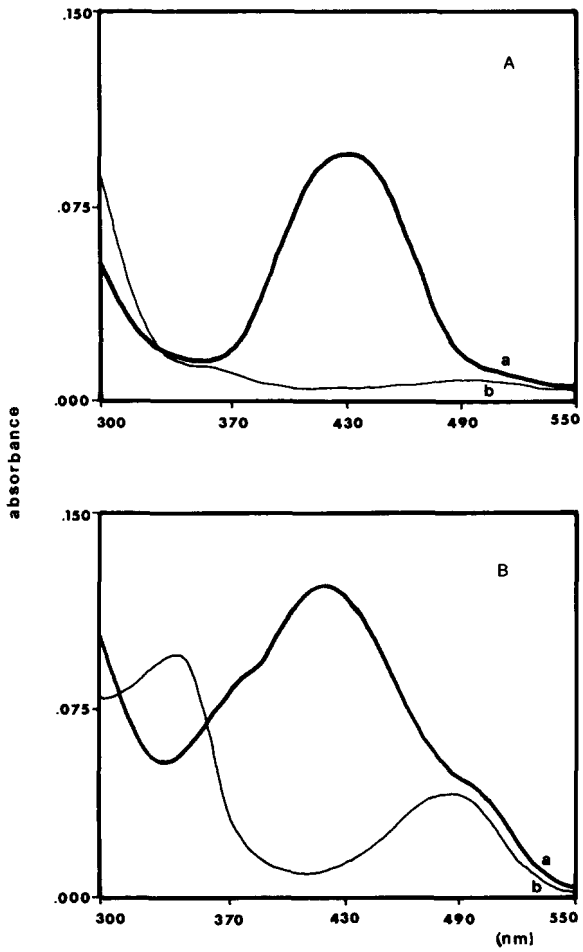

FIG. 2. **Visible spectrum of NBD-Cl-modified GST.** *A*, GST (15 μM) was reacted with 2 mM NBD-Cl in 0.01 M sodium acetate buffer, pH 5.0, and 1 mM EDTA (1 ml, final volume) for 3 h at 25 °C. After Sephadex G-25 chromatography, the modified enzyme was analyzed as such (*a*) and after reaction with 50 mM DTT for 10 min (*b*). Protein concentration was 0.15 mg/ml. *B*, GST (15 μM) was reacted with 0.4 mM NBD-Cl in 0.01 M potassium phosphate buffer, pH 8.0, and 1 mM EDTA for 30 min at 25 °C. After Sephadex G-25 chromatography, the modified enzyme was analyzed as such (*a*) and after reaction with 50 mM DTT for 10 min (*b*). Protein concentration was 0.12 mg/ml.

TABLE I

Fluorescence of NBD-modified GST

The NBD-modified GST at pH 5.0 and 8.0 were prepared as reported under "Experimental Procedures."

pH	DTT treatment	Thiol group[a]	Amino group[b]
5.0	−	+	−
5.0	+	−	−
8.0	−	+	+
8.0	+	−	+

[a] Excitation at 420 nm; emission at 545 nm.
[b] Excitation at 475 nm; emission at 535 nm.

activity was assayed after restoration of protein sulfhydryls by DTT treatment. From the data shown in Fig. 6*A*, it appears that within 1 h of incubation with TNM, about 4 tyrosines were nitrated and that this reaction was accompanied by a complete loss of activity. The kinetic inactivation pattern is markedly biphasic and suggests that the nitration of a first tyrosine allows a 50% inactivation and the modification of a second tyrosine results in no appreciable loss of activity, whereas during the nitration of the third amino acid, the activity decreases to about 6% of the initial amount (Fig. 6*B*).

TABLE II

Protection of GST inactivation

GST (1.5 μM) was incubated with NBD-Cl (30 μM) in the presence or absence of 5 mM CDNB or 5 mM *S*-methylglutathione (CH_3-SG). The reaction was performed in 0.4 ml (final volume) of 0.1 M sodium acetate buffer, pH 5.0, and 1 mM EDTA. At fixed times, 10 μl were assayed for the enzymatic activity, as reported under "Experimental Procedures."

	Residual activity		
	15 min	30 min	60 min
		%	
NBD-Cl (30 μM)	82	74	55
CDNB (5 mM) + NBD-Cl (30 μM)	95	90	88
CH_3-SG (5 mM) + NBD-Cl (30 μM)	91	83	79
CDNB (5 mM)	100	100	100
CH_3-SG (5 mM)	100	100	100

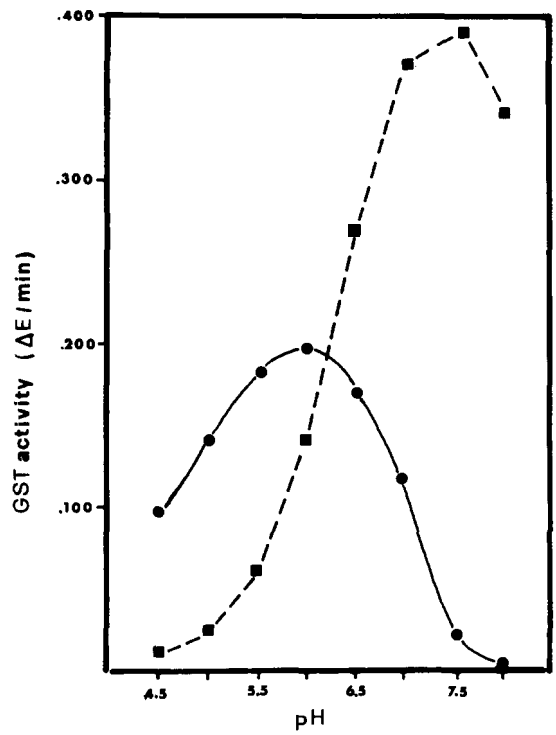

FIG. 3. **pH dependence of the enzymatic conjugation of GSH with NBD-Cl.** One unit of GST was incubated with GSH and NBD-Cl as described under "Experimental Procedures" in the presence of 0.1 M sodium acetate buffers, pH 4.5–5.5, or potassium phosphate buffers, pH 6.0–8.0. The reaction was followed spectrophotometrically continuously at 420 nm. *Broken line*, nonenzymatic conjugation of GSH with NBD-Cl. *Solid line*, enzymatic conjugation corrected of the spontaneous reaction.

DISCUSSION

Results described in this paper show a correlation between the inactivation of GST and NBD-Cl modification under conditions where this reagent is specific for thiol groups (*i.e.* pH 5.0), as well as under conditions where lysines and tyrosines are also modified (*i.e.* pH 8.0). At pH 5.0, only about two reactive SH groups are blocked by NBD-Cl. The identity and the quantitation of the cysteine-NBD adduct were determined on the basis of spectral and fluorometric properties of the reacted enzyme. This modification allows an inactivation higher than 90%, which can be reversed by DTT treatment. The correlation between the blocked thiol and loss of activity clearly indicates that about 50% of inactivation is attained

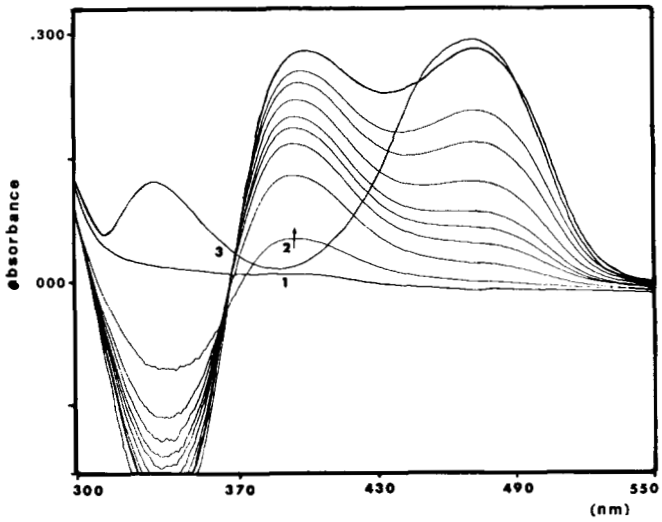

FIG. 4. **Spectral change of GST-SS-cysteamine during the reaction with NBD-Cl at pH 8.0.** GST-SS-cysteamine (13 μM) was incubated with NBD-Cl (0.25 mM) in the presence of potassium phosphate buffer, pH 8.0, and 1 mM EDTA. *Curve 1*, GST-SS-cysteamine against blank without enzyme; *curve 2*, GST-SS-cysteamine after 0.5 min of reaction with NBD-Cl; subsequent *curves* indicated by the *arrow* are after 5, 10, 15, 20, 30, 40, 45, and 60 min of reaction against blank containing all reagents except GST. *Curve 3*, the modified enzyme after 1 h of incubation was passed through a Sephadex G-25 column equilibrated with 0.01 M potassium phosphate buffer, pH 8.0, and concentrated (16 μM, final concentration). After reaction with 50 mM DTT for 10 min at 25 °C, the spectrum was recorded against blank with the same buffer.

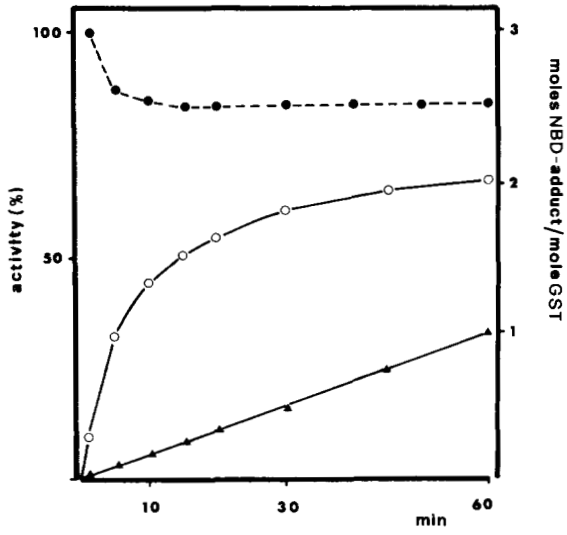

FIG. 5. **Time course of tyrosine and lysine modifications and recovery of GST activity after DTT treatment.** From the spectral data of Fig. 4, the amount of the tyrosine-NBD adduct (ϵ_M = 11,000 at 380 nm) (○) and the lysine-NBD adduct (ϵ_M = 26,000 at 475 nm) (▲) were replotted *versus* time function. The values reported are subtracted from the contribution of each curve on the other at these wavelengths. At each time, aliquots of the modified enzyme were assayed for the enzymatic activity after reaction with DTT (50 mM) for 10 min at 25 °C (●).

when only one sulfhydryl group was modified. A more accurate kinetic treatment demonstrates that these thiols have different reactivities with NBD-Cl; the existence of one fast and one slow reacting thiol whose integrity is essential for the activity has been previously observed for this isoenzyme with other thiol reagents (10) and is confirmed by the present data. Although the primary structure of this isoenzyme is not known, up to now, several attempts were made to identify the

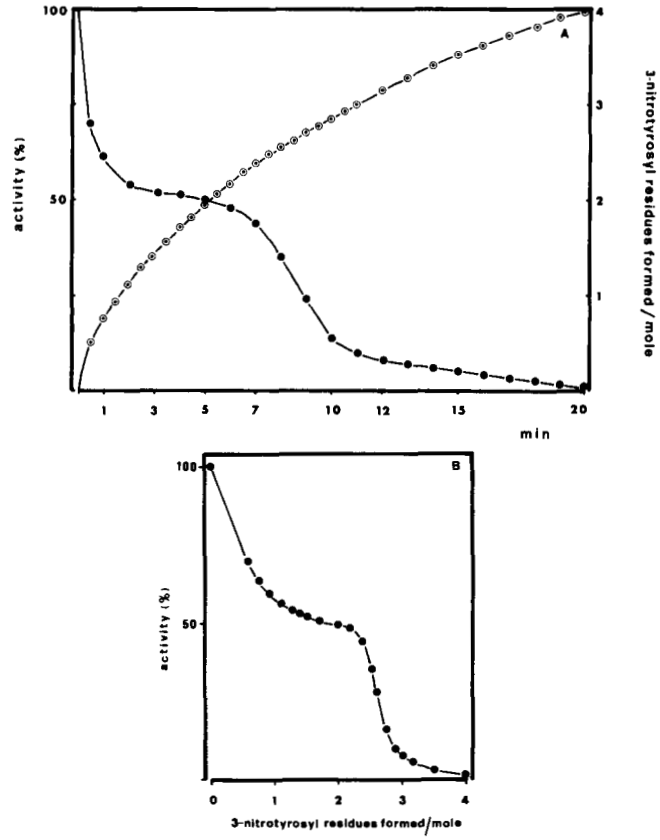

FIG. 6. **Reaction of GST with tetranitromethane.** *A*, GST (10 μM) was reacted with TNM (130 μM) in 0.01 M potassium phosphate buffer, pH 8.0. The reaction of tyrosine with TNM was followed at 428 nm (19) (⊙). At fixed times, aliquots of 0.1 ml were incubated with 50 mM DTT for 10 min at 25 °C, and then the enzymatic activity was determined in the standard assay conditions (●). *B*, the percentage of residual activity was replotted as a function of the modified tyrosines.

amino acid sequence of the reactive–SH group-containing peptide. For this purpose, we used a fluorescent and irreversible SH reagent (ANM) instead of NBD-Cl, which forms a reversible covalent bond with the cysteine residue. High pressure liquid chromatography analysis of the trypsin digestion of the ANM-modified enzyme shows only one fluorescent peak. The detection of a single ANM-containing peptide in this dimeric protein, which has been doubly labeled with this reagent, indicates the identity of the two subunits near the reactive sulfhydryl. The amino acid sequence of this peptide indicates a complete homology with that containing the reactive cysteine 47 of the placental GST (23). Another interesting feature of this reactive sulfhydryl is that it is protected towards the NBD-Cl inactivation by the presence of *S*-methylglutathione or CDNB. This may be an indication that it is probably located between the G and H subsites. However, the protection by *S*-methylglutathione is not a safe indication of the localization of this sulfhydryl near the G subsite since glutathione analogs are known to cause conformational changes in several transferases. An unexpected property of NBD-Cl is that it can be used as substrate by this enzyme in the presence of GSH. This reaction can be followed at 430 nm, where the *S*-NBD-glutathione adduct absorbs. The K_m value for NBD-Cl is 30 μM at pH 5.0, with a V_{max} at a saturating concentration of GSH, corresponding to 3.3% of that observed with CDNB. At the same pH value, the K_m value for CDNB is 0.4 mM; therefore, very similar V_{max}/K_m values can be calculated for these substrates. The fact that

NBD-Cl can replace CDNB in the catalyzed conjugation with GSH suggests that this new substrate probably binds at the H subsite and, therefore, that the reactive sulfhydryl group is near this subsite.

In this regard, several considerations must be made; the low K_m value of 30 μM seems not to be in agreement with the proportionality of pseudo-first order inactivation constants against NBD-Cl concentration observed up to 1.5 mM. One possible explanation is that NBD-Cl can interact with the protein cysteine only in an approaching movement and not in the final bound form.

From a more general point of view, the discovery of a new substrate for GST can be useful; preliminary evidence shows that Mu and Alpha class isoenzymes also use NBD-Cl as a substrate with a V_{max} comparable or higher than with CDNB. Therefore, NBD-Cl may be a useful tool for new spectrophotometric and fluorometric assays being developed in our laboratory.

Other interesting information is obtained from the reaction of NBD-Cl with GST at pH 8.0; among 20 tyrosines and 24 lysines present in this isoenzyme, only 2 tyrosines and 1 lysine, as well as 2 cysteines can be modified under our experimental conditions. A useful procedure for a spectrophotometric identification and quantitation of the involved animo acids is obtained by using an SH-protected enzyme with cystamine. Results obtained seem to indicate that the reaction with lysine does not result in any appreciable change of activity. The loss of about 15% activity obtained with the lysine-modified enzyme after restoration of the 2 tyrosines and cysteines with DTT may be due to some irreversible structural change due to tyrosine modifications. On the other hand, more important data are obtained by nitration experiments. With tetranitromethane, about 4 tyrosines are nitrated, with a complete loss of the enzymatic activity. The inactivation follows a peculiar inactivation pattern and is due to the modification of only 2 reactive tyrosines. It may be supposed that, after the modification of the first 2 reactive tyrosines, a conformational change of this enzyme occurs, which makes the last two tyrosines more accessible to tetranitromethane. Whatever the reason, the importance of 2 tyrosines for the enzymatic activity of GST appears to be a new finding not previously described for any of the isoenzymes of this transferase.

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REFERENCES

1. Chasseaud, L. F. (1979) *Adv. Cancer Res.* **20,** 175–293
2. Jakoby, W. B. & Habig, W. H. (1980) in *Enzymatic Basis of Detoxification* (Jakoby, W. B., ed) Vol. 2, pp. 63–94, Academic Press, New York
3. Mannervik, B. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol.* **57,** 357–417
4. Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M. & Jornvall, H. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7202–7206
5. Mannervik, B. & Danielson, U. H. (1988) *CRC Crit. Rev. Biochem.* **23,** 281–334
6. Carne, T., Tipping, E. & Ketterer, B. (1979) *Biochem. J.* **177,** 433–439
7. Mannervik, B., Guthenberg, C., Jakobson, I. & Warholm, M. (1978) in *Conjugation Reactions in Drug Biotransformations* (Aitio, A., ed) pp. 101–110, Elsevier-North Holland, Amsterdam
8. Shasteen, C. S., Krivak, B. M. & Reed, D. J. (1983) *Fed. Proc.* **42,** 2036–2041
9. Awasthi, J. C., Bhatnagar, A. & Singh, S. V. (1987) *Biochem. Biophys. Res. Commun.* **143,** 965–970
10. Ricci, G., Del Boccio, G., Pennelli, A., Aceto, A., Whitehead, E. P. & Federici, G. (1989) *J. Biol. Chem.* **264,** 5462–5467
11. Ghosh, P. B. & Whitehouse, M. W. (1968) *Biochem. J.* **108,** 155–156
12. Aboderin, A. A., Boedelfeld, E. & Luisi, P. L. (1973) *Biochim. Biophys. Acta* **328,** 20–30
13. Birkett, D. J., Price, N. C., Radda, K. G. & Salmon, A. G. (1970) *FEBS Lett.* **6,** 346–348
14. Ferguson, S. J., Lloyd, W. J. & Radda, G. K. (1975) *Eur. J. Biochem.* **54,** 117–126
15. Ferguson, S. J., Lloyd, W. J. & Radda, G. K. (1975) *Eur. J. Biochem.* **54,** 127–133
16. Del Boccio, G., Casalone, E., Sacchetta, P., Pennelli, A. & Di Ilio, C. (1986) *Biochem. Med. Metab. Biol.* **36,** 306–312
17. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77
18. Simons, P. & Vander Jagt, D. L. (1981) *Methods Enzymol.* **77,** 235–237
19. Sokolovsky, M., Riordan, J. F. & Vallee, B. L. (1966) *Biochemistry* **5,** 3582–3589
20. Turano, C., Barra, D., Bossa, F., Ferraro, A. & Giartosio, A. (1971) *Eur. J. Biochem.* **23,** 349–354
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
22. Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238,** 622–627
23. Lo Bello, M., Petruzzelli, R., De Stefano, E., Tenedini, C., Barra, D. & Federici, G. (1990) *FEBS Lett.* **263,** 389–391
24. Persson, B., Hartog, A. F., Rydstrom, J. & Berden, J. A. (1988) *Biochim. Biophys. Acta* **953,** 241–248
25. Frost, A. & Pearson, R. G. (1961) in *Kinetics and Mechanism*, pp. 263–269, Wiley, New York