Control of Activity through Oxidative Modification at the Conserved Residue Cys$^{66}$ of Aryl Sulfotransferase IV*

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A. David Marshall‡, John F. Darbyshire§, Ann P. Hunter§§, Peter McPhie¶, and William B. Jakoby‡‡‡

From the §Section on Enzymes, Laboratory of Biochemistry and Metabolism, NIDDK, National Institutes of Health, Bethesda, Maryland 20892 and the ¶Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle, Washington 98195

Oxidation at Cys$^{66}$ of rat liver aryl sulfotransferase IV alters the enzyme’s catalytic activity, pH optima and substrate specificity. Although this is a cytosolic detoxification enzyme, the pH optimum for the standard assay substrate 4-nitrophenol is at pH 5.5; upon oxidation, the optimum changes to the physiological pH range. The principal effect of the change in pH optimum is activation, which is manifest by an increase in $K_{cat}$ without any major influence on substrate binding. In contrast, with tyrosine methyl ester as a substrate, the enzyme’s optimum activity occurs at pH 8.0; upon oxidation, it ceases to be a substrate at any pH. The presence of Cys$^{66}$ was essential for activation to occur, thereby providing a putative reason underlying the conserved nature of this cysteine throughout the phenol sulfotransferase family. Mapping of disulfides by mass spectrometry showed the critical event to be the oxidation of Cys$^{66}$ to form a disulfide with either Cys$^{232}$ or glutathione, either one is effective. These results point to a mechanism for regulating the activity of a key enzyme in xenobiotic detoxication during cellular oxidative stress.

A sulfotransferase prepared in homogeneous form from the cytosol of rat liver incongruously has at least two very different pH optima depending on the test substrate. The activity toward the phenolic substrates, 2-naphthol and 4-nitrophenol for example, is highest near pH 5.5 but very poor in the physiological range where sulfation of the substrate tyrosine methyl ester is optimum (1, 2). The enzyme, designated variously as aryl sulfotransferase IV (1, 3, 4) and tyrosine-ester sulfotransferase (EC 2.8.2.9) (5, 6), catalyzes the transfer of the sulfonyl group of PAPS$^1$ to any of a wide range of low molecular weight phenols, hydroxylamines, alcohols, and amines (1, 3, 4, 7). This broad specificity and affinity for lipophilic compounds is characteristic of the enzymes of detoxication, the group of enzymes that prepares xenobiotics for ready excretion (8). The availability of the recombinant liver enzyme from Escherichia coli (6) has allowed the extension (4) of previous studies of mechanism (3) and provided the impetus for an examination of the apparently low activity and inappropriately low pH optimum of an enzyme believed to function in the cytosol.

The sulfotransferase reaction can be measured by two spectrophotometric assays reliant on the absorbance of 4-nitrophenol at pH 7. The first assay is in the physiological direction with PAPS as the sulfate donor and 4-nitrophenol as the acceptor resulting in the products 4-nitrophenyl sulfate and PAP (Equation 1) (2). The second assay is the two-stage transfer of the sulfonyl group from 4-nitrophenyl sulfate to form PAPS in the presence of PAP and the subsequent transfer to 2-naphthol as the acceptor phenol (Equation 2) (2). Under the assay conditions used, activity in the physiological direction was almost 10-fold lower than in the artificial transfer reaction.

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PAPS + 4\text{-nitrophenol} \rightleftharpoons PAP + 4\text{-nitrophenylsulfate} \quad (\text{Eq. 1})
\]

\[
4\text{-Nitrophenylsulfate} + 2\text{-naphthol} \rightleftharpoons 4\text{-nitrophenol} + 2\text{-naphthylsulfate} \quad (\text{Eq. 2})
\]

Upon prolonged storage, enzyme preparations were found to modestly increase their physiological activity at the expense of the transfer activity. These changes were abolished by addition of EDTA or thiols and were induced by disulfides. Significantly, glutathione disulfide, the major form of cellular oxidized disulfide (9, 10), was an efficient activator.

Each monomer of the homodimeric sulfotransferase contains five cysteine residues. The first of these from the amino-terminal, Cys$^{66}$, is highly conserved among phenol sulfotransferases (11, 12). Although a role for the cysteine residues in these enzymes has been suspected, their function has remained elusive. In N-ethylmaleimide inactivation of a rat liver enzyme, two of the five cysteine residues were found to react with the reagent and were protected from reacting by both PAPS and 4-nitrophenol (13). Replacement of Cys$^{70}$, the first cysteine residue from the amino-terminal end in the human form of phenol sulfotransferase, with serine resulted in the loss of activity profile brought about by oxidation of the cysteine residues of aryl sulfotransferase IV. Replacement of each of the five cysteine residues by site-directed mutagenesis, in combi-
nation with mapping of disulfides by mass spectrometric methods, has provided details of the underlying mechanism.

EXPERIMENTAL PROCEDURES

Materials—Hydroxynaphtoate-agarose (SpectraGe/H4A) was purchased from Spectrum (Los Angeles, CA); 4-aminobenzenesulfonyl fluoride and diithiothreitol from ICN Biomedicals (Aurora, OH) and bis-tris propane from Calbiochem; sucrose and glyceral were from J. T. Baker Inc., and all other compounds not designated were at the highest grade available from Sigma. Thin layer chromatography plates of DRA-cellulose (number 13255) and Silica Gel (number 13179) were from Eastman Kodak Co.

Sulfotransferases—Recombinant wild type and mutant sulfotransferases were isolated from E. coli BL21(DE3) bearing the pET3c11 vector (6). The enzyme isolation procedure was the same for all of the sulfotransferase preparations (2) and resulted in greater than 95% homogeneity for each as determined by SDS-polyacrylamide gel electrophoresis (14). Cys → Ser mutants were prepared by site-directed mutagenesis using polymerase chain reaction amplification of oligomers of 30 nucleotides with a single base substitution as the mutant primer (6). The expected mutation was confirmed by sequencing the entire mutant cDNA. Of the two forms of the enzyme, only the β form, which is free of PAP (2), was used here. Since the enzyme was not expressed by E. coli bearing the plasmid for mutant C232S, a double mutant containing C232S and M228W was used.

Enzyme Assays—The details of the spectrophotometric standard transfer assay and the physiological assay have been presented (2). The standard assay (Equation 2) measures the exchange of the sulfuryl group from 4-nitrophenyl sulfate to 2-naphthol in the presence of 20 μM PAP at pH 7.0 and is determined by measurement of the formation of 4-nitrophenol (ε280 = 10.5 × 103 M cm−1 at pH 7.0). Note that two reactions are actually involved: the initial generation of PAPS, the sulfate of which, in turn, is transferred to a second phenol (2). In the physiological assay (Equation 1), 4-nitrophenol is conjugated with PAPS as the donor. All reactions are all carried out at 25 °C in a Hitachi U3110 spectrophotometer in cuvettes of 1-cm light path. Rates were linear with time and protein concentration when average absorbance changes at 400 nm of less than 0.025/min were followed for 3 min.

Both of these assays are limited to the pH range of 6.5–7.5, because 4-nitrophenol has a pKa of 7.14. This limitation was avoided in the physiological trace assay by the inclusion of radioactive [35S]PAPS in Reaction 1 along with a 1 μM acceptor substrate in a final volume of 100 μl containing succinate-NaOH or bis-tris propane–HCl at the required pH (15). The reaction was initiated by addition of PAPS (740 μCi [35S]PAPS per vessel and carried out at 37 °C for 10 min before termination with 25 μl of 2 M acetic acid. After centrifugation, a 3-μl aliquot was used for the separation of [35S]-labeled product from [35S]PAPS by thin layer chromatography (4, 15). Radiolabeled peptides obtained from tryptic cleavage were separated by reverse phase HPLC using an UltraspHERE C18 column (2.0 mm × 25 cm; Beckman, Fullerton, CA). Elution of the peptides was accomplished with solvent A (0.05% trifluoroacetic acid in water) and B (0.05% (v/v) trifluoroacetic acid in 90% acetonitrile) and water; the initial concentration of solvent B was increased linearly from 5 to 60% in solvent A during 80 min at a flow rate of 200 μl/min. The column eluate was monitored at 215 nm using a Shimadzu SPD-10AV module detector.

ESI-MS instrument settings were as follows: source temperature = 100 °C; N2 drying gas = 500 liters/h; nebulizing gas = 20 liters/h; probe voltage = 3.6 kV; cone voltage = 35–50 V; probe position: off axis. Acquisition was carried out from m/z 200–2000 Da over 4.5 s in the CENTROID scanning mode, with unit resolution up to about 1500 Da based on calibration and resolution optimization using polyethylene glycol at M, 300, 600, and 1000.

The identity of GSH-conjugated peptides was confirmed by LC/MS and tandem MS/MS, using multiple reaction monitoring to specifically look for the neutral ion loss of 129 Da from the parent ion under collision-induced dissociation conditions (19).
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RESULTS

Effect of GSH and GSSG—The aryl sulfotransferase catalyzed the transfer of sulfate from 4-nitrophenyl sulfate to 2-naphthol in the transfer assay system (Equation 2) with a specific activity of approximately 600 nmol/mg/min. In comparison, the activity toward 4-nitrophenol with PAPS as the sulfate donor in the physiological assay system (Equation 1) was 10-fold lower at about 60 nmol/mg/min. Modest increases in the physiological activity could be achieved by incubation of enzyme in buffer A at 25 °C for several hours. These increases were eliminated by inclusion of 1 mM concentrations of EDTA, DTT, or GSH, suggesting involvement of an oxidant process.

When the enzyme was incubated with 1 mM GSSG, however, a large rise occurred in the physiological activity within 30–60 min, achieving a maximum of 600 μmol/mg/min; thereafter, activity very slowly and steadily declined (Fig. 1A). In contrast, transfer activity decreased upon oxidation with GSSG: the fall was initially rapid, but then became parallel to the slow decline phase of the physiological reaction (Fig. 1A).

Incubation of 7.5 μM enzyme with 5 and 10 μM GSSG achieved specific activities in the physiological reaction of 350 and 460 μmol/mg/min, respectively. With these low concentrations of oxidant, maximum activity was sustained but took longer to attain. Using 20 μM GSSG, a ratio slightly in excess of 2 mol/enzyme dimer, the decline phase in the activity profile was regained and, at concentrations higher than 50 μM GSSG, all activity curves showed essentially similar phases of rise and decline (Fig. 1B).

Altering the redox ratio of GSSG to GSH at a constant 1 mM total glutathione concentration also changed the timing and maximum activities achieved, but only at 1:3 GSSG-GSH (250 μM GSSG) and above was there a later decline in activity (Fig. 1C). Addition of 0.2 μM bovine liver protein disulfide-isomerase (EC 5.3.4.1) (Sigma) to 7 μM sulfotransferase increased the rate of formation of the activated enzyme (not shown). After 30 min of incubation, and using a 1:99 ratio of GSSG to GSH, a 4-fold increase in activity was observed due to inclusion of the isomerase; after 60 min of incubation, the increase was 9-fold.

Inclusion of PAP in the enzyme/GSSG incubation mixture blocked activation. With 1 mol of PAP/dimer or greater, there was no activation at 1 h in the presence of 1 mM GSSG. It was not possible to determine the effect of PAPS on GSSG activation, since PAPS is rapidly hydrolyzed by the enzyme at physiological pH (3). Excess PAP, 200 μM, added at any time after exposure to GSSG, arrested all further activity changes and, in the presence of a redox mixture of 1:1 GSSG-GSH, led to slow but complete reversal of the activity changes that had occurred: physiological activity declined and the transfer activity was regained (data not shown).

GSSG and the Cysteine Mutants—Five mutant enzymes, one for each of the cysteine residues of the protein, were examined for the effects of oxidation with GSSG. Three of them, C232S, M228I, C238S, and C289S, responded with changes in the activity profile identical to those shown for the wild type enzyme upon incubation with GSSG. Mutant C289S attained between 60 and 80% of the maximal specific activity. Mutant C66S was unique in lacking the activation response; instead, C66S exhibited only a gradual time-dependent decline in both physiological and transfer activities (Fig. 1A). As was observed with the wild type enzyme, 200 μM PAP blocked the activity changes seen for all of the cysteine mutants in GSSG-containing buffer and caused reversal of the effects in redox buffer containing 1:1 GSSG-GSH (data not shown).

Changes in pH Optima—The tracer physiological assay was used to determine pH activity profiles toward 4-nitrophenol, tyrosine methyl ester, and 1-hexanol as representative acceptor substrates. Assays were performed either in the presence of 1 mM DTT or after treatment of the enzyme with 1 mM GSSG for 1 h at 25 °C. When in the presence of the reducing agent DTT, optimum activity for 4-nitrophenol was observed at pH 5.2, whereas activity in the physiological pH region was minimal. From Fig. 2A it can be seen that treatment with 1 mM GSSG leads to a 17-fold increase in optimum activity and a shift in pH optimum to between 6.5 and 7.5 (Fig. 2A). Each of the cysteine mutant enzymes displayed such a change in activity with the exception of the mutant C66S which, although minimally activated by oxidation, remained active only in the acid pH range (Fig. 2B). Tyrosine methyl ester, an eponymous substrate for the enzyme, and 1-hexanol are dissimilar to 4-nitrophenol in that there is extensive and optimal activity in the physiological pH range (Fig. 2, C and E) under reducing conditions; both compounds cease to be substrates upon oxidation. Mutant C66S displayed pH optima and sulfation rates toward tyrosine methyl ester and 1-hexanol similar to those of the wild type enzyme but differed in that 1-h treatment with GSSG resulted in only a 50% reduction and not abolition of activity (Fig. 2, D and F).

Dissociation Constants—The apparent Kd values for the nucleotides PAP and PAPS were determined by displacement of the binding of the hydrophobic fluorescent dye ANS. Fluorescence changes induced by binding were both positive and negative depending upon the ligand tested. Binding of PAP elicited a slight decrease in fluorescence at very low concentrations (<90 nM for reduced enzyme and 800 nM for oxidized); above these concentrations, very large increases in fluorescence occurred, reaching a maximum at about 100 μM for enzyme with DTT and 200 μM for the oxidized form. Large decreases in fluorescence were observed upon binding of 2-naphthol and 2-naphthyl sulfate. Attempts at determining the dissociation
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Prior to oxidation, 4.0 of the five known cysteine groups per subunit were titratable with DTT for the wild type enzyme, and between 3.5 and 3.7 of the four thiols were found for the mutants in which a cysteine was replaced by serine. After incubation of the enzymes with 1 mM GSSG, there was a decrease in the number of free thiol groups titratable. After 1 h of incubation, wild type and mutants C82S, C283S, and C298S lost almost two of the initial free thiol groups, but mutants C66S and C323S,M228I only lost about one. After prolonged incubation with GSSG (24 h), virtually all of the initial free thiol groups of the sulfotransferases were unavailable.

Electrophoresis of Sulfotransferase—When wild type and cysteine mutant enzymes were reduced with DTT and subjected to electrophoresis in SDS under conditions free of reducing agent, each of the enzymes migrated as a single band of 34 kDa. Following overnight oxidation with GSSG, the pattern of electrophoretic migration changed to reveal two major bands of equal density. For wild type and for mutants C66S and C283S, the new bands were at 35 and 31.5 kDa with a minor one at 32 kDa; for cysteine mutants C283S and C298S, the new bands were at 35 and 32 kDa with minor bands at 31.5 and 34 kDa (Fig. 3). These major new bands were barely visible at 1 h, becoming apparent only after prolonged oxidation. Mutant C223S,M228I was exceptional as it showed no changes in electrophoretic pattern upon oxidation (Fig. 3). No significant species of greater M<sub>r</sub> was observed at any time period for any enzyme species.

Mass Spectral Analyses of Glutathione Conjugates—In an attempt to ascribe the activation phenomenon of sulfotransferase to specific cysteine residues, the enzymes were subjected to electrospray ionization mass spectral analysis following timed oxidation by GSSG. A multiply charged envelope of ions was obtained which was deconvoluted using the "MaxEnt" program to yield a molecular mass for the wild type protein reacted with GSSG that was 305 Da higher than the original mass. This clearly established the covalent addition of one glutathione to each enzyme subunit over a 24-h time period (Fig. 4). Formation of this new species was slow but progressive with the ion intensity being relatively low at 1 h, the time at which activation of the enzyme was optimum. Additional low intensity ions at approximately 34,510 Da (addition of 2 GSH) and 34,915 Da (3 GSH) were observed after 24 h incubation with GSSG (Fig. 4).

The sulfotransferase mutants C66S and C323S,M228I underwent the same mass spectral analysis after oxidation by GSSG and yielded identical profiles. The ions obtained again established the addition of a single glutathione to each of these proteins, but with these mutants the reactions occurred rapidly and were near completion after 1 h (Fig. 4). Upon further incubation, ions indicating the addition of a second glutathione to the proteins were detected as shown for the mutant

![Image](http://www.jbc.org/)

**Fig. 2.** pH activity curves for 4-nitrophenol, tyrosine methyl ester, and 1-hexanol. Wild type and mutant C66S sulfotransferases were assayed in the physiological tracer assay either with reduced (1 mM DTT; open symbols) or oxidized (GSSG for 1 h; closed symbols) in sodium succinate (○, ●) or bis-tris propane-HCl ([□], ■) buffers.

**TABLE I**

| Ligand       | Treatment | First K<sub>d</sub><sup>a</sup> | Second K<sub>d</sub><sup>a</sup> |
|--------------|-----------|-------------------------------|-------------------------------|
| PAPS         | DTT       | ND                            | ND                            |
|              | GSSG      | 1.6                           | 35.7                          |
| PAP          | DTT       | ST                            | 0.8                           |
|              | GSSG      | ST                            | 27.0                          |
| 2-Naphthol   | DTT       | ST                            | 179                           |
|              | GSSG      | ST                            | 141                           |
| 2-Naphthylsulfate | DTT   | ST                            | 156                           |
|              | GSSG      | ST                            | 101                           |

<sup>a</sup> ND, not determined; ST, stoichiometric binding.

Constant for PAPS were not effective because of time-dependent changes in fluorescence believed to result from the futile hydrolysis of PAPS by the enzyme to yield PAP (3).

The association of enzyme and PAP could be analyzed with the PCMLAB program, revealing the best fit to be attained by a two binding site model for each of the ligands. Stoichiometric binding was found to occur at the first site for PAP, 2-naphthol and 2-naphthyl sulfate regardless of oxidation state of the enzyme. At the second site, however, oxidation of the protein with 1 mM GSSG for 1 h elicited a 30-fold increase in K<sub>d</sub> for PAP, but only small decreases in K<sub>d</sub> for 2-naphthol and 2-naphthyl sulfate (Table I). In each instance, the total fluorescence change was greater using the completely reduced enzyme than that treated with GSSG.

**Determination of K<sub>m</sub> and K<sub>cat</sub>**—The spectrophotometric physiological and transfer assays were used to compare apparent K<sub>m</sub> and K<sub>cat</sub> for substrates with enzyme in the presence of DTT or after incubation with GSSG for 1 h. Upon oxidation there were changes in the K<sub>m</sub> and K<sub>cat</sub> for the wild type and mutant enzymes with the sole exception of the mutant C66S for which both of these parameters remained constant. For the physiological assay system, partial oxidation caused K<sub>m</sub> increases for 4-nitrophenol and PAPS. Despite the higher K<sub>m</sub>, K<sub>cat</sub> was 6–15-fold greater upon GSSG oxidation (Table II). Similar changes in the apparent K<sub>m</sub> for PAP and 4-naphthyl sulfate were observed using the transfer assay system, whereas K<sub>cat</sub> values, in contrast, decreased 2–3-fold in all but mutant C82S for which K<sub>cat</sub> doubled (data not presented).

**Enzyme Thiols**—The change in the number of titratable enzyme thiol groups, estimated by reaction with DTTN, is recorded in Table III as a function of time of exposure to GSSG.

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The values were determined under physiological assay conditions. The $K_{m}$ that is presented is based on data with 4-nitrophenol.

**Table II**

Comparison of the kinetic parameters for wild type and Cys→Ser mutant enzymes

| Mutant     | $K_{m}$ for PAPS (μM) | $K_{m}$ for 4-nitrophenol (μM) | $K_{cat}$ (s⁻¹) |
|------------|-----------------------|--------------------------------|-----------------|
| Wild type  | DTT GSSG              | DTT GSSG                        | DTT GSSG        |
| C66S       | 8.8 58.4              | 0.3 30.8                        | 0.08 0.71       |
| C82S       | 6.2 6.7               | 0.6 0.5                         | 0.06 0.06       |
| C283S, M228I | 8.7 46.7         | 0.6 75.8                        | 0.03 0.29       |
| C283S      | 3.5 10.6              | 0.8 32.9                        | 0.08 0.51       |
| C289S      | 10.6 40.7             | 0.2 82.4                        | 0.04 0.62       |
| C232S, M228I | 9.6 21.7         | 0.3 17.3                        | 0.08 0.51       |

**Table III**

Titration of enzyme thiols with DTNB

| Mutant     | Initial number of free thiols (0 h) | Number of thiols lost after GSSG (0–1 h) | Number of thiols lost after GSSG (0–24 h) |
|------------|-------------------------------------|------------------------------------------|------------------------------------------|
| Wild type  | 4.1                                 | 1.8                                      | 3.9                                      |
| C66S       | 3.6                                 | 1.2                                      | 3.5                                      |
| C82S       | 3.5                                 | 1.9                                      | 3.5                                      |
| C283S, M228I | 3.6                                 | 1.3                                      | 3.5                                      |
| C283S      | 3.5                                 | 1.9                                      | 3.5                                      |
| C289S      | 3.7                                 | 1.8                                      | 3.5                                      |

**Fig. 3.** SDS-gel patterns for wild type and mutant sulfotransferase proteins after complete oxidation. Enzymes were treated with GSSG for 24 h, diluted with SDS sample buffer, and subjected to electrophoresis under nonreducing conditions on a 12% Tris-glycine SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250.

C232S, M228I (Fig. 4). The reaction of mutants C283S and C289S with GSSG yielded ions at 1 h that were similar in mass and intensity to those obtained with the wild type enzyme, indicating slow and incomplete addition of a single glutathione to both mutants, however, acquired a second glutathione after 24 h of incubation (not shown).

Mutant C82S was the exception because it yielded an ion representing the addition of two glutathione molecules (+610 Da) without passing through a stage at which only a single addition was evident; the intensity of this new ion increased slowly over the 24-h incubation period. When fully oxidized, the protein was equally divided between two peaks representing the original molecular weight and the diglutathiolated monomer.

**Mapping of Disulfides**—The possibility of formation of intra-protein disulfides was examined by tryptic digestion and peptide mapping of the GSSG-treated protein. The reaction of GSSG with the enzyme was terminated by the addition of the alkylating agent 4-vinylpyridine to prevent further disulfide interchange. Following alkylation and tryptic cleavage, peptides were separated by liquid chromatography and analyzed by mass spectrometry. Tryptsin was chosen for proteolysis because it generates separate peptides for each of the five cysteine residues. The cysteine-bearing tryptic peptides and their masses, based on the known sequence (6), are listed in Table IV after calculation with the GPMAW V2.12 (Lighthouse Data, Odense, Denmark) program.

LC/MS analysis of the wild type enzyme tryptic digests, after incubation with GSSG for 24 h yielded peaks that corresponded in mass to peptides containing cysteine residues Cys²⁸⁹ and Cys²⁹⁴ linked to Cys²³² and Cys²³⁵ linked to Cys²⁸⁹ by disulfide bonds.

The elution of the peak at 3175.5 at 50.8 min with multiply charged ions at $m/z$ 1588.94 (2⁺), 1059.35 (3⁺), and 794.86 (4⁺) represents the linkage of peptides incorporating Cys²⁶⁶ and Cys²⁷³ (Fig. 5C), that occurred within 1 h, the time of maximal enzyme activity. The total ion count for this peak remained almost constant in intensity thereafter. The appearance of the peak at 39.8 min, represented by ions at $m/z$ 1624.77 (1⁺), 812.56 (2⁺), and 542.22 (3⁺), representing disulfide linked peptides 32 and 34 incorporating Cys²⁶⁶ and Cys²⁷³ (Fig. 5A), was minor at 1 h, but increased in intensity with time. This peak represents peptides 32, 33, and 34, i.e. the disulfide bond formation prevents the usual tryptic cleavage between the three peptides; this conclusion is supported by the reciprocally diminished peak for peptide 32 incorporating Cys²⁶⁶ with 4-vinylpyridine.

The single glutathione addition to the wild type enzyme took place at Cys²⁸⁹ as confirmed by a peak of mass 2156.5 at 40.4 min with a mass 305 Da higher than that predicted for unmodified peptide 9 (Fig. 5B). This formation of the glutathione conjugate was observed as a gradual time-dependent transition from the ions representing peptide 9 incorporating Cys²⁸⁹ conjugated to 4-vinylpyridine $m/z$ 979.3 (2⁺) and 653.3 (3⁺) to the ions at $m/z$ 1079.3 (2⁺) and 719.8 (3⁺) (Fig. 5B). The presence of the glutathione conjugate was verified by LC/MS and tandem MS/MS as the characteristic neutral loss ion of 129 Da under CID conditions.

Disulfide mapping of the peptides derived from mutant C82S indicates that a disulfide bond formed between Cys²⁸³ and Cys²⁹⁴ as it did with wild type enzyme. The formation of the disulfide between Cys²⁶⁶ and Cys²³² did not occur on all of the protein, however. This incomplete formation of the latter disulfide bond may explain the presence of the two peaks observed with mass spectral analysis of the intact C82S protein, representing nonconjugated and diglutathiolated species.

Both mutants, C66S and C232S, M228I, neither of which is able to form the disulfide bond of the wild type enzyme between Cys²⁶⁶ and Cys²³², underwent complete addition of a single glutathione within 1 h of incubation with GSSG. A second glutathione was slowly added to Cys²⁸⁹ of these mutants upon prolonged incubation with GSSG in a manner similar to that with the wild type enzyme. A mass increase of 305 to peptide 27 confirmed the S-glutathiolation of Cys²³² in the mutant enzyme C66S. Glutathione addition to peptide 6, containing Cys²⁶⁶, in the mutant C232S, M228I required indirect detection. The 4-vinylpyridine conjugate and the S-glutathiolated form of peptide 6 were not resolved by HPLC and eluted too close to the solvent front; this was confirmed using a custom synthesized peptide of sequence CGR conjugated with 4-vinylpyridine and, separately, with glutathione. The observation of a neutral ion loss of 129 Da under CID conditions from the peptides in the peak eluting close to the solvent front using LC/MS and tandem
MS/MS confirmed that mutant C232S,M228I contained S-glutathiolated Cys66 after 1-h incubation with GSSG. This conjugate was not observed at zero time for the mutant enzyme or at any time in the wild type preparations.

**DISCUSSION**

The characteristics of tyrosine-ester sulfotransferase include several curious elements. For one, the PAP-dependent transfer of sulfate from 4-nitrophenyl sulfate to 2-naphthol (Equation 1) was catalyzed at a rate 10 times greater than the reaction in the physiological direction, i.e. the sulfation of 4-nitrophenol using PAPS as the sulfate source (Equation 2). The reason for this difference emerged when the two activities were found to change upon moderate oxidation of the enzyme. Incubation of enzyme with GSSG led to both rapid loss of the transfer activity and the time-dependent activation of the sulfation of 4-nitrophenol. Further oxidation produced complete loss of activity. There was a direct relationship between the extent and rate of the activation and the concentration of GSSG, but, the subsequent loss of activity was observed only if GSSG exceeded 2 mol/enzyme dimer. In redox buffers containing both oxidized and reduced glutathione, activation was dependent on the redox ratio and not the absolute concentration of GSSG. Below a 1:4 ratio of GSSG:GSH, high activity was sustained, suggesting that oxidation and reduction of the enzyme were in a stable equilibrium under these conditions. The equilibrium was disrupted by intervention with PAP, which binds extraordinarily tightly to the enzyme (2), but only under reducing conditions. PAP halts, but does not reverse, the effects of oxidation with GSSG alone. In a redox system, however, PAP bound to the reduced form of the enzyme and prevented reoxidation, thereby reversing the physiological activation and restoring the transfer activity. The binding of PAP (and PAPS) is believed to be at a site close to Cys66 (12), consistent with the capability of PAP to block oxidation.

A second enigmatic element of this sulfotransferase involves the diverse range of pH optima for different substrates. Sulfation of tyrosine methyl ester was optimum at about pH 8, but that for 4-nitrophenol and 2-naphthol (1) was patently inconsistent with the presumption that the enzyme has a role in the detoxication process. Resolution to this paradox was made possible by the

![Fig. 4. ESI mass spectra of intact wild type and mutant C232S,M228I proteins, treated with GSSG for increasing periods of time. The ions obtained were deconvoluted using the "Maximum Entropy Program" to yield peaks, indicating the average molecular mass of each protein species.](http://www.jbc.org/)

**TABLE IV**

| Tryptic peptide | Amino acid residues | Cysteine residue included | Average predicted M<sub>r</sub> |
|-----------------|---------------------|--------------------------|-----------------------------|
|                 |                     |                          | Unmodified | 4-Vinylpyridine conjugate | Glutathione conjugate |
| 6, 27           | 66–68 and 230–253   | 66 and 232               | 3175.59    | 3122.87                   |
| 6, 27, (33), 34 | 279–291             | 283 and 289              | 1622.87    | 1622.87                   |
| 9, 27           | 82–99               | 82                       | 1852.06    | 1957.06                   |
| 27              | 230–253             | 232                      | 2844.21    | 2949.21                   |
| 32              | 279–286             | 283                      | 959.07     | 1064.07                   |
| 34              | 289–291             | 289                      | 362.42     | 467.42                    |
|                 |                     |                          |             | 667.42                    |
observation that partial oxidation of the enzyme leads to a
dramatic change from an acid pH optimum to one in the phys-
ological pH range for these substrates and results in a large
increase in the rate of sulfation. Nevertheless, two other sub-
strates, 1-hexanol and tyrosine methyl ester, both of which had
activity for the neutral pH range in the reduced enzyme, es-
sentially lost activity at all pH values upon oxidation. We have
observed, therefore, changes in substrate specificity as a func-
tion of the redox state of the enzyme’s environment.

The steps underlying the activation process can be under-
stood only in part by considering the information obtained with
the cysteine mutants. There are three strong candidates for the
mechanism of activation: S-glutathiolation of a critical cysteine
residue, formation of a disulfide between two cysteine residues
within a subunit, and disulfide bond formation between the two
subunits of the homodimer. The last of these can be dismissed
because SDS-electrophoresis provided no evidence for the for-
mation of intersubunit disulfides: higher molecular weight spe-
cies, greater than one subunit, were not formed. The slightly
smaller $M_r$ species, which were observed on electrophoresis
after treatment with GSSG, are probably due to conformational
changes resulting from disulfide formation among the five SH
groups of each subunit. Data from thiol titration with DTNB
following incubation of the enzymes with GSSG indicated only
that mutants of Cys66 and Cys232 were different in their be-
havior (Table III) but did not distinguish between the other two
candidates.

An answer to the mechanism of GSSG activation required
mass spectrometric analysis and disulfide mapping. ESI-MS
analysis of the intact protein disclosed that a single glutathione
had added to the wild type enzyme, but only on prolonged
incubation, far after maximum activation had been achieved.

Although S-glutathiolation of a protein at a conserved cysteine
is known to regulate the activity of several enzymes, including
human immunodeficiency virus type 1 protease (20), glutathi-
one transferase (21), and the phosphatase activity of carbonic
anhydrase III (22), the timing of conjugate formation for the
wild type sulfotransferase did not coincide with activation.
Disulfide mapping of the tryptic peptides derived after incuba-
tion with GSSG confirmed that the event occurring for the wild
type enzyme at the time of maximum activation consists of the
formation of a disulfide bond between Cys66 and Cys232. The
S-glutathiolation of Cys82, as well as disulfide bond formation
between Cys283 and Cys289, occur subsequently during the
period that leads to inactivation. These later events are prob-
ably without normal physiological function, since it is unlikely
that the enzyme will encounter such high ratios of GSSG to
GSH for the protracted periods of time required.

Mutant enzymes C283S and C289S displayed the same pro-
file of changes in activity as the wild type. The only difference
observed among these enzymes was the addition of a second
glutathione after 24-h incubation with GSSG, presumably be-
cause the disulfide between Cys283 and Cys289 does not exist.
Mutant C82S differed in its lower maximal physiological activ-
ity than the wild type; mass spectral data imply that only half
of the enzyme forms a disulfide between Cys66 and Cys232,
whereas the other portion incorporates 2 mol of glutathione.

The critical element in the activation appears to be centered
on the oxidation of Cys66. Simply removing the charge due to
the thiolate anion at Cys66, as in the substitution of cysteine by
serine in mutant C66S, does not lead to permanent activation;
in fact, that mutant can not be activated. One mode of activa-
tion is achieved by formation of a disulfide bond between Cys66
and Cys232. The creation of that disulfide bond is not an abso-
lute requirement for activation, however, since a mutant in which Cys$^{232}$ is replaced by serine also can be activated; the latter occurs by S-glutathiolation as documented by the finding of a disulfide with glutathione at Cys$^{66}$. In contrast, the S-glutathiolation at Cys$^{232}$ is ineffective in activating mutant enzyme C66S. The importance of Cys$^{66}$ for activation provides a rational for the conserved nature of Cys$^{66}$ throughout the phenol sulfotransferase family (11, 12).

The demonstration that the activity of a major detoxication enzyme is critically altered by the redox potential of its environment has potential significance in times of cellular oxidative stress. The activation that has been described for the sulfotransferase occurs within the expected cellular ratios of GSSG:GSH experienced under oxidative stress (23) and is indicated here as being accelerated by cellular enzymes catalyzing protein disulfide exchange reactions (24, 25). Furthermore, it has been postulated that under conditions of oxidative stress, even without significant rises in the concentration of cellular GSSG, there will be direct, nonenzymatic coupling of GSH to cysteine thiols through oxygen-radical catalysis (26–28). The survival advantages conferred on the cell by this means of regulating aryl sulfotransferase IV remain to be investigated as does the mechanism determining the switch in substrate specificity.

REFERENCES

1. Sekura, R. D., and Jakoby, W. B. (1981) Arch. Biochem. Biophys. 211, 352–359
2. Yang, Y. S., Marshall, A. D., McPhie, P., Guo, W. X., Xie, X., Chen, X., and Jakoby, W. B. (1996) Protein Expression Purif. 8, 423–429
3. Duffel, M. W., and Jakoby, W. B. (1981) J. Biol. Chem. 256, 11123–11127
4. Gu, W. X., Yang, Y. S., Chen, X., McPhie, P., and Jakoby, W. B. (1994) Chem. Biol. Interact. 92, 25–31
5. Mattock, P., and Jones, J. G. (1970) Biochem. J. 116, 797–803
6. Chen, X., Yang, Y. S., Zheng, Y., Martin, B. M., Duffel, M. W., and Jakoby, W. B. (1992) Protein Expression Purif. 3, 421–426
7.Ramawatam, S. G., and Jakoby, W. B. (1987) J. Biol. Chem. 262, 10039–10043
8. Jakoby, W. B., and Ziegler, D. M. (1990) J. Biol. Chem. 265, 20715–20718
9. Meister, A., and Anderson, M. E. (1983) Annu. Rev. Biochem. 52, 711–760
10. Gilbert, H. F. (1980) Adv. Enzymol. 63, 96–172
11. Falany, C. N., Zhuang, W., and Falany, J. L. (1994) Chem. Biol. Interact. 92, 57–66
12. Zheng, Y., Bergold, A., and Duffel, M. W. (1994) J. Biol. Chem. 269, 30313–30319
13. Borchardt, R. T., Schasteen, C. S., and Wu, S. E. (1982) Biochim. Biophys. Acta 708, 280–283
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. Ramaswammy, S. G., and Jakoby, W. B. (1987) Methods Enzymol. 143, 201–207
16. Ketley, J. N., Habig, W. H., and Jakoby, W. B. (1975) J. Biol. Chem. 250, 8670–8673
17. Knott, G. D. (1995) Am. Lab., March, 48–54
18. Jocelyn, P. C. (1970) Methods Enzymol. 143, 44–67
19. Baille, T. A., and Davis, M. R. (1993) Biochim. Biophys. Acta 332, 288–294
20. Davis, D. A., Dorsey, K., Wingfield, P. T., Stahl, S. J., Kaufman, J., Fales, H. M., and Levine, R. L. (1996) Biochemistry 35, 2482–2488
21. Dafre, A. L., Sies, H., and Akerboom, T. (1996) Arch. Biochem. Biophys. 332, 228–232
22. Cabidze, E., and Levine, R. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1170–1174
23. Gilbert, H. F. (1995) Methods Enzymol. 251, 8–28
24. Gravina, S. A., and Mieyal, J. J. (1993) Biochemistry 32, 3368–3376
25. Noiva, R., and Lennarz, W. J. (1992) J. Biol. Chem. 267, 3553–3556
26. Rokutan, K., Thomas, J. A., and Johnston, R. B. J. (1991) J. Immunol. 147, 260–264
27. Chai, Y. C., Ashraf, S. S., Rokutan, K., Johnston, R. B. J., and Thomas, J. A. (1994) Arch. Biochem. Biophys. 310, 273–281
28. Thomas, J. A., Poland, B., and Honzatko, R. (1995) Arch. Biochem. Biophys. 319, 1–9
Control of Activity through Oxidative Modification at the Conserved Residue Cys66 of Aryl Sulfotransferase IV

A. David Marshall, John F. Darbyshire, Ann P. Hunter, Peter McPhie and William B. Jakoby

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