Oxidative Inhibition of Human Soluble Catechol-O-methyltransferase

Naomi J. H. Cotton\textsuperscript{1,2}, Barry Stoddard\textsuperscript{2} & William W. Parson\textsuperscript{1}

\textsuperscript{1}Department of Biochemistry, Box 357350, University of Washington, Seattle, WA 98195-7350

\textsuperscript{2}Fred Hutchinson Cancer Research Center, Box 358080, Seattle, WA 98109-8080

Corresponding author: William W. Parson, parsonb@u.washington.edu, phone (206) 543-1743, fax (206) 685-1792

running title: Oxidative Inhibition of Catechol-O-methyltransferase
ABSTRACT

A common polymorphism in the human gene for catechol-O-methyltransferase results in replacement of Val 108 by Met in the soluble form of the protein (sCOMT), and has been linked to breast cancer and neuropsychiatric disorders. The 108M and 108V variants are reported to differ in their thermal stability, with 108M COMT losing catalytic activity more rapidly. Because human sCOMT contains seven cysteine residues and includes CXXC and CXXS motifs that are associated with thiol-disulfide redox reactions, we examined the effects of reducing and oxidizing conditions on the enzyme. In the absence of a reductant 108M sCOMT lost activity more rapidly than 108V, whereas in the presence of 4 mM dithiothreitol (DTT) we found no significant differences in the stability of the two variants at 37 C. DTT also restored most of the activity that was lost upon incubation at 37 C in the absence of DTT. Mass spectrometry showed that cysteines 188 and 191 formed an intramolecular disulfide bond when sCOMT was incubated with oxidized glutathione, while cysteines 69, 95, 157 and 173 formed protein-glutathione adducts (PSSG). Replacing C95 by serine protected 108M sCOMT against inactivation in the absence of a reductant; C33S and C188 mutations had little effect, and C69S was destabilizing. The sequences surrounding the reactive cysteine residues of human sCOMT and other proteins that form glutathione adducts at identified sites all include Pro and/or Gly and most include a hydrogen-bonding residue, suggesting that glutathiolation at conserved sites plays a physiologically important role.
The enzyme catechol-\(O\)-methyltransferase (COMT, E.C. 2.1.1.6) modifies a variety of endogenous and exogenous catechol substrates by transferring a methyl group from S-adenosylmethionine (SAM)\(^1\) to either the meta or the para hydroxyl group of the catechol ring. It plays important roles in the metabolism of catechol estrogens and the degradation of the catecholamine neurotransmitters dopamine and epinephrine.

COMT is produced as both a soluble protein with 221 residues (s-COMT, 25 kDa) and a membrane-bound protein with an additional 50 residues at the N-terminus (mb-COMT, 30 kDa) (1). A single gene on human chromosome 22q11.2 encodes both proteins, but separate promoters initiate their expression (2). The levels of expression of the two forms are tissue-specific: in the rat, s-COMT accounts for 95-99\% of the enzyme in liver and most other tissues (3), while mb-COMT is the major species in the adrenal medulla and some parts of the brain (4). mb-COMT is found in the endoplasmic reticulum and the nuclear membrane (5); s-COMT, in the cytosol and nucleus (6).

A common single-nucleotide polymorphism (SNP) in the coding region of the human COMT gene results in substitution of methionine for valine at position 158 of mb-COMT and position 108 of s-COMT (7,8). In the general U.S. population, the frequencies of the Met/Met, Met/Val, and Val/Val genotypes are approximately 0.17, 0.45 and 0.38, respectively (9). The COMT activity in erythrocyte lysates or liver biopsy samples from individuals with the Met/Met genotype is about 1/4 of that in people with the Val/Val genotype, while heterozygous individuals have intermediate levels of activity. The 108M and 108V variants of s-COMT have similar kinetic properties (10,11), but appear to differ in thermal stability (11-19). Lotta et al. (11) found that recombinant human108M s-COMT lost 80\% of its activity in 30 minutes at physiological temperature, while the 108V variant remained fully active. SAM protected the108M enzyme against this loss of activity (11). Qualitatively similar differences between the apparent stabilities of the two variants have been seen in enzyme preparations from a variety of tissues (11-19).

The 108/158M allele has been associated with increased risk of breast cancer (20-23) and a wide spectrum of mental disorders, including obsessive-compulsive disorder (24,25), ultra-rapid-cycling bipolar disorder (26,27), certain manifestations of schizophrenia (28-30), anxiety (31), and adult-onset alcoholism (32-34). It also has been
linked to decreased responses of the µ-opioid system to pain (35). A haplotype that combines the 108/158V allele with particular SNPs in two non-coding regions of the gene is strongly associated with schizophrenia (36), possibly because it results in decreased expression of the protein (37). The association of the 108/158M allele with mental dysfunction is particularly strong in patients with velocardiofacial syndrome, who lack the gene for COMT entirely on one copy of chromosome 22 and thus may be especially sensitive to the allele on the other copy (8,38).

No high-resolution structures of human COMT have been described. However, crystal structures of rat s-COMT in complex with SAM and catechol inhibitors are known (39-41), and the human protein probably is similar in structure because the amino acid sequences are 81% identical (see Figs. 1 and 2). The central structural motif, a seven-stranded β-sheet with helices on either side, is also characteristic of other SAM-dependent methyltransferases (42). The SAM-binding domain of human histamine methyltransferase, for example, is superposable on rat s-COMT with an RMS deviation of 2.9 Å over 156 C–β atoms (42). The variable residue 108 of s-COMT is located in a loop between a helix and β-strand whose distal ends are close to the SAM-binding site (Fig. 1). The rat protein has a leucine residue at position 108 and no known polymorphism at that location. Remarkably, however, histamine-N-methyltransferase has a common SNP (105Thr/Ile) at almost exactly the same position. In that case, the two variants of the enzyme have somewhat different kinetic properties but similar thermal stabilities (43).

There has been little discussion of structural differences that might explain the different stabilities or activities of the 108V and 108M variants of human s-COMT. Whether the inactivation of the 108M enzyme reflects gross unfolding or a more subtle change in the structure is unknown. Weinshilboum and coworkers (17,19) examined the proteins by polyacrylamide gel electrophoresis, isoelectric focusing, immune fixation, and photoaffinity labeling, and found no significant differences between the Met and Val variants. However, a possible clue comes from early observations that rat s-COMT loses activity on storage but can be reactivated by the reducing agent dithiothreitol (DTT) (44).

Of the 20 common amino acids, cysteine is the most sensitive to oxidation; the thiol groups of exposed cysteines readily oxidize to form intra- or intermolecular disulfide
bridges. Cysteine is one of the least abundant amino acids in proteins and also has the highest sequence conservation. Rat s-COMT contains four cysteines (residues 33, 69, 157 and 188), while human s-COMT has seven (33, 69, 95, 157, 173, 188 and 191). Two of the three additional cysteines in the human protein (C95 and C173) are close to the active site and may contribute to, or modulate substrate binding (see Fig. 1). The third (C191) along with C188 forms a CXXC motif, which is found in thioredoxins, glutaredoxins, DsbA and protein disulfide isomerase, proteins that undergo reversible thiol/disulfide oxidation/reduction reactions (45-48) (see Fig. 2). In addition, C69 is part of a CXXS motif that is found in many proteins with thiol/disulfide oxido-reductase activities (49). Fomenko et al. (49) have suggested that the local secondary structure and hydrogen bonding with the serine hydroxyl group makes cysteines in this motif particularly prone to oxidation. Vilbois et al. (12) have shown that cysteines 33, 69, 95 and 173 of human s-COMT react readily with the thiol reagent 5-iodoacetamide fluorescein and that this treatment inactivates the enzyme. They further showed that SAM and MgCl₂ decrease the reaction with cysteines 69 and 95 and partially protect the enzyme against inactivation by the thiol reagent, suggesting that cysteine 69, 95, or both, are essential for catalytic activity (12). However, no intramolecular disulfide linkages were detected via mass spectroscopy in those studies.

With the above observations in mind, it occurred to us that the reported differences between the thermal stabilities of the 108V and 108M variants of human s-COMT could reflect differences in susceptibility to oxidation or in the effects of oxidation on catalytic activity. The presence of two redox-associated motifs further suggested that cysteine oxidation and/or glutathiolation might be part of a mechanism for regulating COMT activity or, in the case of glutathiolation, for protecting the protein under conditions of oxidative stress. Although many investigators have included DTT or β-mercaptoethanol in assays for COMT activity, there have been no published studies of the effects of oxidants or reductants on the difference between the thermal stabilities or activities of the Val and Met variants. To our knowledge, mutations of Cys residues in human COMT also have not been investigated. However, Männistö et al. (50) have reported without details that a C33A mutation destroys the activity of rat s-COMT; the C69A, C157A and
C191A mutant enzymes were said to be active. (As mentioned above, rat s-COMT does not have cysteines at positions 95, 173, and 191.)

In the present work, we have compared the enzymatic activities of the 108V and 108M human s-COMT variants at physiological temperature in the presence or absence of DTT. Additionally, we have studied the effects of C33S, C69S, C95S and C188S mutations on the enzymatic activity and stability of the 108M and 108V variants of recombinant human s-COMT. We also have looked for changes in the redox states of the cysteine residues when the 108M and 108V enzymes are incubated under oxidizing or reducing conditions, and have examined the reactivation of the oxidized enzymes by DTT.

**EXPERIMENTAL PROCEDURES**

*Chemicals.* S-(5'-Adenosyl)-L-methionine (SAM), 4'-hydroxy-3'-methoxyacetophenone, 1,4-dithio-L-threitol, L-glutathione, isopropylthiogalactoside (IPTG), dithiothreitol (DTT) and phenylmethylsulfonylfluoride (PMSF) were obtained from SIGMA-ALDRICH, 4'-methoxy-3'-hydroxyacetophenone from Taizhou Dongdong Pharmachem (China), and 3,4'-dihydroxyacetophenone from Oakwood Products, Inc.

*Cloning, Expression & Purification.* Dr. David Eaton and Helen Smith (Department of Environmental Health, University of Washington) provided a c-DNA clone of 108V human s-COMT in the Novagen pET22b(+) vector, which contains a carboxy-terminal histidine tag. Starting with the 108V clone, we mutated Thr39 to Ala to agree with the NCBI sequence for human s-COMT (NP_009294.1), and then introduced the V108M mutation followed by C33S, C69S, C95S, and C188S mutations for both the 108V and 108M variants. All the mutations were made using the “QuickChange” site-directed mutagenesis kit (Stratagene). DNA from each strain was sequenced to verify the mutation and ensure that no unintended changes had occurred. Recombinant s-COMT was expressed in Escherichia coli BL21*(DE3) cells (Stratagene). Cell cultures with an absorbance of ~0.65 at 595 nm were induced with 500 μM IPTG and grown for six hours at 37 C. Cultures were centrifuged at 4000 x g for 15 minutes at 4 C, and the pellets were re-suspended and disrupted by sonication (50% duty, 80% power, for 10 minutes) in 100
mM Tris-HCl pH 8, 300 mM NaCl, 1 mM EDTA, 10% glycerol (v/v), 5 mM β-mercaptoethanol, 5 mM MgCl$_2$, and 10 μM PMSF. The cell extract was centrifuged at 12,000 x g for 20 minutes at 4°C to pellet any insoluble material. The resulting supernatant was passed through a filter with 0.22 μm pores (Millipore) and applied to a 4 ml column of Talon Metal Affinity resin (Clontech). Nonspecifically bound proteins were cleared using 30 ml of wash buffer (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM MgCl$_2$, 5 mM β-mercaptoethanol) followed by 30 ml of wash buffer with 7 mM imidazole. COMT then was eluted using two 6-ml volumes of elution buffer (wash buffer plus 100 mM imidazole) and pooled into 6 ml of ice-cold storage buffer (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 10% glycerol (v/v), 5 mM DTT, 5 mM MgCl$_2$). This procedure yielded up to 15 mg (~0.6 μmol) of COMT per liter of culture. The concentrated protein was dialyzed overnight at 4°C in 2 L storage buffer and stored at -20°C. To remove DTT and mercaptoethanol, enzyme stocks were thawed, exchanged into buffer lacking a reducing agent (50 mM Tris HCl, pH 7.5, 1.5 mM MgCl$_2$) using “Quick Spin” protein columns (Roche), and returned to -20°C for storage.

Protein concentration was determined by the Bradford assay (51) or, in a few cases, ultraviolet absorbance (52). The latter gave somewhat lower enzyme concentrations, and thus higher specific activities.

**COMT Enzymatic Activity.** The assay mixture contained 1 μM recombinant COMT, 50 mM Tris-HCl (pH 7.5), 1.5 mM MgCl$_2$ and, except where indicated otherwise, 4 mM DTT in a total volume of 250 mL. The reaction was initiated by adding 20 μM dihydroxyacetophenone ($K_m$ ~10 μM (53,54)) and 200 μM SAM ($K_m$ ~50 μM (11)), allowed to proceed for 15 or 30 minutes at either 22 or 37°C, and terminated by the addition of 150 μl of saturated NaCl and 250 μl of ethylacetate and vortexing. After the mixture had separated into aqueous and organic phases, 150 μl of the organic phase was removed for analysis by gas chromatography.

The methylated products (4¢hydroxy-3¢methoxyacetophenone and 4¢methoxy-3¢hydroxyacetophenone) were separated, as shown in Figure 3, in an Agilent Technologies 5890B gas chromatography apparatus with a fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness, Agilent Technologies HP5), a 95% methyl-, 5% phenyl-silicone stationary phase, and a mass spectrometer (Agilent Technologies 5970B). 1 μl of
sample was injected in the splitless mode, and helium at a starting pressure of 15 psi was used as the carrier gas in constant-flow mode. The oven temperature was increased from 70 to 250 °C over a total run time of 15 minutes. The solvent delay was 3 minutes. The retention times of the two products were separated by approximately 1 minute. The products were identified by comparison with standards and by mass spectrometry. The mass spectrometer was operated in the electron-impact mode using single-ion-monitoring at m/z = 166. A standard curve relating concentration to the integrated peak area of the gas chromatogram was produced for each product. Enzyme activities are expressed as μM methylated products (3’-hydroxy-4’-methoxyacetophenone plus 3’-methoxy-4’-hydroxyacetophenone) formed per unit time (30 or 15 minutes, as indicated) per μM protein, and the values given are the mean and standard deviation of the mean of three measurements. The meta/para methylation ratio typically was between 1.3 and 1.6.

Reaction with Glutathione, Proteolysis, and MALDI-TOF Mass Spectrometry. Human s-COMT (~20 μM) was incubated for 5 hr at 4 °C in 10 mM Tris HCl (pH 7.5), 2 mM MgCl₂ and 1 mM NaCl, with or without 7 mM DTT or 5 mM oxidized glutathione (GSSG). 10 μl of 5 mM iodoacetamide, an alkylating agent, was then added to 10 μl protein samples. After 45 min in the dark at room temperature, the protein was digested with 200 ng of sequencing grade trypsin (Promega) for 2 hours at 37 °C. The digestion was stopped by the addition of acetic acid to a final concentration of 1%. The proteolyzed material was analyzed with a matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE-Pro, Applied Biosystems). A 1 μL fraction of proteolyzed sample was mixed with 2.0 μL of matrix solution (0.1% trifluoroacetic acid/50% acetonitrile saturated with α-cyano-4-hydroxycinnamic acid) and 1.0 μL of the mixture was spotted onto a stainless steel target and allowed to dry. Positive-ion spectra were collected in the reflector mode. The mass accuracy of fragment ions was ±1 Da. A standard peptide mixture was used for external calibration.

Immunoblotting. After treatment with oxidized glutathione as described above, human s-COMT (20-40 μg) was submitted to SDS-polyacrylamide electrophoresis (SDS-PAGE) under non-reducing conditions and then transferred onto a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). The protein was exposed to an IgG2a mouse monoclonal antibody that recognizes glutathione-protein disulfide adducts.
Adherent antibody was detected by reaction with horseradish peroxidase-conjugated goat anti-mouse secondary antibody and enhanced chemiluminescence detection (Pierce).

Bioinformatics and Homology Modeling. Amino acid sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/). The CYSPRED algorithm (55) was used to predict the oxidation states of cysteine residues in human s-COMT. We used the protein-protein BLAST (blastp) on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) to search the SwissProt database (http://www.us.expasy.org.sprot/) for “short, nearly exact” sequence matches. A homology model of human sCOMT was made by starting with the crystal structure of the rat enzyme (39), replacing the necessary residues, minimizing the vanderWaals, torsional and electrostatic energies of the modified side chains sequentially (56), and then minimizing the energy of the entire structure using the ENCAD (57) force field.

RESULTS

At protein concentrations below approximately 50 µM, all the variants of purified human s-COMT migrated as expected for the monomeric protein on SDS-PAGE under standard reducing conditions (2 mM D-mercaptoethanol). At higher concentrations, the protein migrated predominantly as a dimer, and bands representing higher aggregates also were evident. The protein remained monomeric under these conditions if 3-10 mM DTT was included in the electrophoresis buffer, suggesting that the aggregation resulted from formation of intermolecular disulfide bonds. To avoid such aggregation, the measurements of enzyme stability and activity described below were conducted with s-COMT concentrations of 1 µM. Studies of the formation of intramolecular disulfide bonds and reactions with glutathione were done with 20 µM s-COMT, which was low enough so that relatively little aggregation occurred under the conditions of the experiments.

Figure 1 shows a homology model of human s-COMT based on the rat crystal structure. In this model, the sulfur atom of Cys 69 is approximately 7 Å from that of Cys 33 and about 10 Å from that of Cys 95, while the sulfur atoms of cysteines 188 and 191...
are about 8 Å apart. Relatively minor fluctuations of the structure thus might bring these atoms close enough together to allow formation of an intramolecular disulfide bond. The side chain of Cys 173 is too far from the other cysteines to form an intramolecular disulfide bond without major structural changes. This cysteine is, however, directly adjacent to an active-site residue (Pro172), so that formation of an intermolecular disulfide bond to glutathione or another protein probably would affect the enzymatic activity adversely.

To study the effects of DTT on the stability of s-COMT, we exchanged 108V and 108M protein stocks into buffer lacking DTT by rapid centrifugation through size-exclusion columns (see Methods). The buffer-exchange process itself did not affect enzyme activity if the protein was incubated at 22 C with 200 μM SAM and 4 mM DTT for one hour before it was assayed. 108M s-COMT that was freed of reductants and then incubated with DTT and SAM in this way had an activity of 16.6 ± 0.8 μM methylated products per 30 minutes per μM enzyme at 22 C, as compared to 16.5 ± 0.8 in controls that were not submitted to the buffer exchange; the corresponding activities for 108V s-COMT were 16.6 ± 0.8 and 16.5 ± 1.4. The protocol for the incubation preceding the enzyme assay was chosen because 4 mM DTT consistently gave high activities with both the wild-type enzymes and the mutants described below. We included SAM in some experiments because Lotta et al. (11) found that it protects 108M s-COMT against inactivation, but it proved not to be essential.

By contrast, when the enzyme was assayed in the absence of DTT, 108M s-COMT displayed significantly lower activity than 108V s-COMT (Fig. 4). The loss of activity by 108M s-COMT was manifest if the enzyme was assayed immediately after thawing, and incubation for periods up to 60 minutes in the absence of DTT had little or no further effect. However, addition of 4 mM DTT restored the enzymatic activity of 108M s-COMT, so that the activity became identical to that of the 108V enzyme within experimental error. In the presence of DTT, incubation for 60 minutes at 37 C had no significant effect on the activity of either variant of the enzyme (see Fig. 4). The 108M isoform thus does not appear to be markedly less stable than 108V s-COMT at 37 C if DTT is present.
Similar results (not shown) were obtained when the enzyme was incubated for 15 minutes at 45 C in the presence or absence of DTT, and then returned to 37 C for assay. Furthermore, the activity of 108M s-COMT that had been incubated for 15 minutes at 45 C in the absence of DTT could be fully restored by adding DTT after the incubation. DTT thus not only protects 108M s-COMT from inactivation under these conditions, but can reactivate the inactivated enzyme.

Because cysteine residues often are prone to oxidation, we examined the effects of replacing cysteine 33, 69, 95 or 188 by serine in the 108V and 108M enzymes. Column 2 of Table I shows the activities of all ten variants of the enzyme after incubation for 1 hour at 22 C in buffer containing 4 mM DTT and 200 µM SAM. As stated above, the activities of the 108M and 108V enzymes were essentially identical after this treatment. With the exception of 108M/69S, the activities of all the CysÆ Ser mutants were similar to, or slightly higher than those of the wild-type enzymes.

We also were interested to see whether COMT activity could be restored after longer periods of incubation in the absence of reducing agent. Column 3 of Table I gives the ratio of the activities measured at 37 C in the absence or presence of 4 mM DTT, following incubation for 2.5 hr at 37 C without a reductant. Although the buffer was not exchanged to remove DTT in these experiments, the dilution of the protein stocks into the incubation buffer reduced the DTT concentration to below 100 µM, which would be less than the concentration of dissolved O₂. The activities of most of the mutant enzymes declined upon incubation under aerobic conditions, but could be restored by the addition of 4 mM DTT. The Cys(95)Ser mutation increased the activity measured in the absence of DTT in both the 108V and 108M variants, whereas the Cys(69)Ser mutation appeared to make both variants more sensitive to oxidation.

To examine the sensitivity of the individual cysteine residues to oxidation, we incubated s-COMT under either reducing or oxidizing conditions (7 mM DTT or 5 mM GSSG), alkylated the thiol groups that remained reduced, digested the protein with trypsin, and identified the tryptic peptides by MALDI-TOF mass spectrometry. Immunoblotting with an IgG2a mouse monoclonal antibody (see Methods) showed that the 108V and 108M variants of s-COMT both formed glutathione-protein disulfide adducts when the proteins were incubated with 5 mM GSSG, and neither 108V nor 108M
s-COMT retained measurable enzymatic activity under these conditions. Again, however, the enzymatic activity could be largely or completely restored by the addition of 4 mM DTT (data not shown). Table II gives the calculated masses of the cysteine-containing tryptic peptides, along with the observed \( m/z \) values of the MALDI-TOF peaks assigned to the free peptides and their derivatives formed by alkylation (amidoacetylation) or glutathiolation. Alkylation of a cysteine residue with iodoacetamide increases the \( m/z \) value by 57 Da, and glutathiolation by 305 Da. One or more of the expected peaks were identified for each of the peptides, and the observed \( m/z \) values agreed well with the predicted values. In protein samples that had been incubated with DTT, all seven cysteine residues were predominantly alkylated. After incubation with GSSG, cysteines 69, 95, 157 and 173 remained partially available for alkylation, but also had formed protein-glutathione (PSSG) adducts.

Cys173 was the most sensitive to glutathiolation. Figure 5 shows the regions of the mass spectra representing the peptide comprised of residues 163-184, which includes this cysteine. After incubation with DTT, the alkylated peptide \( (m/z = 2334.6) \) gave a strong peak, whereas the peak representing the free peptide \( (m/z = 2277.6) \) was barely detectable (Fig. 5A). After incubation with GSSG, the peaks representing the glutathiolated and alkylated peptides had similar intensities (Fig. 5B). A miscleaved peptide containing Cys173 (residues 162-184) displayed the same pattern, giving \( m/z \) peaks at 2406, 2463 and 2711 for the free, alkylated and glutathiolated peptides, respectively.

The mass peaks for glutathiolated derivatives of the peptides containing cysteines 69, 95 and 157 also were easily identifiable (Table II). Cysteine 33, though sufficiently exposed to undergo alkylation under reducing conditions, did not form a detectable PSSG derivative in the presence of 5 mM GSSG.

Cysteines 188 and 191 were both fully alkylated in s-COMT that was incubated with DTT, so that the masses of the peptides containing the two cysteines were approximately 114 Da greater than the masses expected for the free peptides (Fig. 6A and Table II). When the protein was incubated with GSSG, the dominant peak moved to the region expected for the free peptides (Fig. 6B). Masses predicted for glutathiolated derivatives of cysteine 188 or 191, or for a doubly glutathiolated peptide, were not observed. However, inspection of the mass peaks in Figure 6B showed that the sub-peaks for
corresponding isotopic compositions in the free and di-alkylated peptides differed by 116 mass/charge units, not 114. This can be seen more clearly by comparing Figs. 6C and 6D, in which the two regions of interest in Fig. 6B are expanded and the region representing larger \( m/z \) is shifted horizontally by -114 mass/charge units. After this shifting, the sub-peaks in Fig. 6C for a peptide with two free thiols should be directly above the subpeaks in Fig. 6D for the corresponding di-alkylated peptide with the same isotopic composition. Note that the sub-peak for the lightest representative of the free peptide (\( m/z = 2053.8 \)) is two mass/charge units smaller than one would expect if two thioacetylamido groups were replaced by free thiols. The peaks at \( m/z = 2053.8 \) and 2054.8 also were seen in samples reconstituted in aerobic buffer without an added reductant or oxidant, but were more intense in buffer containing 5 mM GSSG. They were not seen in MALDI-TOF spectra of the 108V/188S s-COMT mutant under oxidizing conditions (data not shown). The simplest interpretation of these observations is that the free peptide contains an intramolecular disulfide bond between cysteines 188 and 191.

Results similar to those shown in Table II and Figs. 5 and 6 also were obtained with 108M s-COMT. There were no significant differences between 108M & 108V s-COMT in the relative intensities of the mass peaks assigned to the peptide with a disulfide bond between cysteines 188 and 191. The relative intensities for the peaks representing PSSG derivatives also were similar between the two proteins. Masses predicted for combinations of the tryptic peptides cross-linked by a disulfide bond were not detected with either protein.

Although numerous proteins form protein-glutathione adducts under oxidizing conditions, the physiological significance of these adducts has remained unclear (58-61). The particular cysteine residue that is glutathiolated has been identified in approximately 13 proteins, now including human s-COMT. These proteins are listed in Table III along with the amino acid sequences three residues on either side of the reactive cysteine. In all cases, the sequence includes proline and/or glycine, residues that often serve to induce a loop in the protein fold. Cysteines 173 and 95 are located in such loops in s-COMT (see Fig. 1). Hydrogen-bonding residues, especially serine and threonine, also are found in almost all the sequences surrounding the reactive cysteine.
The short sequences surrounding the known sites of the reactive cysteines were searched for nearly exact matches in the SwissProt database, and the results are included in Table III. In many cases, the search retrieved other proteins that are known to form PSSG adducts, but for which the reactive cysteine residue has not been identified. The searches also returned some proteins that have not been identified as reacting with glutathione.

**DISCUSSION**

In spite of numerous clinical studies pointing to correlations of particular COMT genotypes with breast cancer and neuropsychiatric disorders, the structural consequences of replacing Val by Met at position 108 have remained largely unknown. The studies described here indicate that the rapid loss of activity by purified 108M s-COMT at physiological temperature probably does not reflect gross unfolding of the protein, but rather the oxidation of Cys residues to form a disulfide bond or, in the presence of GSSG, a glutathione adduct. The 108M variant evidently either is more susceptible to this oxidation, or suffers a greater loss of enzymatic activity in its oxidized form. In the presence of 4 mM DTT, we found no significant differences between the stabilities of the 108V and 108M variants. Additionally, DTT was able to restore activity to both variants of the enzyme following extended periods at 37 C.

Residue 108 is located in a loop between an \( \alpha \)-helix (a5) and a \( \beta \)-sheet (b3) that terminate near the binding site for SAM on the opposite side of s-COMT (see Fig. 1). Cysteine 95 resides at the distal terminus of helix a5. It therefore seems possible that structural changes in the region of residue 108 would cause Cys 95 to move, changing its distance from Cys 33 or 69 and thus increasing the likelihood of forming a disulfide bond. An illustration of such a structural change can be seen in crystal structures of human transthyretin and its amyloidogenic V30M variant (62). In that case, replacing Val 30 by Met forces the surrounding \( \beta \)-sheets apart by as much as 1 Å, distorting the substrate-binding cavity and increasing the solvent exposure of Cys 10 (63).

In the light of their locations in the protein, we chose cysteines 33, 69 and 95 as initial targets for site-specific mutations. The Cys(95)Ser mutants of both 108M and 108V s-
COMT proved to be more resistant to the loss of activity in the absence of reductants, in agreement with the hypothesis suggested above (Table II). Replacing Cys 69 by Ser destabilized the protein, suggesting that this residue also is important for maintaining the protein in an enzymatically active conformation. Because Cys 69 is in a particularly strongly conserved region of the protein, an algorithm that considers multiple sequence alignments (55) picked out this residue as likely to be oxidized in the native protein. However, MALDI-TOF spectra gave no evidence for formation of a disulfide bond involving Cys 33, 69 or 95. Cysteines 69, 95, 157 and 173 did form PSSG adducts under oxidizing conditions, but this in itself would not explain the loss of activity in the absence of GSSG. In addition, although replacing Cys 95 by serine stabilized the enzymatic activity, it did not protect the enzyme completely. Studies of multiple mutants will be needed to explore the significance of this point, as replacing any one of the seven cysteines by serine would leave the others available for oxidation.

The MALDI-TOF spectra show that an intramolecular disulfide bond between cysteines 188 and 191 forms under oxidizing conditions. Although the peak intensities in the spectra must be interpreted cautiously, the C188-C191 disulfide appears to predominate over the dithiol in the presence of GSSG (Fig. 6B). The disulfide is seen in both the 108V and 108M proteins, and under aerobic conditions in the absence of added reductants or oxidants as well as in solutions containing GSSG. Whether the C188-C191 disulfide bond occurs to a significant extent in vivo remains to be determined. It may be pertinent in this context that the membrane-bound form of COMT (mb-COMT) is localized to the nuclear membrane and the endoplasmic reticulum, where many proteins destined for secretion undergo oxidation in eukaryotic cells (64). Although the kinetic properties of mb-COMT have been described, there are no reported studies of the thermal stabilities or sensitivity to oxidation of the 158M and 158V variants of mb-COMT.

Mutation of Cys 188 to Ser had little effect on the enzymatic activity or stability of either 108Val or 108Met s-COMT, but also could be of greater importance in mb-COMT.

In addition to cysteines, methionine residues in many proteins are susceptible to oxidation (65,66). Met108 is accessible to the solvent in the homology model of human s-COMT (Fig. 1), and thus could be disposed to oxidation. Oxidation to the sulfoxide, if it occurred, might amplify the putative disruptive effects of a methionine at this position.
However, we did not observe any peptide masses indicative of methionine oxidation in the MALDI-TOF spectra.

The physiological significance of the reactions of s-COMT with GSSG also requires further exploration. Recent work on other proteins suggests that glutathiolation may serve to protect proteins temporarily from irreversible damage, or to regulate protein activity during oxidative stress. Protein glutathiolation may play a role in redox signaling, as some PSSG complexes undergo glutaredoxin-dependent deglutathiolation while others do not (58,61). As Table III shows, the propensities of particular cysteine residues for glutathiolation depend on the local sequence. All the cysteine residues known to undergo glutathiolation are flanked by one or more of the turn-inducing residues proline and glycine, and almost all the surrounding sequences include Thr, Ser or another hydrogen-bonding residue. Although it is not surprising that Cys residues in exposed loops would be most vulnerable to reaction with GSSG, the fact that some of these sequences are highly conserved suggests that glutathiolation is physiologically important for at least some of these proteins. Glutathiolation has been shown to inactivate some of these proteins (67-71) or to introduce new functions such as phosphatase activity (72).

Fomenko et al. (49) have shown that the CxxS motif is associated with cysteine redox functions in proteins with a variety of overall folds. The CxxS sequence usually is sandwiched between a \( \beta \)-sheet and an \( \alpha \)-helix, as is the CGYS sequence that contains C69 in s-COMT. The motif CxxS tends to be strongly conserved in other proteins (49), and the CGYS motif in s-COMT falls within one of the two sequences that are completely conserved in all the available mammal, amphibian and fish sequences of this protein (see Fig. 2). In addition, although the CxxS motif has not previously been related specifically to glutathiolation, C69 was one of the cysteines that we found to react with GSSG.

Table III illustrates an approach that may be useful for identifying specific cysteine residues that undergo glutathiolation in other proteins. Protein BLAST searches for nearly exact sequence matches to known glutathione binding sites identified potential sites of glutathiolation in a number of other proteins, including some proteins that have been shown experimentally to form PSSG adducts but where the reactive cysteines have not been identified.
ACKNOWLEDGEMENTS

We thank Helen Smith and Dave Eaton for kindly providing a clone of human s-COMT, Brian Phillips for guidance pertaining to GC-MS analysis, Alaina Forbes for help with mutagenesis and the categorization of PSSG adducts, Karen Rutherford help with mutagenesis and creating Fig. 1, Brian Bennion for help with the homology model, Shayna Davis for help with mutagenesis, Philip Gafken, Angela Norbeck, and Nick Vincent-Maloney for help with MALDI-TOF analyses, Vladimir Vigdorovich for guidance on Western blot analyses, and Alan Weiner and Jeffrey Posakony for helpful suggestions. This work was supported in part by NIH grant R01GM49857. Naomi Cotton was supported by an NIH pre-doctoral training grant in molecular biophysics (5-T32-GM08268) and by a grant from the University of Washington Alcoholism and Drug Abuse Institute.
Table I. Enzyme activity of s-COMT mutants in the presence and absence of DTT

<table>
<thead>
<tr>
<th>s-COMT</th>
<th>Activity after 1hr at 22 C with DTT &amp; SAM</th>
<th>Activity in absence/presence of DTT, after 2.5 hr at 37 C without DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>108V</td>
<td>7.1 +/- 0.3</td>
<td>0.7 (5.4/7.4)</td>
</tr>
<tr>
<td>108V /33S</td>
<td>13.2 +/- 0.1</td>
<td>0.8 (9.6/12.4)</td>
</tr>
<tr>
<td>108V /69S</td>
<td>11.0 +/- 0.8</td>
<td>0.2 (1.2/6.9)</td>
</tr>
<tr>
<td>108V /95S</td>
<td>8.2 +/- 0.1</td>
<td>1.0 (6.2/6.2)</td>
</tr>
<tr>
<td>108V /188S*</td>
<td>17.5 +/- 0.3</td>
<td>0.3 (4.0/12.3)</td>
</tr>
<tr>
<td>108M</td>
<td>7.5 +/- 0.7</td>
<td>0.7 (2.7/4.1)</td>
</tr>
<tr>
<td>108M /33S</td>
<td>12.2 +/- 0.1</td>
<td>0.3 (3.4/10.1)</td>
</tr>
<tr>
<td>108M /69S</td>
<td>4.8 +/- 0.4</td>
<td>0.0 (0.0/5.3)</td>
</tr>
<tr>
<td>108M /95S</td>
<td>13.0 +/- 0.3</td>
<td>0.9 (5.7/6.5)</td>
</tr>
<tr>
<td>108M /188S*</td>
<td>15.9 +/- 1.1</td>
<td>0.5 (4.1/7.8)</td>
</tr>
</tbody>
</table>

*Purified s-COMT (1 M) was incubated for 1 hr at 22 C with 4 mM DTT and 200 M SAM (column 2), or (in a separate experiment) for 2.5 hr at 37 C without DTT or SAM (column 3), and then was assayed at the incubation temperature. The incubation medium included 50 mM Tris HCl (pH 7.5) and 1.5 mM MgCl₂ in all cases. Enzyme activities are expressed as M of methylated products formed per 30 minutes per M protein. Column 3 gives the ratio of the enzyme activities measured in the absence or presence of DTT, following incubation at 37 C without DTT. Because of differences in the experimental conditions, including the time between the addition of DTT and the beginning of the assay period, the data in columns 2 and 3 cannot be compared directly.

*Protein concentration measured by ultraviolet absorbance (see Methods).
Table II. Predicted and observed masses of tryptic peptides containing cysteine

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Residues</th>
<th>Calculated mass (Da)</th>
<th>Observed m/z (Da/atomic charge unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>free thiol</td>
</tr>
<tr>
<td>ILNHVLQHAEPGNA-QSVLEAIDTYCEQK</td>
<td>9-36</td>
<td>3120.54</td>
<td>nd b</td>
</tr>
<tr>
<td>IVDAVIQEHQPSVLL-ELGAYCGYSAVR</td>
<td>49-75</td>
<td>2930.51</td>
<td>2930.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LITIEINPDCAAITQR</td>
<td>86-101</td>
<td>1770.94</td>
<td>1770.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YLPDTRLLEECGLLR</td>
<td>147-161</td>
<td>1747.93</td>
<td>1747.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTVLLADVNCPGAP-DFLAHVR</td>
<td>163-184</td>
<td>2278.20</td>
<td>2277.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSSCFECTHYQSFLEYR</td>
<td>185-201</td>
<td>2056.85</td>
<td>2055.8</td>
</tr>
</tbody>
</table>

aHuman 108V s-COMT was incubated with either 7 mM DTT or 5 mM oxidized glutathione (GSSG), and then alkylated with iodoacetamide, proteolyzed with trypsin, and submitted to MALDI-TOF mass spectrometry as described in Methods. The calculated masses of peptides containing cysteine are given in column 3, and the observed m/z of peaks assigned to the free peptide or its products of alkylation or glutathiolation (PSSG) in columns 4-6. Alkylation (acetamidylation) should increase m/z by 57 Da, or 114 Da for the di-alkylated derivative of the peptide containing cysteines 188 and 191; glutathiolation should increase m/z by 305 Da. The italicized numbers in column 6 are the ratio of the intensities of the m/z peaks for the glutathiolated and alkylated products. Similar results were obtained with 108M s-COMT.

bnot observed.
<table>
<thead>
<tr>
<th>Protein, NCBI Number, Sequence</th>
<th>Short, Nearly Exact Sequence Matches</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaredoxin [4504025] (73)</td>
<td>KPSCPYC: HIRA protein [1708202]</td>
</tr>
<tr>
<td>KPTCPYC</td>
<td>GEVCPA: Hydroxyperoxidase II [115722]</td>
</tr>
<tr>
<td>Carbonic anhydrase III [313937985] (74)</td>
<td>DPSCLF: Long transient receptor channel 2 [668045]</td>
</tr>
<tr>
<td>DPSCLFP</td>
<td>DPSCLSP: Nuclear factor activated T-cell [8928220]</td>
</tr>
<tr>
<td></td>
<td>PSCLF: Glutathione-S-transferase P1-1 [544445]</td>
</tr>
<tr>
<td></td>
<td>PSCVFP: Pyruvate kinase [7404396]</td>
</tr>
<tr>
<td></td>
<td>PSCLLP: Tubulin tyrosine ligase-like [2045371]</td>
</tr>
<tr>
<td></td>
<td>DPSCL: Adenylate kinase [24418463]</td>
</tr>
<tr>
<td></td>
<td>EPSCLDAFP: Glutathione-S-transferase Mu2 [232204]</td>
</tr>
<tr>
<td>Glutathione-S-transferase A1-1 [121730] (75)</td>
<td>LPVCPP: ATP-binding cassette ABC1 [13123945]</td>
</tr>
<tr>
<td>LPVCPPE</td>
<td>LPVCPP: Mucin 5B precursor [23821885]</td>
</tr>
<tr>
<td></td>
<td>LPVCSPE: HTH-type transcription regulator [2506549]</td>
</tr>
<tr>
<td></td>
<td>LPVCTPE: Vit. K-dependent prot. Z precursor [131092]</td>
</tr>
<tr>
<td></td>
<td>PVCPP: Keratin, type II cytoskeletal 5 [125105]</td>
</tr>
<tr>
<td></td>
<td>VCPPPE: Mitosis inducer protein kinase cdr1 [19855079]</td>
</tr>
<tr>
<td>Glutathione-S-transferase [6226628] (76)</td>
<td>GACSLA: Glutamyl-tRNA reductase [13432157]</td>
</tr>
<tr>
<td>VGACSLA</td>
<td>VGPCSLA: Cyclin puc1 [116155]</td>
</tr>
<tr>
<td>H-Ras P21 [131869] (77)</td>
<td>GPRCMSC: Arginine deiminase AD1 [10719888]</td>
</tr>
<tr>
<td>GPGCMSC</td>
<td>PGCMS: Histone-lysine N-methyltransferase [25091206]</td>
</tr>
<tr>
<td></td>
<td>PGCMS: Lim domain kinase 1 [3915762]</td>
</tr>
<tr>
<td>PTP-1B [4506289] (68)</td>
<td>HCSAG: Ubiquitin-conjugating enzyme E2 [1717848]</td>
</tr>
<tr>
<td>VIHCSAG</td>
<td>WHCSAG: Leukocyte antigen precursor [3312650]</td>
</tr>
<tr>
<td>Guanidinoacetate N-methyltransferase [2498404] (71)</td>
<td>GENCSSPA: Inositol triphosphate receptor 1 [17366467]</td>
</tr>
<tr>
<td>GENCSSPA</td>
<td>GSCSPA: Ubiquitin-activating enzyme E1 [24418865]</td>
</tr>
<tr>
<td></td>
<td>GESCSP: Lymphotoxin-a-precursor TNF-b [549087]</td>
</tr>
<tr>
<td>HIV-1 protease [37105846] (67)</td>
<td>IEVCGH: Na⁺/Ca²⁺ exchanger [10720116]</td>
</tr>
<tr>
<td>IEICGHK</td>
<td>DICGH: Testis-specific PTP-BL related Y [27151660]</td>
</tr>
<tr>
<td>QIGCTLN</td>
<td>IGCTL: Syntaxin 1B [2501085]</td>
</tr>
<tr>
<td></td>
<td>IGCTL: Transcript Y11 protein [27805760]</td>
</tr>
</tbody>
</table>
Ca-ATPase SERCA1 [1586563] (78)  
**TLGCTSV**  
CTSV: K-ras oncogene-associated protein [13431900]  
**SVICTSDK**  
STICSDK: Sodium/Potassium ATPase [23830899]

Creatine kinase [125305] (70)  
**VLTCPNSN**  
VLTCPNS: Arginine kinase [41017422]  
VLTCP: Centaurin alpha 2 [27923749]

cAMP protein kinase [4506055] (69)  
**WTLCGTP**  
WLSCGVP: B-lymphocyte antigen C19 [1705708]  
WTLGTP: Serine/Threonine protein kinase [41017453]  
WTFCGTP: cGMP protein kinase [125379]

Annexin A2 [30962842] (79)  
**EILCKLS**  
TCKLS: Glyceraldehydephosphate dehydrogenase [6016079]  
**EIICSRT**  
SGSCSRT: Nucleolar GTP-binding protein [17368711]

s-COMT (Cys69) (This work)  
**GAYCGYS**  
GAYCGYS: 6-phosphofructokinase [9988075]  
GAYCGYS: N-acetylgalactosamine kinase 2 [399518]  
GAYCGYS: Tyr protein kinase precursor [13878706]  
CGYS: SAM decarboxylase precursor [4502069]

s-COMT (Cys95) (This work)  
**NPDCAAI**  
DCAAII: Alcohol dehydrogenase (113600)  
PDAGCAAI: Amine oxidase (MAO-B) [113981]  
CAAITSR: Serine hydroxymethyltransferase [20138316]

s-COMT (Cys157) (This work)  
**LEECGGLL**  
LEECGL: DNA polymerase III alpha subunit [14195653]  
EECGLLL: Thyroid receptor interacting prot [20981729]  
LEECGFL: Fascin 3 (Testis fascin) [18203315]

s-COMT (Cys173) (This work)  
**NVICCPGA**  
SCPGAP: Arginine N-methyltransferase [20137529]  
NAICPG: hydroxyprostaglandin dehydrogenase [129889]  
ICPGA: Calcium-dependent protein kinase C [6225593]  
PCPGAP: Myosin IXb [14548118]  
DVTCPGA: Mitotic checkpoint regulator [37537845]  
CPGAPG: Serine/Threonine kinase NIK [14285588]

---

*Identified glutathiolation sites in proteins (columns 1 and 2) and short, nearly exact sequence matches found in other proteins (column 3). Numbers in brackets are NCBI acquisition (Gi) numbers. In column 2, the reactive cysteines are underlined and Pro, Gly,
Ser, and Thr residues are highlighted with bold type. Underlined proteins in column 3 have been shown to form PSSG adducts (59,60,80), but their reactive sites have not been identified experimentally. Where sequence matches were found in the same protein from more than one species, only one is listed.
Figure Legends

FIG. 1. Homology model of human s-COMT bound to Mg$^{2+}$, dinitrocatechol (a substrate analog and inhibitor) and SAM. The seven Cys residues are colored yellow and the location of residue 108 is shown in red.

FIG. 2. Amino acid sequences of human, pig, rat, mouse, frog, fish and protozoan s-COMT as aligned by ClustalW. Cysteine residues are indicated by bold C. The total number of cysteines in each protein is indicated and CxxS and CxxC motifs are labeled.

FIG. 3. Separation of 3¢-methoxy-4¢-hydroxyacetophenone and 3¢-hydroxy-4¢-methoxyacetophenone (the methylated products formed from 3¢,4¢-dihydroxyacetophenone) by gas chromatography.

FIG. 4. Dependence of the enzymatic activity of 108M and 108V s-COMT on the presence of a reductant. Protein stocks were exchanged into buffer containing 50 mM Tris HCl (pH 7.5) and 1.5 mM MgCl$_2$ but no DTT, and stored at –20 C (see Methods). Protein samples from these stocks were incubated for 0, 30 or 60 minutes at 37 C in 50 mM Tris HCl (pH 7.5), 1.5 mM MgCl$_2$ with or without 4 mM DTT as indicated in the figure, and then assayed for 15 minutes at 37 C with no further additions of DTT.

FIG. 5. Alkylation and glutathiolation of Cys173. Human 108V s-COMT was incubated in 10 mM Tris-HCl (pH 7.5), 2 mM MgCl$_2$, and 1 mM NaCl with either 7 mM DTT ($A$) or 5 mM GSSG ($B$) and then alkylated and digested with trypsin as described in Methods. The figure shows regions of the MALDI-TOF mass spectra representing peptide fragments with mass/charge ($m/z$) ratios between 2200 and 2600 Da per atomic charge unit. Peaks assigned to the peptide comprised of residues 163-184 are labeled ($free$ = free peptide, $alkylated$ = amidoacetylated peptide, $PSSG$ = glutathiolated peptide). The peaks at $m/z$ = 2406 and 2463 Da per atomic charge unit are assigned to free and alkylated forms of a miscleaved peptide containing residues 162-184.
FIG. 6. Analysis of a peptide containing a potential disulfide bond. Human 108V s-COMT was incubated with 7 mM DTT (A) or 5 mM GSSG (B) and then alkylated and digested with trypsin as for Fig. 5. Panels A and B show regions of the MALDI-TOF mass spectra representing peptide fragments with mass/charge (m/z) ratios between 2000 and 2300 Da per atomic charge unit. Peaks assigned to the peptide comprised of residues 185-201 are labeled. Portions of spectrum B are expanded in panels C and D, and are shifted horizontally to align subpeaks that differ in m/z by 114 Da per charge unit (the difference expected if the labeled peaks in C represent peptides with two free thiol groups and those in D represent corresponding peptides with two thioacetylamido groups). Oxidation of the dithiol to form an intramolecular disulfide bond decreases the mass of the free peptide (C) by 2 Da.
REFERENCES


---

1 Abbreviations: DTT, dithiothreitol; GSH and GSSG, reduced and oxidized glutathione; IPTG, isopropylthiogalactoside; MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; PMSF, phenylmethylsulfonylfluoride; PSSG, protein-glutathione adduct; SAM, S-adenosylmethionine; s-COMT and mb-COMT, the soluble and membrane-bound forms of catechol-O-methyltransferase, respectively.
Figure 2

CxxS
DAVI#EHQPSVNLLELGAYCGYSAVRMARLLSPGARLITIEINPDCAITQRMVEFAGVDKVTLLVVGASQD---1EPQLKKK-YDVTLD
DTTVQQEQRRPSVNLLELGAYCGYSAVRMARLLPSARLLTIELNPNAAIQAQVVDFAQLQDVRTVVGASQD---1EPQLKKK-YDVTLD
DAVIREYPSLVLLELGAYCGYSAVRMARLLQPGARLTMEMNDYAATQQMLNFAGLQDKVTILNGASQD---1LPQLKKK-YDVTLD
DAVIREYPSLVLLELGAYCGYSAVRMARLLPGARLTMEMNDYAATQQMLNFAGLQDKVTILNGASQD---1LPQLKKK-YDVTLD
DNIVKETNPSTLLLEGTYGYSAIRICRSLKPGARFFTVEFNPAYAAYAVQMMIEFAGLKDVKVQVLEGSTSD---1LPQLKKK-YEVETLD
DSVSENLPKELLELGTYGYSTVRIARLLPPGARLTLLEFNPNYAIVAREQVIZAWGREDKQLVEGASED---WPRMKEH-FGIETF
DAAVRRADPALALELGLYGALRIAR-AAPPRVYVSVELAEANASSRAWAHAGVDDRVCVGTTIDGGRITFDALTEHGFATGTL

CxxC
MVFLDHKDRYLPDTNLEECGLRRKGTVLLEAIDNVICPA#DFHALVHRGS---CFECTIONQSEFLEREV-DGLEKAIYKPGSAGP- 221
MVFLDHKDRYLPDTNLEECGLRRKGTVLLEAIDNVICPA#DFHALVHRGC---RFECTHYSSLEYSQMV#DGLEKAVKPGSPAQP- 186
MVFLDHKDRYLPDTNLEECGLRRKGTVLLEAIDNVICPA#DFHALVHRGS---SFECTHYSSLEYSQMV#DGLEKAIYQGSPSPDKS 221
MVFLDHKDRYLPDTNLEECGLRRKGTVLLEAIDNVICPA#DFHALVHRGS---SFECTHYSSLEYSQMV#DGLEKAVQGSPSPVKS 222
FVFVTDHV#KDPDQ#LECNLLLRRKG#VLLEAIDNVICPA#DFL#VHRGC---KYM#CTNFSL#YMD#E-DLEKAVFR- 214
LVFLDHKDRYLPDTKLMGGLLEAIDNVICPA#P#LYEY#VNSR---SYKSCYFKHLEYTRA-DLEKSVFLG- 207
FVFVTDHKAYLPDLQSFIDGRLHPGSIVVADVNRELPGAPKRYA#R-QGMSWNTIELEK#HLEQ-YTLPDLESEYLG- 220
Figure 4
Figure 5
**Figure 6**

(A) Mass spectrum of GSSCFeCTHYQSFLEYR<sup>201</sup> with peaks at 2053.8, 2054.8, 2055.8, 2056.8, 2057.8, and 2058.8 m/z. The peak at 2169.5 m/z is labeled as di-alkylated.

(B) Mass spectrum of GSSG with peaks at 2055.8 m/z (Cys SH or S-S) and 2169.5 m/z (di-alkylated).

(C) Expanded view of the GSSG spectrum showing peaks at 2053.8, 2054.8, 2055.8, and 2056.8 m/z.

(D) Mass spectrum of GSSG with peaks at 2169.8 and 2170.8 m/z (di-alkylated).
Oxidative inhibition of human soluble catechol-O-methyltransferase
Naomi J.H. Cotton, Barry Stoddard and William W. Parson

J. Biol. Chem. published online March 18, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401086200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2004/03/18/jbc.M401086200.citation.full.html#ref-list-1