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ARTICLE *in* BIOCHEMISTRY · MAY 2002

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Properties and Utility of the Peculiar Mixed Disulfide in the Bacterial Glutathione Transferase B1-1[†]

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Received October 9, 2001; Revised Manuscript Received January 17, 2002

ABSTRACT: Bacterial glutathione transferases appear to represent an evolutionary link between the thiol:disulfide oxidoreductase and glutathione transferase superfamilies. In particular, the observation of a mixed disulfide in the active site of *Proteus mirabilis* glutathione transferase B1-1 is a feature that links the two families. This peculiar mixed disulfide between Cys10 and one GSH molecule has been studied by means of ESR spectroscopy, stopped-flow kinetic analysis, radiochemistry, and site-directed mutagenesis. This disulfide can be reduced by dithiothreitol but even a thousand molar excess of GSH is poorly effective due to an unfavorable equilibrium constant of the redox reaction ($K_{eq} = 2 \times 10^{-4}$). Although Cys10 is partially buried in the crystal structure, in solution it reacts with several thiol reagents at a higher or comparable rate than that shown by the free cysteine. Kinetics of the reaction of Cys10 with 4,4′-dithiodipyridine at variable pH values is consistent with a pK_a of 8.0 ± 0.1 for this residue, a value about 1 unit lower than that of the free cysteine. The 4,4′-dithiodipyridine-modified enzyme reacts with GSH in a two-step mechanism involving a fast precomplex formation, followed by a slower chemical step. The natural Cys10-GSH mixed disulfide exchanges rapidly with free [^{3}H]GSH in a futile redox cycle in which the bound GSH is continuously replaced by the external GSH. Our data suggest that the active site of the bacterial enzyme has intermediate properties between those of the recently evolved glutathione transferases and those of the thiol:disulfide oxidoreductase superfamily.

Glutathione transferases (GSTs)[1] (EC 2.5.1.18) are a ubiquitous superfamily of enzymes which are involved in the detoxification metabolism of xenobiotics. They catalyze the conjugation of GSH to a number of toxic electrophilic compounds, thus promoting their excretion (*1*). The cytosolic GSTs are dimeric proteins grouped in at least ten gene-independent classes named Alpha, Beta, Delta, Kappa, Mu, Pi, Sigma, Theta, Zeta, and Omega, which display different primary structures and substrate specificities (*2*−*10*). One common feature of all GSTs is the ability to activate the GSH molecule by lowering its pK_a from 9.0 to about 6.0−6.7 (*11*−*15*). A critical residue involved in this activation is a tyrosine which is located in the GSH binding site (G-site) and which is strictly conserved in the Alpha, Pi, Mu, and Sigma GSTs. The hydroxyl group of this residue stabilizes the thiolate group of GSH by forming a hydrogen bond with its sulfur atom. A serine residue plays a similar role in the Theta, Delta, and Zeta GSTs (*9*). Several crystal structures, representative of each class, have been solved in the last years, revealing that all GSTs have very similar tertiary structures and active site topologies (*10*, *16*−*24*). The recently determined crystal structure of the bacterial GSTB1-1, however, revealed some novel structural properties of this enzyme. Notably, in the bacterial enzyme from *Proteus mirabilis*, peculiar is the presence of a mixed disulfide between GSH and the sulfhydryl group of Cys10 which is located in the G-site. The GSH molecule is bound in the G-site in a fashion similar to that observed in the active site of all other GSTs, and it is also anchored to the protein by means of 11 polar bonds and several hydrophobic interactions (*23*). The presence of this mixed disulfide, demonstrated by both X-ray diffraction data and by mass spectrometry (*23*), is almost paradoxical since the enzyme preparations were

[†] G.R., C.D.I., and N.A. were partially supported by MURST PRIN2000. G.R. and G.A. were partially supported by the National Research Council of Italy (Target Project on Biotechnology).

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[1] Abbreviations: GST, glutathione *S*-transferase; GSTB1-1$_{ox}$, oxidized bacterial glutathione *S*-transferase; GSTB1-1$_{red}$, reduced bacterial glutathione *S*-transferase; GSH, glutathione; GSSG, glutathione disulfide; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, 1,4-dithio-L-threitol; DPDS, 4,4′-dipyridyl disulfide; PS$^-$, 4-thiopyridinate anion; BPA, 3-bromopyruvic acid; MTSL, 1-oxy-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methylmethanethiolsulfonate.

10.1021/bi0158425 CCC: $22.00
Published on Web 03/07/2002

carried out in the presence of reducing compounds. It is also unclear how, in this oxidized state, the GSH molecule participates in the nucleophilic attack to the electrophilic center of the substrate. Nevertheless, this enzyme is able to catalyze the classical GST-conjugating activity in which GSH is attached to the cosubstrate 1-chloro-2,4-dinitrobenzene (CDNB) (*25*). Site-directed mutagenesis of Cys10 did not reveal any critical involvement of this residue in catalysis (*26*), and thus the presence of this disulfide remains an unsolved puzzle. Interestingly, the recently discovered human Omega class GST displays a similar propensity to form a GSH mixed disulfide with Cys32 (*10*), so the presence of a mixed disulfide in the GST active site is not confined to the bacterial enzyme. This paper describes our attempts to discover both the molecular origin of the preferential oxidized state of Cys10 and some possible utilities of the existence of the mixed disulfide with GSH. For the first time, we have prepared the enzyme in the reduced state, and this has allowed us to characterize some chemical properties of Cys10 in both the reduced and oxidized form.

MATERIALS AND METHODS

Materials. Glutathione (GSH), glutathione disulfide (GSSG), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 1,4-dithio-L-threitol (DTT), 4,4′-dipyridyl disulfide (DPDS), and 3-bromopyruvic acid (BPA) were Sigma products; 1-oxy-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methylmethanethiolsulfonate (MTSL) was purchased from Toronto Research Chemicals Inc. (Canada); [^{3}H]GSH (50 Ci/mmol) was purchased from NEN (Boston) products.

Enzymes. The recombinant GSTB1-1 and C10A mutant were expressed in *Escherichia coli* and purified as previously described (*25*, *26*). The protein concentration was obtained from the protein absorbance at 280 nm assuming an $\epsilon_{1mg/mL}$ of 1.02. The extinction coefficent was calculated on the basis of the amino acid sequence as reported by Gill et al. (*27*) and confirmed by the bicinchoninic acid method. A molecular mass of 22.5 kDa per GST subunit was used in the calculation (*3*).

Reduction of the Mixed Disulfide between Cys10 and GSH. The mixed disulfide of the native enzyme (GSTB1-1$_{ox}$) was reduced after 30 min incubation at pH 7.0 and 37 °C with 50 mM DTT. The excess of reagents was removed from the reduced enzyme (GSTB1-1$_{red}$) by a Sephadex G-25 column equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. Cysteine groups were titrated at 25 °C in 0.1 M potassium phosphate buffer, pH 8.0, with 0.1 mM Ellman reagent (DTNB) (*28*). Attempts to reduce the mixed disulfide with GSH have been performed by incubating GSTB1-1$_{ox}$ (0.05 mM) with variable GSH concentrations (from 10 to 500 mM) in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0 at 25 °C. The amount of spurious GSSG in the GSH stock solutions was determined enzymatically with the NADPH−glutathione reductase assay procedure.

Reaction of GSTB1-1$_{red}$ with MTSL. The kinetics of the MTSL binding to GSTB1-1$_{red}$ (115 μM) were determined in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0, by adding MTSL at 1:1 subunit:label ratio to the enzymatic sample. The reaction between MTSL and GSTB1-1$_{red}$ was monitored by recording ESR spectra every 40 s using an ESP 300 Bruker spectrometer operating at 9 GHz. The temperature was maintained at 25 °C with a Bruker constant temperature device. The MTSL modified enzyme was then reacted with 5 mM GSH. The thiol−disulfide exchange between GSH and the spin-label molecule was followed by means of ESR measurements.

Reaction of GSTB1-1$_{red}$ with the Alkylating Agents BPA and CDNB. GSTB1-1$_{red}$ (1 μM) was incubated with a 20-fold excess of BPA at 25 °C in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0. The reaction was followed by recording the enzymatic activity at various times. The activity was assayed at 340 nm by the addition of 1 mM CDNB and 5 mM GSH directly in the incubation mixture. Reaction of GSTB1-1$_{red}$ (34 μM) with a 20-fold excess of CDNB was performed in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0, at 25 °C, and at different times, aliquots of the incubation mixture were assayed for the residual activity. The activity was followed at 340 nm and 25 °C in 1 mL of 0.1 M potassium phosphate buffer, pH 6.5, containing 1 mM CDNB and 5 mM GSH. Kinetics of the enzyme inactivation were fitted to a single-exponential equation, and pseudo-first-order rate constants were calculated.

Reaction of Free Cysteine with BPA and CDNB. A solution of 1 μM cysteine in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0, was incubated at 25 °C with a 20-fold excess of BPA. The reaction was followed at 300 nm where the cyclic imino acid lanthionine ketimine (ϵ_{300nm} = 6.2 mM^{-1} cm^{-1}), formed spontaneously after the conjugation of cysteine with BPA (*29*), adsorbs. A 5 cm light-path cuvette has been used for a more accurate spectrophotometric determination. Reaction of 34 μM cysteine in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0, with a 20-fold excess of CDNB at 25 °C was followed at 340 nm where the Cys-DNB adduct absorbs. The experimental traces were fitted to a single-exponential equation, and pseudo-first-order rate constants were calculated.

Reaction of GSTB1-1$_{red}$ with DPDS. GSTB1-1$_{red}$ (20 μM), in 0.01 M potassium phosphate buffer, pH 7.0, was rapidly mixed with the same volume of DPDS (200 μM) dissolved in (50:50:50) mM phosphate−acetate−borate buffers in the pH 5.0−9.0 range. Rapid kinetic experiments were run on an Applied Photophysics kinetic spectrometric stopped-flow instrument equipped with a 1 cm light-path observation chamber thermostated at 25 °C. The formation of the mixed disulfide was monitored by following the increase of the absorbance at 324 nm (ϵ_{324nm} = 19.8 mM^{-1} cm^{-1}) due to the release of the 4-thiopyridinate anion (PS$^-$). Experimental traces were fitted to a single-exponential equation, and pseudo-first-order rate constants were calculated. The pK_a value of the Cys10 was calculated by fitting the pH dependence of the rate constants to the equation:

$$k_{obs} = k_{obs}^{lim}/(1 + 10^{pK_a - pH}) \quad (1)$$

where k_{obs}^{lim} is the limiting pseudo-first-order rate constant at alkaline pH values.

Kinetics of the Thiol−Disulfide Interchange. GSTB1-1$_{red}$ (200 μM) was incubated at pH 7.0 with 2 equiv of DPDS, followed by a Sephadex G-25 chromatographic step to remove the excess of reagent. Stopped-flow experiments were then performed by rapidly mixing the DPDS-modified enzyme (20 μM in 0.01 M potassium phosphate buffer, pH

7.0) with different amounts of GSH (from 0.1 to 80 mM) in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0. Displacement of PS^- was monitored by following the increase of the absorbance at 324 nm. Experimental traces were fitted to a single-exponential equation, and pseudo-first-order kinetic constants were calculated. The nonlinear dependence of k_{obs} on GSH concentration was fitted to a hyperbolic binding equation.

Exchange of GSTB1-1$_{ox}$ with [^{3}H]GSH. Commercial [^{3}H]-GSH contains 10 mM DTT as reducing agent. One microliter of [^{3}H]GSH solution (0.02 nmol of tritiated GSH and 10 nmol of DTT) was incubated for 2 min at 25 °C with 30 nmol of GSSG in 20 μL of 0.01 M potassium phosphate buffer, pH 7.0. At the end of the incubation, the resulting solution contained 20 nmol of [^{3}H]GSSG, 20 nmol of [^{3}H]-GSH, and 10 nmol of oxidized DTT. One hundred microliters of GSTB1-1$_{ox}$ (68 μM in 0.01 M potassium phosphate buffer, pH 7.0) was incubated with the above prepared mixture (120 μL final volume) at 25 °C. After suitable incubation times (between 1 and 60 min) the sample was acidified with 0.1 M sodium acetate buffer, pH 5.0, and the enzyme was separated from the reagents on a Sephadex G-25 column equilibrated with the same buffer. The enzyme fractions were pooled, and the radioactivity was measured in a Packard (Tri-Carb 2100TR) liquid scintillation counter. The value obtained was normalized to the total radioactivity of the sample, and the relative amount of labeled GSTB1-1$_{ox}$ was calculated.

Reaction of GSTB1-1$_{red}$ with GSSG. GSTB1-1$_{red}$ (50 μM) in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0, was incubated 30 min at 37 °C with 10 mM GSH and variable amounts of GSSG (from 0.2 to 1 mM). After a Sephadex G-25 chromatographic step, the reactive thiols of the protein were titrated with DTNB.

Proteolytic Digestion of GSTB1-1. The native and the reduced enzyme (9 μM) was subjected to proteolytic digestion with trypsin, at a trypsin:enzyme mass ratio of 0.1, in 0.1 M ammonium bicarbonate buffer, pH 7.8, at 37 °C. At fixed times, aliquots (100 μL) of the incubating mixture were assayed for GST activity with 1 mM CDNB and 5 mM GSH.

Effect of Temperature. GSTB1-1$_{ox}$ and GSTB1-1$_{red}$ (0.7 μM) were incubated for 15 min at different temperatures ranging between 36 and 85 °C in (50:50:50) mM phosphate−acetate−borate buffer, pH 6.0. GST activity was measured at 340 nm and at 25 °C by adding 5 mM GSH and 1 mM CDNB directly in the incubation mixture.

GSTB1-1 Oxidation by Cupric Ions. GSTB1-1$_{ox}$ and GSTB1-1$_{red}$ (40 μM) were incubated for 30 min at 37 °C with $CuSO_4$ (200 μM) in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0. At the end of the incubation, the free −SH groups of the protein were reacted with BPA (200 μM) for 30 min. After this time, no free −SH groups were detected by DTNB reagent, and the sample was analyzed by SDS−PAGE in the absence and presence of the reducing agent 2-mercaptoethanol.

RESULTS

The Sulfydryl Groups in the Native GSTB1-1. Three cysteine residues per subunit are present in GSTB1-1 (Cys10, Cys145, Cys147). The crystal structure of the native enzyme indicates that Cys145 and Cys147 are shielded from the solvent, localized in the dimer interface, and their sulfhydryl groups are not involved in a disulfide bond (*23*). Conversely, the sulfhydryl group of Cys10 is covalently bound to a GSH molecule. Titration of the native enzyme with the DTNB shows about two slowly reacting −SH groups (1.7) per GST subunit ($t_{1/2}$ = 29 min at 25 °C and pH 8.0) (Figure 1). As expected, the C10A mutant enzyme gives a similar titration pattern with DTNB, confirming that the two reactive cysteines in the native GSTB1-1 are Cys145 and Cys147. Preincubation of GSTB1-1$_{ox}$ with 10 mM GSH (pH 7.0) did not cause any appreciable change of the number of titratable −SH groups, confirming a strong propensity of Cys10 to remain in the oxidized state even under reducing conditions. A different result is obtained when the native enzyme is reacted with the more powerful reducing agent DTT. After 30 min incubation with 50 mM DTT, followed by a Sephadex G-25 chromatographic step to remove the excess reagent, the enzyme has now one additional fast DTNB-reacting −SH group ($t_{1/2}$ < 0.5 min), likely Cys10, followed by the two slow-reacting thiols ($t_{1/2}$ = 26 min) (Figure 1). GSTB1-1 with a reduced Cys10 residue (GSTB1-1$_{red}$) is a stable enzyme when stored at neutral pH values in the presence of EDTA. This allowed us to characterize for the first time this form of the enzyme.

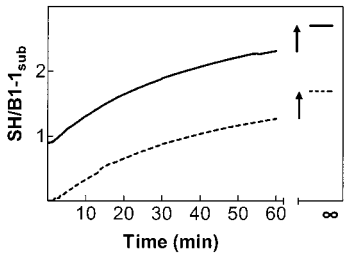

FIGURE 1: Reaction of GSTB1-1$_{ox}$ and GSTB1-1$_{red}$ with DTNB as a function of time. 8 μM enzyme in 0.1 M potassium phosphate buffer, pH 8.0, and 1 mM EDTA was reacted at 25 °C with 0.1 mM DTNB. The reaction was followed at 412 nm, and the number of DTNB-reacted thiol groups was calculated using a thionitrobenzoate extinction coefficient of 13.6 mM^{-1} cm^{-1}. About 1.7 DTNB-titratable −SH groups for GSTB1-1$_{ox}$ (dashed line) and 2.7 for GSTB1-1$_{red}$ (solid line) were observed at the end of the reaction.

GSH Is Ineffective in the Reduction of GSTB1-1$_{ox}$. GSTB1-1$_{ox}$ (0.05 mM) was incubated with variable GSH concentrations ranging from 10 to 500 mM. After 30 min of incubation, the amount of reduced Cys10 was calculated by titration with DTNB. Up to 100 mM GSH, the recovery of reduced enzyme is negligible, and even 500 mM GSH only yields 10% of the reduced enzyme. An estimation of the equilibrium constant for reaction 1, $K_{eq} = 2 \times 10^{-4}$, has been made, taking into account the presence of 0.15% of spurious GSSG in the GSH stock solutions. The propensity of Cys10 to exist as a mixed disulfide with GSH has been also verified on the basis of the reverse reaction i.e., by testing the facility of the reduced enzyme to be oxidized by GSSG even in the presence of an excess of reduced GSH. Incubation of 0.05 mM GSTB1-1$_{red}$ with 10 mM GSH in the presence of variable GSSG concentrations (from 0.2 to 1 mM) invariably yields a quantitative recovery (from 90% to 100%) of GSTB1-1$_{ox}$.

reaction 1

$$GSTS{-}SG + GSH \leftrightarrows GSTSH + GSSG$$

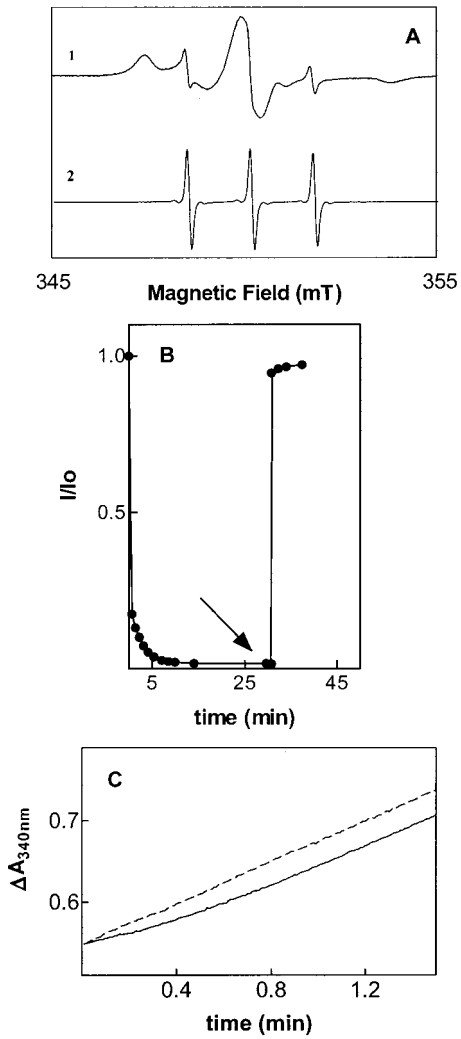

FIGURE 2: Reaction of GSTB1-1_{red} with MTSL. (A) ESR spectra of the free and bound MTSL. Spectrum 1: 115 μM spin-labeled GSTB1-1 in (50:50:50) mM phosphate−acetate−borate buffer, pH 7.0. Spectrum 2: free MTSL in the same buffer. ESR conditions: power, 20 mW; modulation amplitude, 1.0 G; frequency, 9.8 GHz; temperature, 25 °C. (B) Fractional free label signal versus time for the reaction of MTSL with GSTB1-1_{red} at a 1:1 label subunit ratio. An arrow indicates the addition of 5 mM GSH to the spin-labeled GSTB1-1. (C) Activity of the spin-labeled GSTB1-1 (5 mM GSH and 1 mM CDNB in 0.1 M potassium phosphate buffer, pH 6.5, 25 °C) (solid line). The initial rate is about 50% of that of the fully active enzyme. The rate increases with time, reaching the native activity after about 45 s. The activity of the spin-labeled enzyme pretreated for 10 min with 5 mM GSH is also reported (dashed line).

Reactivity of Cys10. A first indication of the reactivity of Cys10 comes from the reaction of the reduced enzyme with MTSL, a spin-label thiol reagent. The ESR spectrum of the immobilized nitroxide group bound to the enzyme is well distinguishable from that of the free label species (Figure 2A), and this property has been utilized to follow the progress of the reaction. The interaction of Cys10 with MTSL is very fast and yields a rapid and quantitative disulfide bond formation even at a MTSL:GSTB1-1 ratio of 1:1. As shown in Figure 2B, the free label signal disappears in about 10 min of incubation at pH 7.0, but about 80% of the free reagent is lost in the first spectrum (about 30 s). The C10A mutant does not react appreciably with MTSL in the same incubation time, confirming that Cys10 is the primary target

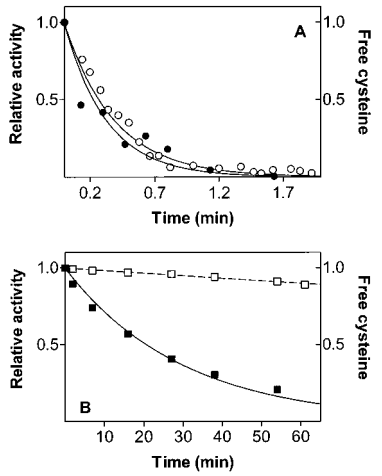

FIGURE 3: Reaction of GSTB1-1_{red} with BPA and CDNB. (A) Time course of the inactivation of GSTB1-1_{red} (1 μM) in the presence of BPA (20 μM) at pH 7.0 and 25 °C (●). For comparison is reported the time course of the reaction between free cysteine (1 μM) and BPA (20 μM) at pH 7.0 and 25 °C (○). The reaction was followed at 300 nm (for details see Materials and Methods section), and data are reported as relative free cysteine as a function of time. (B) Time course of the inactivation of GSTB1-1_{red} (34 μM) in the presence of CDNB (1 mM) at pH 7.0 and 25 °C (■). The time course of the reaction between free cysteine (34 μM) and CDNB (1 mM) at pH 7.0 and 25 °C is also reported (□).

of this interaction. The activity of the modified enzyme (in mixed disulfide with MTSL) using CDNB as cosubstrate is also strongly inhibited. The rate of the enzymatic reaction is not constant but increases with time, and after about 45 s the enzyme recovers all its original activity (Figure 2C). It is likely, under the assay conditions used (5 mM GSH, pH 6.5), that GSH removes MTSL from Cys10 and restores the catalytic efficiency of the enzyme completely. The reaction between Cys10 and MTSL, even performed at a ratio of 1:1, is too fast and does not allow an accurate kinetic analysis. An alternative approach to analyze the kinetics was made by utilizing the alkylating agents BPA and CDNB.

Reaction of GSTB1-1_{red} with BPA and CDNB. Alkylating reagents BPA and CDNB cause an irreversible inactivation of the enzyme, and this feature has been utilized to follow the time course of the reaction. In the presence of an excess of BPA (20 μM, pH 7.0, and 25 °C), the inactivation is fast and follows pseudo-first-order kinetics ($t_{1/2}$ = 14 s) (Figure 3A). CDNB (1 mM, pH 7.0, and 25 °C) reacts slowly, and the inactivation of the enzyme is accomplished only after 1 h of incubation ($t_{1/2}$ = 20 min) (Figure 3B). BPA and CDNB were ineffective when incubated with GSTB1-1_{ox} and with C10A mutant enzymes, confirming that GSTB1-1 inactivation occurs only as a consequence of Cys10 modification. The reactivity of Cys10 with BPA and CDNB was compared to that of the free cysteine incubated in the same experimental conditions (Figure 3). In the case of BPA, the rate of formation of the cysteine derivative is comparable to the inactivation rate of GSTB1-1_{red}. On the contrary, the reaction of CDNB and free cysteine shows a $t_{1/2}$ of about 200 min, indicating a reaction about 10-fold slower than that with GSTB1-1_{red}.

Reactivity of Cys10 with DPDS as a Function of pH. The reaction rate of Cys10 with the neutral aromatic disulfide DPDS at various pH values was utilized to estimate the pK_a value of the protein thiol. In fact, both BPA and CDNB were

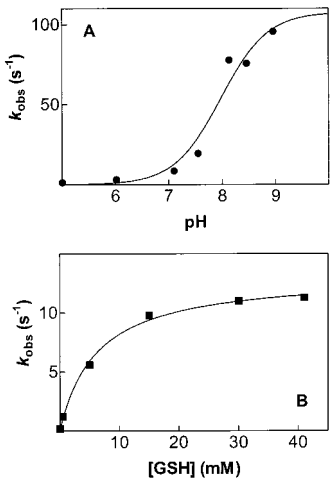

FIGURE 4: Reaction of GSTB1-1_{red} with DPDS. (A) pH dependence of the rate constant for the reaction between GSTB1-1_{red} (10 μM) and DPDS (100 μM) (for conditions see the Materials and Methods section). The k_{obs} versus pH relationships were curve fitted to eq 1, and a pK_a value of 8.0 ± 0.1 was calculated for the Cys10 residue. (B) Rate of PS^- displacement as a function of GSH concentration. The DPDS-modified enzyme (10 μM after mixing) was rapidly mixed with GSH (from 0.05 to 40 mM after mixing) at pH 7.0 and 25 °C. The time course of PS^- displacement was recorded at 324 nm, and pseudo-first-order rate constants (k_{obs}) were calculated at different GSH concentrations. The nonlinear dependence of k_{obs} on GSH concentration was fitted to a hyperbolic binding equation which fulfils the rate constant for the thiol−disulfide exchange reaction, $k_1 = 13 \pm 0.4$ s^{-1}, and the dissociation constant, $K_d = 6.5 \pm 0.6$ mM, for the GSH-labeled enzyme complex.

not considered ideal reagents due to the polarity of their molecules. The reaction was too fast to be followed by a standard spectrophotometric approach, so a stopped-flow apparatus was utilized. Formation of the mixed disulfide Cys10-SP was monitored at 324 nm, where the released 4-thiopyridinate anion (PS^-) absorbs ($\epsilon_{324nm} = 19.8$ mM^{-1} cm^{-1}), and performed at different pH values between 5.0 and 9.0. The resulting pH dependence of the pseudo-first-order rate constants is shown in Figure 4A and yields a pK_a = 8.0 ± 0.1 for the ionization of the Cys10 residue, about 1 unit lower of the pK_a of a free cysteine (pK_a = 8.7) (*30*). Moreover, the reaction at pH 7.0 between 20 μM DPDS and 10 μM GSTB1-1_{red} occurs with a $t_{1/2}$ of about 0.5 s, which is about 10 times lower than the value obtained for the reaction of DPDS with free cysteine in the same conditions ($t_{1/2} = 5.5$ s).

Rate of Thiol−Disulfide Exchange. The purified GSTB1-1 always occurs as a mixed disulfide with GSH, and this finding may be explained by a compact structure of the active site which might prevent the accessibility of reducing agents to the mixed disulfide. To test this hypothesis, GSTB1-1 reacted with 1 equiv of spin-label MTSL was incubated at 25 °C with 5 mM GSH. Addition of GSH to the labeled enzyme restores quickly (within 30 s) the free label signal (Figure 2B), indicating that the label displacement is fast and quantitative in the presence of GSH. This high reactivity of the mixed disulfide was an unexpected finding, and experiments were then repeated using a stopped-flow apparatus in order to measure the rate of thiol−disulfide exchange. DPDS-labeled GSTB1-1 was prepared as reported in the Materials and Methods section and rapidly mixed, at pH 7.0 and 25 °C, with different amounts of GSH. Displacement of PS^- was monitored by following the increase of the absorbance at 324 nm. The k_{obs} dependence on GSH concentration follows saturation kinetics (Figure 4B) and can be fitted to a two-step mechanism according to reaction 2.

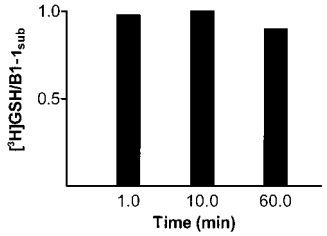

FIGURE 5: Radioactive labeling of GSTB1-1_{ox} as a function of incubation time. The exchange between [^{3}H]GSH and the GSH molecule bound to Cys10 in GSTB1-1_{ox} was determimed as described in the Materials and Methods section.

reaction 2

$$\text{GSH} + \text{E-SSP} \overset{K_d}{\rightleftharpoons} \text{E-SSP·GSH} \xrightarrow{k_1} \text{E-SSG} + PS^-$$

Reaction 2 involves the initial formation of a reversible enzyme−GSH complex followed by the PS^- displacement step which becomes rate limiting at GSH concentrations higher than 20 mM. Analysis of data gives a dissociation constant $K_d = 6.5 \pm 0.6$ mM for the binding of GSH to the labeled enzyme and a rate constant of $k_1 = 13 \pm 0.4$ s^{-1} for the thiol−disulfide exchange reaction.

The high reactivity of the disulfide bond when Cys10 is bound to DPDS or MTSL may be not recovered in the natural mixed disulfide Cys10-SG. By using [^{3}H]GSH, we attempted to verify this possibility by observing the rate of [^{3}H]GSH incorporation in the active site. The oxidized enzyme was incubated at pH 7.0 with labeled GSH and GSSG in a 1:1 molar ratio. At various times (from 1 to 60 min), the fraction of incorporation was evaluated. This ranged between 90% and 100% at all incubation times (Figure 5), indicating that the exchange is fast and complete even after 1 min of incubation.

GSTB1-1 Proteolysis. The proteolytic susceptibility of both GSTB1-1_{ox} and GSTB1-1_{red} was investigated in the presence of trypsin. As reported in Figure 6A, the time course of inactivation by trypsin of GSTB1-1_{red} reaches 14% of residual activity after 1 h of incubation. On the contrary, GSTB1-1_{ox} follows a slower trend of inactivation, retaining about 70% of residual activity after the same time of incubation.

Effect of Temperature on the Stability of GSTB1-1. The reduced enzyme is more easily inactivated by heat treatment than GSTB1-1_{ox}. In fact, GSTB1-1_{ox} retains more than 70% of its activity after 15 min of incubation at 80 °C, whereas the reduced enzyme is completely inactivated after an identical treatment (Figure 6B). These results strongly suggest that the presence of GSH covalently bound to Cys10 is essential for maintaining a fully active conformation even at critical temperatures.

GSTB1-1 Oxidation and SDS−PAGE Analysis. A further confirmation of the protective role of the mixed disulfide was obtained by oxidation experiments performed by incubating both GSTB1-1_{red} and GSTB1-1_{ox} with $CuSO_4$. SDS−PAGE (Figure 6C) shows that the prevalent form of GSTB1-1_{ox}, after treatment with $CuSO_4$, was mainly a monomeric

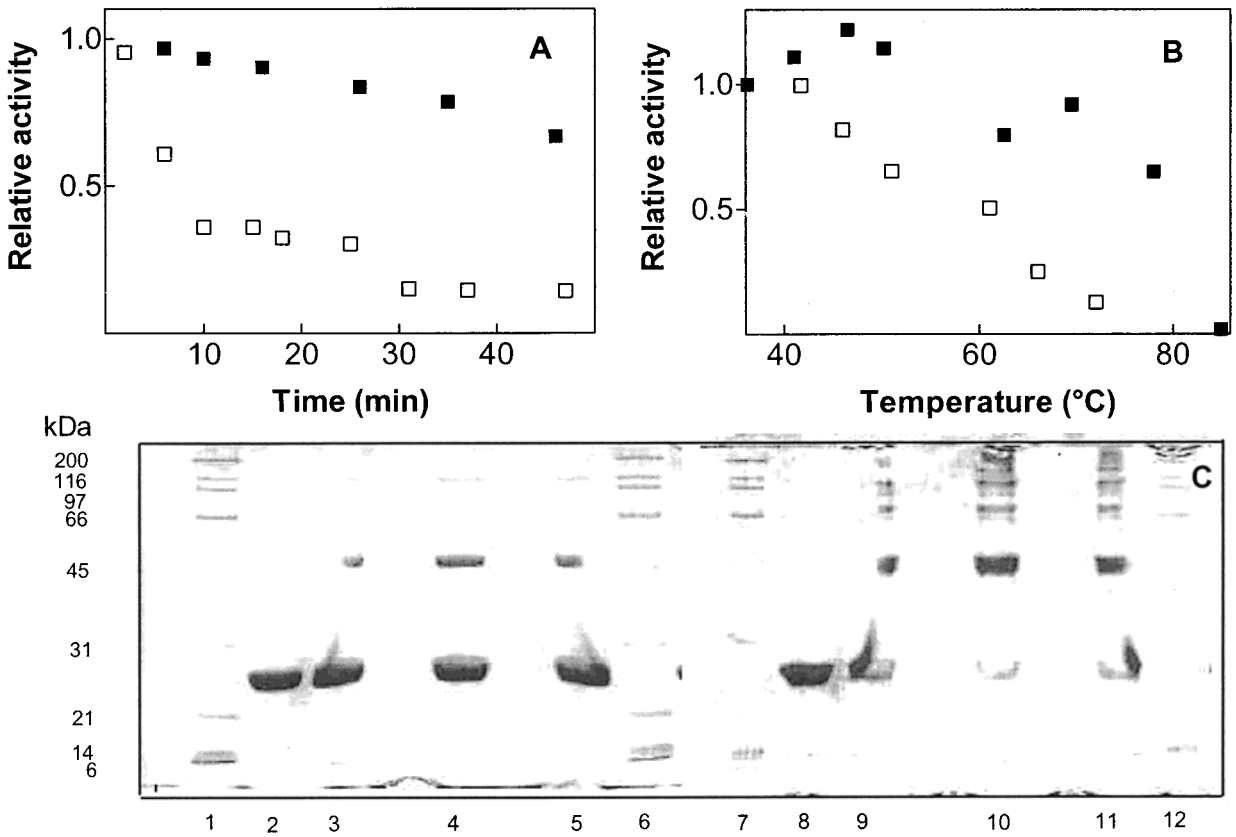

FIGURE 6: Protective role of the Cys10-SG mixed disulfide. (A) Proteolytic inactivation of GSTB1-1_{ox} (■) and GSTB1-1_{red} (□). The native and reduced enzymes (9 μM) were incubated at pH 7.8 with trypsin as reported in the Materials and Methods section. (B) Temperature inactivation of GSTB1-1_{ox} (■) and GSTB1-1_{red} (□). Native and reduced enzymes (0.7 μM) were incubated for 15 min at pH 6.0 at different temperatures, and residual activity was determined as reported in the Materials and Methods section. (C) SDS−PAGE analysis of GSTB1-1_{ox} (10 μg) and GSTB1-1_{red} (10 μg) after treatment with $CuSO_4$. 2-Mercaptoethanol was omitted in the gel and in the running buffer and only added to the sample buffer when indicated. Lanes 1, 6, 7, and 12 are standard proteins (from the top to the bottom) myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa), boiled at 100 °C in a sample buffer containing 2-mercaptoethanol. Lanes 3−5 are GSTB1-1_{ox} and lanes 9−11 are GSTB1-1_{red} treated with $CuSO_4$ as reported in the Materials and Methods section. Lanes 2 and 8 are GSTB1-1_{ox} and GSTB1-1_{red}, respectively, treated with $CuSO_4$ and boiled at 100 °C in the presence of 2-mercaptoethanol. During electrophoresis lateral diffusion of 2-mercaptoethanol from lanes 2, 6, 8, and 12 occurs which results in partial reduction of neighboring nonreduced samples and the creation of arcs of protein which link the high molecular mass species and the 23 kDa species.

species with molecular mass of about 23 kDa. On the contrary, GSTB1-1_{red} gave essentially a dimeric species with a molecular mass of about 46 kDa. Other polymeric components are also visible. Diffusion of 2-mercaptoethanol from lanes 2, 6, 8, and 12 converted the high molecular mass species to the 23 kDa species, indicating that the high molecular mass forms came from disulfide bridge formation involving Cys10 and the same residue or another cysteine on a second subunit. As a control, nontreated GSTB1-1_{ox} and GSTB1-1_{red}, in absence of 2-mercaptoethanol, did not form dimers spontaneously (data not shown).

DISCUSSION

The peculiar mixed disulfide Cys10-SG found in GSTB1-1_{ox} is one of a few significant differences between it and other GST classes. The more common GSTs are in fact widely distributed, present mainly in the cytosol, expressed in high amounts (3−5% of the total cytosolic protein), and display high and broad substrate specificity (*31*). In contrast, GSTB1-1 is present at low levels and is mainly localized in the periplasmic space of the bacterium (*32*). It is also characterized by low specific activity and can utilize only a restricted range of selected substrates (*25*). Thus, it has been suggested that the in vivo role of GSTB1-1 may not be as a conjugating enzyme but possibly as a redox enzyme (*23*). It has been postulated that GSTs have evolved from an ancestral member of the thiol:disulfide oxidoreductase superfamily (*33*). In particular, the $\beta\alpha\beta\alpha\beta\beta\alpha$ motif of the N-terminal domain of all GSTs superimposes well with the structures of thioredoxin and glutaredoxin. These enzymes are also characterized by catalytic mechanisms involving a mixed disulfide as the critical intermediate in catalysis and possess one or two essential cysteines in the active site (*34*, *35*). This redox scenario has been lost in the more recently evolved GSTs, such as the Alpha, Mu, and Pi classes, but not in the GSTB1-1 which shows a cysteine residue in the G-site in a unusual mixed disulfide with GSH. Thus, the active site of GSTB1-1 could represent a "transition structure" between an ancestral redox enzyme and a classical GST-conjugating enzyme. We have previously shown that GSTB1-1 displays a striking superposition with structures from the thiol:disulfide oxidoreductase superfamily (*23*). For example, superposition of GSTB1-1 bound to GSH with human thioredoxin complexed to a target peptide showed

that the target peptide's backbone superimposed closely with GSH, binding in the same antiparallel manner and forming a β-sheet interaction with the residues preceding the *cis*-proline residue in each protein. Although the active site cysteines do not superimpose exactly, the substrate cysteine/cystine is positioned in the same relative location in space. It was noted that a cysteine of another GST class, Delta class GST, did superimpose exactly with a bacterial thioredoxin. Furthermore, a consensus sequence motif based on the superposition successfully found only thioredoxins/glutaredoxins and GSTs in searches of sequence databases. We proposed that GSTB1-1 evolved from a member of the thiol:disulfide oxidoreductase superfamily on the basis of a detailed structural analysis of its structure in comparison to a number of thioredoxin structures, and our findings reported here support this conclusion.

An investigation of the redox properties of the Cys10 is of particular importance in this context as it may reveal how much different the active site of this enzyme is from that of the thiol:disulfide oxidoreductase superfamily.

A preliminary finding is the strong propensity of Cys10 to form a mixed disulfide with GSH. The reduced enzyme can be obtained only after concentrated DTT treatment. Even high GSH concentrations failed to break the Cys10-GSH disulfide, and this accounts for the constant recovery of GSTB1-1_{ox} after purification procedures including 10 mM GSH. Moreover, incubation of the reduced enzyme with an excess of GSH and traces of GSSG invariably yields an oxidized enzyme. The sulfhydryl group of Cys10 is almost buried and shielded from the solvent in the crystal structure of the oxidized enzyme. However, when it is in the reduced form, this residue is the most reactive among the three cysteines of this enzyme. Reaction of GSTB1-1_{red} with a stoichiometric amount of the spin-label reagent MTSL results in an almost quantitative reaction of the sole Cys10 within a few seconds incubation time. Alkylating compounds such as BPA and CDNB confirm the relevant reactivity of Cys10, which reacts with comparable or higher rates than the free cysteine. Significantly, the alkylated enzyme completely loses its conjugating activity toward electrophilic substrates. This inhibition is probably due to a steric hindrance of the reagents. Stopped-flow experiments with the neutral disulfide DPDS confirmed again the high reactivity of Cys10 and allowed us to calculate a pK_a of 8.0 ± 0.1 for its sulfhydryl group. This value, about 1 unit lower than that of free cysteine in solution, is probably caused by the presence of Lys107 and Lys35 in the G-site and partially accounts for the higher reactivity of Cys10 toward alkylating agents, assuming a large solvent accessibility of Cys10. The above evidence conflicts with the buried location of the disulfide in the crystal structure in which the active site does not look that flexible. In fact, helix α2 is locked in position by the extra β-sheet preceding it (*23*). The conflicting data could be explained by assuming that Cys10 is only buried by virtue of the bound GSH molecule or alternatively by the existence of a remarkable flexibility of the active site. Support for the latter hypothesis comes from oxidation experiments (Figure 6C) showing intersubunit disulfide bridge formation involving Cys10 and implying an active site movement.

We also investigated the reactivity of Cys10 when involved in a mixed disulfide. In fact, the permanence of the Cys10-SG disulfide in the presence of an excess of GSH could be merely due to the inaccessibility of the disulfide to the free thiol. However, the very fast displacement of aromatic thiols (such as MTSL or DPDS) by GSH is indicative of a high reactivity of the disulfide group and thus of a good solvent accessibility. Stopped-flow experiments lead to the identification of a multistep mechanism of displacement by GSH, with a first fast phase in which GSH binds to the active site followed by a slower step which represents the chemical nucleophilic reaction. However, the possibility that the solvent accessibility of these mixed disulfides may be artifactual due to the apolarity and large size of MTSL and DPDS was taken into consideration. The fast exchange of [^{3}H]GSH with the natural mixed disulfide Cys10-SG is strong evidence that this disulfide is really exposed to the solvent. In addition, these data suggest that a futile redox cycle is operative in GSTB1-1_{ox} in the presence of physiological GSH concentrations; i.e., GSH linked to the Cys10 residue is continuously replaced by the external GSH, but the enzyme is never recovered in the reduced form. In this context, it appears that the active site is able to accept, transiently, a second GSH molecule, which was not observed in the crystal structure. Given that the disulfide is fully accessible in solution, three different factors may promote the permanence of such mixed disulfide when an excess of GSH is present: (i) The sulfur atom of Cys10 is exposed to the solvent while the sulfur atom of the linked GSH is buried. In this case a sulfur-directed thiol−disulfide exchange may force the persistence of the protein disulfide. (ii) The leaving thiol group of GSH has a lower pK_a value than Cys10 (a lower pK_a of the thiol makes it a good leaving group in the thiol−disulfide exchange reactions as discussed below). (iii) The affinity of the active site for GSSG is much higher than that for GSH. In other words, the effective concentration of GSSG in the active site could be much higher than that in solution, leading an oxidative environment. The current crystallographic data suggest the first possibility not to be feasible. In fact, in the crystal structure the Cys10 sulfur atom is buried, and only the GSH sulfur atom appears partially exposed. The two remaining hypotheses are likely; preliminary data from our laboratory indicate that GSSG has about a 20-fold higher affinity for the G-site than GSH and that the GSH molecule in the G-site shows a p$K_a = 6.5$. Probably both factors contribute to the observed persistence of the mixed disulfide in GSTB1-1_{ox}, but the second one should represent the prominent drawing force. In fact, a much higher affinity for GSSG should be necessary to account for the failure of GSH to reduce the enzyme. The redox properties of GSTB1-1 can now be compared to the redox system found in the active site of the thiol:disulfide oxidoreductase superfamily. This comparison may indicate the crucial step of an evolutionary transition from a redox enzyme into a conjugating enzyme. Thioredoxin (which catalyzes the reduction of protein disulfides) is characterized by the sequence C-X-X-C in the active site. Both of these cysteines are essential for activity as an intramolecular disulfide is formed during catalysis. Among these two sulfhydryls, one shows near-neutral character (p$K_a = 7.5$) and this initiates catalysis, while the second one has a pK_a about 4 units higher (*34*). The neutral−basic properties of these two cysteines favor the formation of the final intramolecular disulfide that can only be enzymatically reduced by the NADPH-dependent thioredoxin reductase. In fact, it is well-known that the acidity

of the sulfhydryl group is essential for making it a good leaving group in thiol−disulfide exchange reactions. In other words, only an acidic protein sulfhydryl group involved in a mixed disulfide is easily reduced by a free thiol (*36*). Glutaredoxins (that catalyze the reduction of both protein mixed disulfides and mixed disulfides with GSH using GSH as reducing agent) display the same motif in the active site C-X-X-C. For specific reactions, only the N-terminal Cys is essential for activity, and its pK_a value is very low (3.5−4) (*35*, *37*). In this case, the low pK_a value favors the reduction of the intermediate GSH-glutaredoxin mixed disulfide by the external GSH forming a reduced glutaredoxin and GSSG as final products. It appears that GSTB1-1 exhibits redox intermediate characteristics between thioredoxin and glutaredoxin families as it has only one sulfhydryl group in the active site and it shares a pK_a similar to that observed in thioredoxin. In regard to the GST superfamily, GSTB1-1 (owning a single cysteine in the active site) appears as a snapshot in the evolution pathway from a redox enzyme toward a conjugating enzyme. The loss of one cysteine residue in the active site of an ancestral glutaredoxin could be the first step which precedes the final disappearance of thiol groups in the active site of a conjugating enzyme as found in the more recently evolved GSTs.

Apart from its possible functional role in catalysis, data reported in this paper clearly show that this atypical mixed disulfide involving Cys10 may provide some structural advantages to GSTB1-1. The increased thermostability of the oxidized enzyme and its higher resistance toward trypsin cleavage are clear indications of a protective role of the disulfide, probably due to a relatively more rigid active site when GSH is present. In addition, the reduced enzyme is more sensitive to oxidative stress as documented by the metal-promoted intersubunit disulfide bond formation involving Cys10 in GSTB1-1$_{red}$.

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BI0158425