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A nano switch mechanism for the redox-responsive sulfotransferase

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ABSTRACT

Cellular redox signaling is important in diverse physiological and pathological processes. The activity of rat phenol sulfotransferase (*r*SULT1A1), which is important for the metabolism of hormone and drug, is subjected to redox regulation. Two cysteines, Cys232 and Cys66, nanometer away from each other and from the enzyme active site were proposed to form disulfide bond to regulate the activity of *r*SULT1A1. A nano switch, composed of a flexible loop from amino acid residues 59–70, explained how this long distance interaction between two cysteines can be achieved. The enzyme properties were investigated through site-directed muatagnesis, circular dichroism, enzyme kinetics and homologous modeling of the *r*SULT1A1 structures. We proposed that the formation of disulfide bond between Cys232 and Cys66 induced conformational changes of sulfotransferase, then in turn affected its nucleotide binding and enzyme activity. This discovery was extended to understand the possible redox regulation of other sulfotransferases from different organisms. The redox switch can be created in other redox-insensitive sulfotransferases, such as human phenol sulfotransferase (*h*SULT1A1) and human alcohol sulfotransferase (*h*SULT2A1), to produce mutant enzymes with redox regulation capacity. This study strongly suggested that redox regulation of drug and hormone metabolism can be significantly varied even though the sequence and structure of SULT1A1 of human and rat have a high degree of homology.

1. Introduction

Cellular redox signaling contributes to the control of cell development, differentiation, growth, death and adaptation, and has been implicated in diverse physiological and pathological processes [1]. The non-equilibrium nature of biological systems necessitates temporal or spatial suppression or enhancement of specific biochemical pathways, either gene regulation or protein modification, according to cellular metabolism or exogenous signals [2]. Many factors, including clinical oxygen treatment, chemical (toxicants) stress, physical stress, aging, virus infection, and different pathological conditions, can cause oxidative stress. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can modify thiol bonds that affect a protein's function [3]. Oxidative stress is a well-known cause of changes in GSSG/GSH ratios and levels *in vivo* [4].

Previous study indicated that stress and oxidative regulation of rat phenol sulfotransferase (*r*SULT1A1) occurs at the level of protein modification, but not at gene expression [5,6]. *In vitro* studies confirmed that a highly conserved Cys66 is important for the regulation of *r*SULT1A1 catalytic activity [7,8]. Redox status and nucleotide binding have been shown to regulate catalytic activity of *r*SULT1A1 and are shown to be responsible for the complete interconversion of two enzyme forms [9]. It is intriguing to observe that cytosolic SULTs can be regulated either at gene level or at protein level through redox treatments [7,8]. What is the distinction between these two types of enzyme and if the redox regulation can be created in all cytosolic SULT are the subjects of this research.

Sulfonation (Fig. 1) catalyzed by cytosolic sulfotransferase in biological system is a popular and important biotransformation that involves in detoxication of a broad range of endobiotics and xenobiotics and activation and deactivation of hormones and carcinogens [10,11]. Macromolecular substrates such as proteins and polysaccharides are metabolized by membrane-bound SULTs [12]. Recent studies have implicated the SULTs in a number of disease states including various forms of cancer [13], entry of the herpes virus [14], enterovirus [15,16] or HIV [17,18], and chronic inflammation [19]. They are novel therapeutic targets to discover new drugs [20].

Our approach to study this phenomenon is to first understand the mechanism for SULT redox regulation according to the sequence, structure and catalytic function of SULTs. It has been proposed that two distanced cysteines, Cys232 and Cys66, in this enzyme may be responsible for the redox regulation of its catalytic

Abbreviations: Bis–tris propane, 1,3-bis[tris(hydroxymethyl) methylamino] propane; CD, circular dichroism; DEAE, diethylaminoethyl; GSSG, glutathione (oxidized form); GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SULT, sulfotransferase; TCEP, tris(2-carboxyethyl) phosphine.[2]

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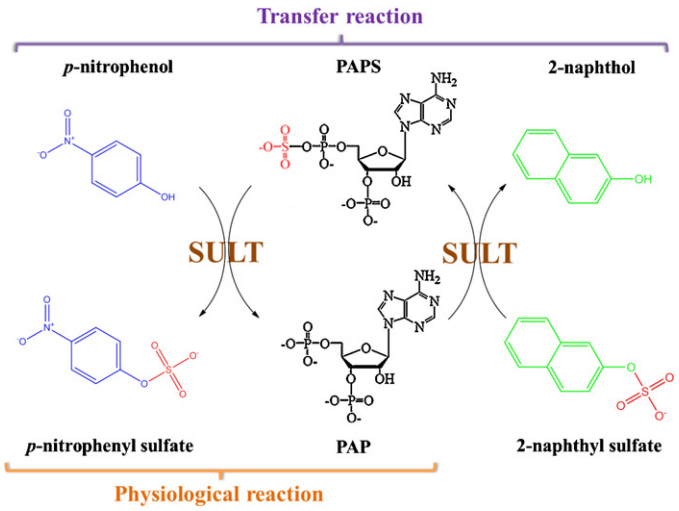

Fig. 1. Reactions catalyzed by SULT1A1.

activity [8]. Neither cysteine is near the enzyme active site. How the formation of potential disulfide bond can affect the *r*SULT1A1 activity is unclear. Furthermore, the involvement of the three other cysteines in *r*SULT1A1 needs to be explained in a redox environment. Sequence and structural analyses of SULTs indicated that the flexible loop (amino acid residue 59–70) and Trp49 of *r*SULT1A1 may closely relate to redox regulation of its activity. How these amino acids coordinate to function as a redox switch is to be explained. Finally, it is important to understand the difference between rat and human SULT1A1 because rat is frequently used as animal model to study human diseases. To understand the structural and mechanistic basis for redox regulation of cytosolic SULTs would be a remarkable progress to elucidate the role in the metabolism of carcinogens, drugs, neurotransmitters and xenobiotics, and may assist in chemical risk assessment and the design of more effective therapeutics.

2. Materials and methods

2.1. Reagents

Bis–tris propane, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol, (ethylenedinitrilo) tetraacetic acid (EDTA), glutathione reduced form (GSH), glutathione oxidized form (GSSG), 4-methylumbelliferone (MU), 4-methylumbelliferyl sulfate (MUS), 2-naphthol, N^6-(6-aminohexyl)adenosine 3′,5′-diphosphate, 3′-phosphoadenosine 5′-phosphate (PAP), PAP agarose, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), *p*-nitrophenol (*p*NP), *p*-nitrophenyl sulfate (*p*NPS) were purchased from Sigma (USA). Tris(2-carboxyethyl) phosphine (TCEP) was obtained from Pierce (USA). Glycerol, potassium phosphate and sucrose were obtained from J.T. Baker (USA). DEAE Sepharose fast flow, glutathione S-transferase sepharose fast flow, and HiTrap™ desalting column was obtained from GE Healthcare (USA). All other chemicals were obtained commercially at the highest purity possible.

2.2. Computational analysis of protein sequence and structure

SULT sequences were obtained from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). SULT structures used in this report were solved by X-ray crystal diffraction and obtained from Protein Data Bank (PDB, http://www.rcsb.org/pdb/). The molecular modeling of *r*SULTT1A1 structure was done at SWISS-MODEL (http://www.expasy.org/swissmod/SWISS-MODEL.html). The first approach mode was run [21] and hSULT1A1 was used as template. Amino acid sequences of SULTs were aligned using ClustalW program at European Bioinformatics Institute (EBI, http://www.ebi.ac.uk/Tools/clustalw/index.html), and their protein structures were compared and aligned using the Combinatorial Extension (CE) method (http://cl.sdsc.edu/ce.html).

2.3. Site-directed mutagenesis of cDNA encoding SULTs

Site-directed mutagenesis was performed with *PfuTurbo*® DNA polymerase using QuickChange (Stratagene, La Jolla, CA). All primers for mutagenesis were purchased from Mission Biotech Co., Ltd. (Taiwan). The cDNA of *r*SULT1A1 and human SULT incorporated in the pET-3c and pGEX-2TK expression vector were used as templates in conjunction with specific mutagenic primers, respectively. Mutated cDNA sequences were confirmed using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA) following the standard protocol. Six mutant clones and their primers for mutagenesis were prepared: *r*SULT1A1 mutant C66S (forward primer, 5′-G CTA GAG AAG **AGT** GGC CGC GCC CCC-3; reverse primer, 5′-G GGC GCG GCC **ACT** CTT CTC TAG CTT GCC-3′), *r*SULT1A1 mutant C232S (forward primer, 5′-G AAA GAG AAC **AGC** ATG ACT AAC TAC AC-3′; reverse primer, 5′-A GTT AGT CAT **GCT** GTT CTC TTT CAT TTT C-3′), *h*SULT1A1 mutant P236C (forward primer, 5′-G AAG AAG AAC **TGC** ATG ACC AAC TAC ACC-3′; reverse primer, 5′-A GTT GGT CAT **GCA** GTT CTT CTT CAT CTC-3′), *h*SULT2A1 mutant I66C (forward primer, 5′-T GCC AAG TGG **TGC** CAA TCT GTG CCC ATC-3′; reverse primer, 5′-G CAC AGA TTG **GCA** CCA CTT GGC ATC CCC-3), *h*SULT2A1 mutant K227C (forward primer, 5′-G AAA GAA AAC **TGC** ATG TCC AAT TAT TCC C-3′; reverse primer, 5′-A ATT GGA CAT **GCA** GTT TTC TTT CAT GCT C-3′), and *h*SULT2A1 mutant I66C/K227C. Bold and underlined nucleotides indicated the designated positions for site-directed mutagenesis.

2.4. Expression and purification of SULT recombinant enzymes

Recombinant wild type and mutant SULT clones were transformed into *Escherichia coli* BL21 (DE3) and over-expressed. Detail methods were described previously [22–24]. SULTs were extracted by sonication, and purified by DEAE Sepharose chromatography and GST gene fusion system (GE Healthcare, USA). The two forms of SULT1A1 were separated by PAP-agarose chromatography as described previously [9]. All enzymes used in this study were purified and the purity was at least 95% homogeneity according to SDS-PAGE.

2.5. Preparation of reduced and oxidized SULTs

SULTs were continuously reduced, oxidized and reduced again to prepare enzymes in different redox stages and to demonstrate that these enzymes were reversible under redox environments. Purified SULTs were passed through HiTrap™ desalting column with exchange buffer (1 mM EDTA, 10% glycerol, and 125 mM sucrose in 100 mM bis–tris propane at pH 7.0) to remove possible interfering reagents. SULTs (1 ml, 20 μM dimmer) were then incubated in 1 mM TCEP for 60 min at 25 °C to give the reduced SULTs. Excess amount of the reducing agent was removed by desalting chromatography. The oxidized SULTs were prepared by incubating the reduced SULTs (1 ml, 10 μM dimmer) with GSSG (1 mM) at 25 °C for 60 min. The excess amount of GSSG was removed through HiTrap™ desalting column. The oxidized SULTs

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(1 ml, 5 μM dimmer) were again incubated in 1 mM TCEP for 60 min at 25 °C, and then following by desalting chromatography to obtain reduced SULTs from their oxidized forms.

2.6. Titration of protein thiol

The number of free cysteine thiol groups of SULT was determined according to previously described method [25]. After passing through the desalting column with exchange buffer, SULT (1 μM) was incubated with 50 μM DTNB in 100 mM bis–tris propane (pH 7.0) at 25 °C in a final volume of 1 ml. Formation of the 2-nitro-5-thiobenzoate ion was followed at 412 nm (ε = 14,150 M^{-1} cm^{-1}). Each titration was performed in triplicate together with controls without the addition of the enzyme or DTNB, respectively.

2.7. Dissociation constant of PAP and SULT

The K_d was determined by fluorescence as described previously [26]. An aliquot amount of PAP was added into the solution containing SULT (0.06 or 1 μM), 50 mM potassium phosphate buffer at pH 7.0. The decrease in intrinsic fluorescence of protein was measured at 340 nm upon excitation at 280 nm and 25 °C with a Hitachi spectrofluorometer (F-4500, Japan). The dissociation constant was obtained by the equation: $\Delta F = \Delta F_{max} - K_d$ (ΔF/[PAP]). The K_d was calculated by using nonlinear regression by SigmaPlot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS Inc., Chicago, IL).

2.8. Enzyme assay

The activities of *h*SULT2A1 were determined by fluorescence based on a coupled-enzyme assay method [22]. The reaction mixture consisted of 100 mM potassium phosphate buffer at pH 7.0, 20 μM PAPS, 4 mM MUS and 3.2 mU *r*SULT1A1 mutant K65E/R68G. In this assay, the increase of MU reflected the usage of PAPS by SULT2A1 and was determined at 450 nm upon excitation at 360 nm. SULT1A1 assay was carried out *via* the change of absorbency due to free *p*-nitrophenol ($\varepsilon_{400\ nm}$ = 10,500 M^{-1} cm^{-1} at pH 7.0) as described previously [27]. Two types of SULT1A1 activities were determined [8,24]. The physiological assay followed the decrease in absorbance of *p*NP (100 μM) when PAPS (300 μM) was the sulfuryl group donor. The reaction mixture consisted of 300 μM PAPS, 100 μM *p*NP, and 100 mM bis–tris propane at pH 7.0. The transfer reaction determines the rate of sulfuryl group exchange between two phenols in the presence of PAP which served as a cofactor (Fig. 1). The reaction mixture consisted of 1 mM *p*NPS, 50 μM 2-naphthol, 2 μM PAP and 100 mM bis–tris propane at pH 7.0.

2.9. Determination of kinetic constants

Measurements of the kinetic constants for each substrate were performed by varying the concentrations of one substrate, while keeping the other substrates at a fixed and near saturating concentrations. The apparent K_m and k_{cat} were determined using nonlinear regression by SigmaPlot 2001, V7.0 and Enzyme Kinetics Module, V1.1 (SPSS Inc., Chicago, IL).

2.10. Circular dichroism measurements

Data were collected by a Jasco J-715 spectropolarimeter and processed with J-700 standard analysis version 1.33.00 (Jasco, Japan). A 10 mm light path was used in the near-UV range (240–340 nm) and 1 mm light path in the far-UV (200–240 nm). SULT enzymes were passed through a desalting column with potassium phosphate buffer (10 mM) at pH 7.0 following by centrifugation to assure clarity.

2.11. Structural simulation illustrating mechanism of redox-responsive SULTs

We selected *h*SULT1A1 (PDB code 1LS6) and *h*SULT2A1 (PDB code 1EFH) that contain PAP as the protein templates for modeling their mutant structures, P236C and I66C/K227C, respectively, by SWISS-MODEL [28]. PAP containing template structures are important because the binding of this nucleotide appeared to be significantly affected in various redox states. The disulfide bond structure located in between Cys66 and Cys232 of the redox-sensitive SULTs were modeled and energy optimized according to their theoretical conformations by using SYBYL® [29].

3. Results

3.1. Identification of amino acids involved in redox regulation of rSULT1A1

Structure and sequence of SULTs provided important information for the function of each amino acid. Fourteen rat and human cytosolic SULT protein sequences were analyzed (Fig. S1). The PAP/PAPS binding pocket, which contains 5′-phosphosulfate binding domain (5′-PSB, green and yellow) and 3′-phosphate binding domain (3′-PB, blue and purple), is conserved in all SULTs (highlighted in red, yellow, and purple in Fig. S1). Cys66 of *r*SULT1A1 is also conserved in other SULTs except for SULT2 and SULT4 family that contain an isoleucine (deep gray). On the contrary, Cys232, the proposed amino acid that forms disulfide bond with Cys66, in *r*SULT1A1 is not conserved among SULTs. The corresponding residues (light gray) in other SULTs are proline, lysine, and glutamine in SULT1 family, *h*SULT2A1, and SULT4A family, respectively. The enormous variety of residue observed indicated that this position is very flexible to admit different amino acid without affecting its enzymatic function.

Structural comparison among SULT1A1s confirmed that the locations of the three-dimensional structures of the above mentioned amino acids were consistent with sequence alignment result. The two targeted amino acids, Cys66 and Cys232 and their corresponding amino acids in other SULTs, highlighted in Fig. 2 were about 12.7 Å apart. The root-mean-square deviation (RMSD) of these amino acids in different SULTs indicated that their positions were conserved (Table S1). The RMSD values of C_α atoms of Cys66 in *r*SULT1A1 between Cys70 in *h*SULT1A1 and Ile66 in *h*SULT2A1 are 0.47 Å (backbond atoms, 0.51 Å) and 2.29 Å (backbond atoms, 2.48 Å), respectively. Furthermore, the RMSD values of C_α atoms of another corresponding residue (Cys232 in *r*SULT1A1) between Pro236 in *h*SULT1A1 and Lys227 in *h*SULT2A1 are 0.41 Å (backbond atoms, 0.56 Å) and 0.95 Å (backbond atoms, 0.99 Å), respectively. These values revealed the locations and flexibility of the regions that may directly involve the redox regulation of SULT activity. The functions of each amino acid were further confirmed by site-directed mutagenesis and kinetic studies in this report.

3.2. Determination of the number of free thiols in SULTs

The change of enzyme thiol groups in the reduced and oxidized forms of SULTs may indicate the number of thiols available for redox manipulation. Cysteine thiols in SULTs were first determined by DTNB when the enzymes were isolated from a reducing condition (Table 1(A)). Prior to GSSG treatment, 4.5 of the five known cysteine groups per subunit of *r*SULT1A1 were titrated with DTNB. The single mutants, C66S and C232S, of *r*SULT1A1 gave 3.5

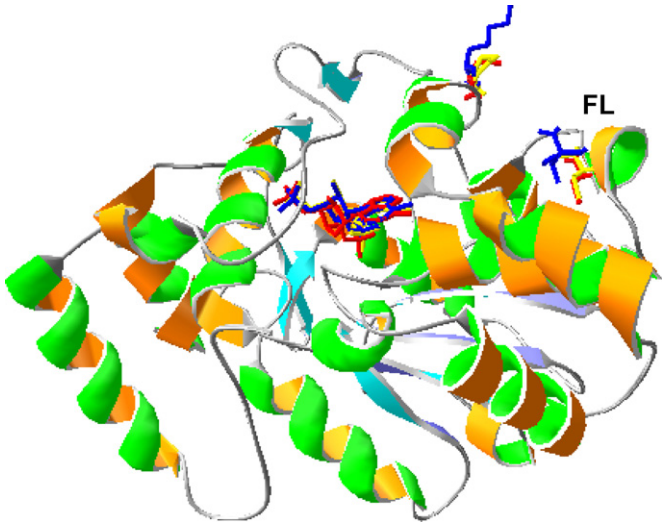

Fig. 2. Structure of SULT1A1. The three dimensional structure was obtained by homologous modeling (SWISS-MODEL) using *h*SULT1A1 (PDB code, 1LS6) as template. Identity between *r*SULT1A1 and *h*SULT1A1 is 80%. PAP and the two amino acids corresponding to Cys66 and Cys232 (upper right) of *r*SULT1A1 from other SULTs are denoted as molecular structure. The two amino acid residues and PAP of *r*SULT1A1, *h*SULT1A1 (Cys70 and Pro236), and *h*SULT2A1 (Ile 66 and Lys227) were indicated in red, yellow, and blue, respectively. Structure comparisons of the two amino acids in *r*SULT1A1, *h*SULT1A1 (1LS6), and *h*SULT2A1 (1EFH) were analyzed by Combinatorial Extension (CE) as shown in Table S1. FL indicates flexible loop. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and 3.2 titrated thiols, respectively. These data indicated that most of the thiols in *r*SULT1A1 can be determined by DNTB titration, which was consistent with previous finding [8]. Similar results were obtained for *h*SULT1A1 and *h*SULT2A1 and their mutants (Table 1(A)). Wild-type and P236C mutant of *h*SULT1A1, which contain 2 and 3 cysteines, gave 1.7 and 2.8 titrated thiols per subunit, respectively. Wild-type and I66C, K227C, I66C/K227C of *h*SULT2A1, which contain 1, 2, 2, and 3 cysteines, respectively, gave 1, 1.7, 1.8 and 2.8 thiols titrated by DTNB. Our result indicated that, prior to GSSG treatment, nearly all the cysteines in SULTs were in the reduced form and can be titrated by DTNB.

The number of remaining thiols following GSSG treatment are given in Table 1(B). Wild-type *r*SULT1A1, P236C mutant of *h*SULT1A1 and I66C/K227C double mutant of *h*SULT2A1, lost nearly two thiols following GSSG treatment. No more than two cysteines could be oxidized by GSSG even though there are more than two free cysteine thiols in *r*SULT1A1, P236C of *h*SULT1A1 and I66C/K227C of *h*SULT2A1. Also, the two cysteines were effectively oxidized by GSSG only when both targeted cysteines were present (Table 1). Other cysteine thiol was insensitive to GSSG. It is likely that these two cysteine thiols formed a disulfide linkage facilitated by the oxidation of GSSG.

Table 1
Titration of free thiols in SULTs.[a]

SULT (no. of cysteine)	Number of free thiols		
	(A) Reduced[b]	(B) Oxidized[b]	(A) − (B)
*r*SULT1A1			
Wild-type (5)	4.5 ± 0.12	2.7 ± 0.07	1.8
C66S (4)	3.5 ± 0.08	2.4 ± 0.05	1.1
C232S (4)	3.2 ± 0.09	2.0 ± 0.06	1.2
*h*SULT1A1			
Wide type (2)	1.7 ± 0.04	1.4 ± 0.05	0.3
P236C (3)	2.8 ± 0.08	1.2 ± 0.04	1.6
*h*SULT2A1			
Wide type (1)	1.0 ± 0.02	0.9 ± 0.02	0.1
I66C (2)	1.7 ± 0.03	1.4 ± 0.05	0.3
K227C (2)	1.8 ± 0.04	1.2 ± 0.02	0.6
I66C/K227C (3)	2.8 ± 0.07	1.1 ± 0.03	1.7

[a] Enzymes were titrated with DTNB for the thiols in the absence of PAP as described in Section 2.
[b] The reduced and GSSG treated enzymes were prepared with TCEP (1 mM) and GSSG (1 mM), respectively, as described in Section 2.

Table 2
Dissociation constants (K_d) of PAP and SULTs.[a]

	Reduced[b]		Oxidized[b]	
	K_d1 (μM)	K_d2 (μM)	K_d1 (μM)	K_d2 (μM)
*r*SULT1A1				
Wild-type	0.03 ± 0.001	187 ± 15	–[c]	252 ± 21
C66S	0.03 ± 0.003	184 ± 13	0.03 ± 0.003	265 ± 19
C232S	0.03 ± 0.001	218 ± 20	0.02 ± 0.002	223 ± 23
W49A	–[c]	140 ± 14	–[c]	163 ± 10
*h*SULT1A1				
Wild-type	0.11 ± 0.021	117 ± 15	0.09 ± 0.03	120 ± 18
P236C	0.01 ± 0.002	126 ± 16	–[c]	115 ± 12
*h*SULT2A1				
Wild-type	0.02 ± 0.001	290 ± 24	0.01 ± 0.001	156 ± 12
I66C	0.02 ± 0.01	290 ± 28	0.02 ± 0.002	147 ± 22
K227C	0.19 ± 0.08	174 ± 11	0.05 ± 0.02	104 ± 7.9
I66C/K227C	0.05 ± 0.02	264 ± 22	–[c]	146 ± 22

[a] Dissociation constants were determined by fluorescence as described under Section 2. The mean ± standard error was obtained with nonlinear regression. The PAP-free SULTs (60 nM and 1 μM dimmer) were used for the determination of K_d1 and K_d2, respectively.
[b] The reduced and GSSG treated enzymes were prepared with TCEP (1 mM) and GSSG (1 mM), respectively, as described in Section 2.
[c] The tight binding site could not be observed.

3.3. Redox effect on the affinity of PAP and SULTs

PAP can be the substrate, product or cofactor of SULT dependent on how the enzymatic reaction is designed (Fig. 1). The affinity of PAP and SULT can be a good indicator to observe the redox effect on SULT functions. Two binding constants, K_d1 and K_d2, which differ about four orders of magnitude, were observed (Table 2) for all SULTs in their reduced state except W49A mutant of *r*SULT1A1. These data were consistent with previous finding for some SULTs [8,26]. The W49A mutant is known to have low binding affinity with PAP even at its reduced state [30]. This mutant is included in this study because it is consistent with our proposal that Trp49 of *r*SULT1A1 was important for the PAP binding and its conformation was altered under different redox states.

Following GSSG treatment, the nano molar range K_d1 of some SULTs could not be observed (wild-type *r*SULT1A1, P236C of *h*SULT1A1 and I66C/K227C of *h*SULT2A1 in Table 2). These enzymes all contain the two targeted cysteines that are located at positions corresponding to those of Cys66 and Cys232 of *r*SULT1A1. No such redox effect could be observed when one or two of the targeted cysteines were absent (C66S and C232S of *r*SULT1A1, wild-type and P236C of *h*SULT1A1, and wild-type, I66C and K227C of *h*SULT2A1 in Table 2). It was proposed that SULT tight binding site for PAP was significantly altered with the formation of a disulfide bond between the two targeted cysteines.

3.4. Redox effect on the transfer reaction catalyzed by SULT1A1

The transfer reaction of SULT1A1 is defined as shown in Fig. 1, in which PAP is functioning as a cofactor to transfer sulfuryl group between two phenol compounds. To complete a transfer reaction, PAP does not need to be released from SULT enzyme which is the rate-limiting step [9,26,30–32]. Thus, activity of the transfer reaction can be a good measure for determining the binding efficiency of the nucleotide as a cofactor for SULT1A1 catalyzed

Table 3
Kinetic constants of transfer reaction catalyzed by rat and human SULT1A1s and their mutants.[a]

	Reduced[b]			Oxidized[b]		
	K_m (PAP) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (PAP)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
*r*SULT1A1						
Wild-type	0.03 ± 0.01	2.31 ± 0.32	77	3.80 ± 0.50	0.57 ± 0.02	0.15
C66S	0.06 ± 0.02	2.55 ± 0.16	43	0.15 ± 0.05	1.93 ± 0.11	12.9
C232S	0.06 ± 0.02	2.04 ± 0.08	34	0.24 ± 0.02	1.69 ± 0.04	7.04
W49A	5.74 ± 0.66	2.53 ± 0.07	0.4	90.6 ± 13.3	0.62 ± 0.02	0.01
*h*SULT1A1						
Wild-type	0.05 ± 0.01	1.51 ± 0.05	30	0.04 ± 0.01	0.86 ± 0.03	21.5
P236C	0.06 ± 0.01	1.32 ± 0.05	22	1.25 ± 0.10	0.51 ± 0.01	0.41

[a] Enzyme activities were determined as described under Section 2. The mean ± standard error was obtained with nonlinear regression.
[b] The reduced and GSSG treated enzymes were prepared with TCEP (1 mM) and GSSG (1 mM), respectively, as described in Section 2.

reaction. The redox effect on the transfer reaction of SULT1A1 is shown in Table 3. Similar K_m (for PAP) and k_{cat} were obtained for all the reduced form of SULT1A1s, except for W49A mutant of *r*SULT1A1. As mentioned above, PAP binding site of W49A is known to be impaired [30]. This result was consistent with the data shown in Table 2 that, in the reduced state, the presence of either one (wild-type *h*SULT1A1, and C66S and C232S of *r*SULT1A1) or two (wild-type *r*SULT1A1 and P236C of *h*SULT1A1) targeted cysteines did not affect either the dissociation constant of PAP or the transfer activity of SULT1A1.

Following the treatment with GSSG, the oxidized SULT1A1 exhibited significant variations in catalytic efficiency (k_{cat}/K_m), especially when both targeted cysteines (wild-type *r*SULT1A1 and P236C of *h*SULT1A1) were present (Table 3). Elimination of Cys66 or Cys232 of *r*SULT1A1 by site-directed mutagenesis greatly diminished the redox effect, about 90 and 50 folds differences, respectively, in catalytic efficiency compared to that of wild-type enzyme. For *h*SULT1A1, which contains only one cysteine and was less sensitive to the change of redox environment, the redox effect was greatly enhanced (about 50 folds) by introducing additional cysteine (P236C) at location corresponding to Cys232 of *r*SULT1A1 (Table 3).

3.5. Redox effect on SULTs observed by circular dichroism (CD) spectropolarimetry

A three order of magnitude variation in dissociation constants for some SULTs shown in Table 2 indicated that their PAP/PAPS

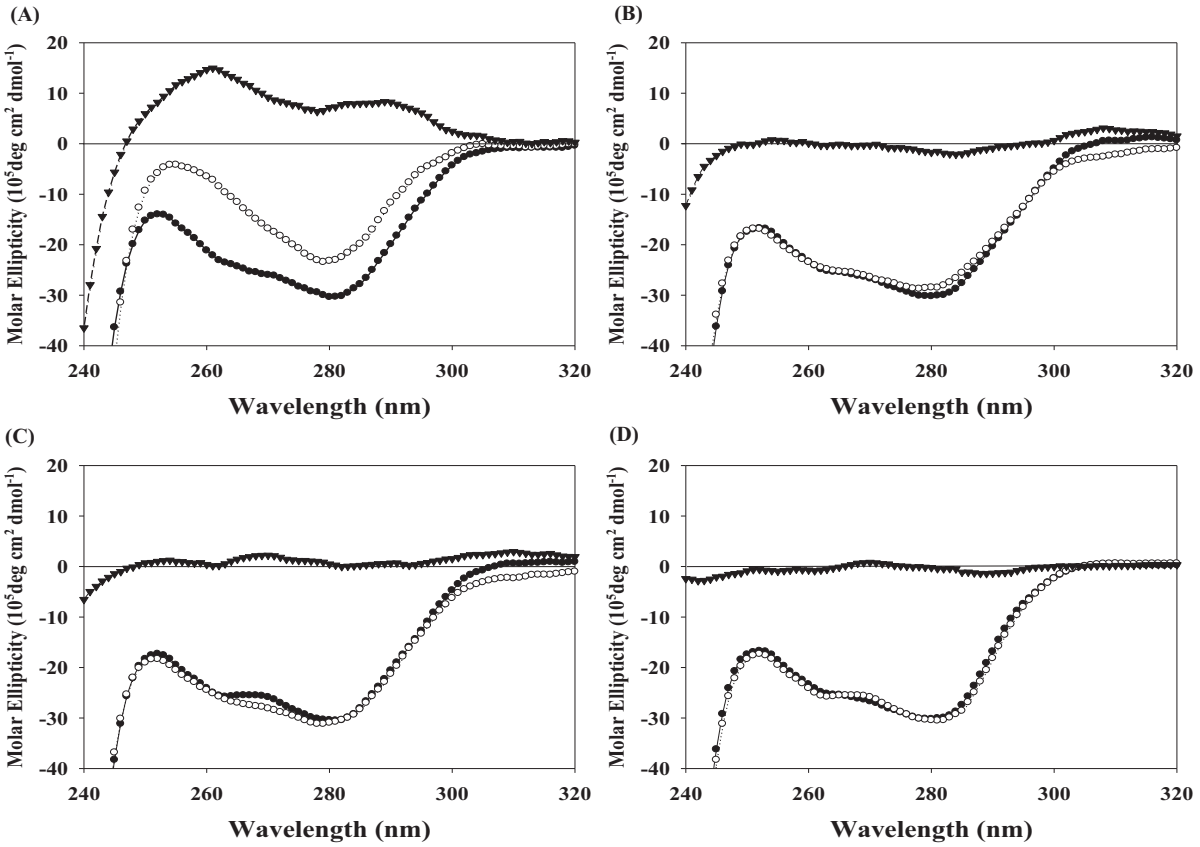

Fig. 3. Effect of redox on CD spectra of *r*SULT1A1 and its mutants. (A) wild-type *r*SULT1A1. (B) C66S mutant of *r*SULT1A1. (C) C232S mutant of *r*SULT1A1. (D) W49A mutant of *r*SULT1A1. Filled circle (●) and open circle (○) denote TCEP-treated and GSSG-treated enzymes, respectively, and filled triangle (▼) denotes their spectral differences. The TCEP-treated enzymes (30 μM) were incubated with equivalent amount of PAP and TCEP (1 mM) at 25 °C for 60 min in a potassium phosphate buffer (10 mM, pH 7.0). The GSSG-treated enzymes were incubated with GSSG (1 mM) at 25 °C for 60 min following by desalting chromatography to remove GSSG. The desalted enzymes (30 μM) were incubated with equivalent amount of PAP at 25 °C in potassium phosphate buffer (10 mM, pH 7.0). The spectra were taken at room temperature from an average of 25 scan in potassium phosphate buffer (10 mM) at pH 7.0.

Table 4
Kinetic constants of redox-responsive SULTs.[a]

Transfer reaction	*r*SULT1A1 wild-type			*h*SULT1A1 mutant P236C			*h*SULT2A1 mutant I66C/K227C		
	K_m (PAP) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (PAP) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (PAP) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
I. Reduced[b]	0.03 ± 0.002	2.31 ± 0.32	77	0.06 ± 0.008	1.32 ± 0.05	22	–[d]	–	–
II. Oxidized[b]	3.80 ± 0.50	0.57 ± 0.023	0.15	1.25 ± 0.10	0.51 ± 0.008	0.41	–	–	–
III. Reduced[c]	0.03 ± 0.002	2.54 ± 0.11	85	0.02 ± 0.006	0.56 ± 0.006	28	–	–	–

Physiological reaction	K_m (PAPS) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ s^{-1} μM^{-1})	K_m (PAPS) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ s^{-1} μM^{-1})	K_m (PAPS) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ s^{-1} μM^{-1})
I. Reduced[b]	3.90 ± 0.30	0.083 ± 0.012	21	0.64 ± 0.04	0.022 ± 0.0003	34	0.84 ± 0.15	0.031 ± 0.001	37
II. Oxidized[b]	30.0 ± 5.02	0.283 ± 0.012	9.3	4.86 ± 0.99	0.005 ± 0.0003	1.0	8.04 ± 1.22	0.032 ± 0.001	4.0
III. Reduced[c]	4.50 ± 0.60	0.102 ± 0.011	22	0.89 ± 0.10	0.018 ± 0.001	20	1.05 ± 0.18	0.028 ± 0.001	27

[a] Enzyme activities and rate constants were determined as described in Section 2. The mean ± standard error was obtained with nonlinear regression.
[b] The reduced and GSSG treated enzymes were prepared with TCEP (1 mM) and GSSG (1 mM), respectively, as described in Section 2.
[c] The reduced enzymes were prepared in the presence of TCEP (1 mM) using the oxidized enzyme.
[d] The transfer assay was developed for phenol sulfotransferase (SULT1A) only and cannot be used for alcohol sulfotransferase (SULT2A).

binding environments were significantly altered in different redox states. CD spectra (200–240 nm) of SULTs and their mutants listed in Table 2 revealed that their secondary structures remained intact before and after GSSG treatment (Fig. S2). However, the variation in CD spectra at longer wavelength range (250–300 nm) revealed that environment around some aromatic residue might significantly vary in wild-type *r*SULT1A1 before and after GSSG treatment. As show in Fig. 3, interaction between PAP and *r*SULT1A1 gave two distinct absorptions at 260 and 285 nm, contributed by PAP and aromatic residue of the enzyme, respectively. These distinct absorptions varied significantly when *r*SULT1A1 was treated with GSSG (Fig. 3A). However, the CD spectra of *r*SULT1A1 became insensitive to GSSG treatment upon the mutation of Cys66 (C66S, Fig. 3B), Cys232 (C232S, Fig. 3C) and W49 (W49A, Fig. 3D), respectively. This observation was consistent with other kinetic data that the presence of both targeted cysteines (Cys 232 and 66) was needed for manipulating SULT1A1 activity through redox regulation. It also indicated that the SULT1A1 binding site of PAP/PAPS was altered upon change of redox environment. Similar changes of CD spectra were also observed for the *h*SULT1A1 and *h*SULT2A1 enzymes when the two targeted cysteines were introduced through site-directed mutagenesis (Fig. S3). The change of CD spectra reveal that PAP binding (260 nm) was important and indicated that PAP and other nucleotides may be an antioxidant of SULT enzyme.

3.6. Reversible redox effect on SULT

The reversibility of the redox-responsive SULTs was demonstrated by continued redox treatments on enzymes that contain the two targeted redox-active cysteines either at their native form (*r*SULT1A1 wild-type) or through genetic engineering (P236C of *h*SULT1A1 and I66C/K227C of *h*SULT2A1). Kinetic constants of both the transfer reaction and the physiological reaction (illustrated in Fig. 1) were obtained (Table 4). Distinct variations in catalytic efficiency (k_{cat}/K_m) were observed when redox treatments were imposed. The catalytic efficiencies of the transfer reaction catalyzed by *r*SULT1A1 wild-type and *h*SULT1A1 mutant (P236C) decreased 510- and 54-fold, respectively, following GSSG treatment. Their catalytic efficiencies were fully recovered following treatment with TCEP of the oxidized enzyme. Similar results were observed for physiological reactions catalyzed by *r*SULT1A1 wild-type, P236C of *h*SULT1A1 and I66C/K227C of *h*SULT2A1 (Table 4). The catalytic efficiencies of the three redox-responsive enzymes were 2.3- to 34-fold lower in oxidative state than those in reducing condition and were fully reversible. It is interesting to point out that the k_{cat} of *r*SULT1A1 wild-type significantly increased in oxidative state. This observation was consistent with previous findings [32] that the release of the PAP was not the rate-limiting step of *r*SULT1A1 catalyzed physiological reaction in the oxidative state.

4. Discussion

It has been shown that oxidation of *r*SULT1A1 is critically important for the enzymatic functions [8,9], but the detailed mechanism regarding how the enzymatic activity is regulated remained to be elucidated. In particular, how two cysteines far away from each other, and both far away from enzyme active site, can interact and regulate enzyme activity through redox mechanism has been an open question in literature for some times [33]. S-Glutathiolation of a protein at a conserved cysteine is known to regulate the protein activities [34–37]. In this report, we also used GSSG as an oxidizing reagent to produce the proposed intramolecular disulfide linkage in sulfotransferase. S-Glutathiolation may help to facilitate the formation of intramolecular disulfide bond in

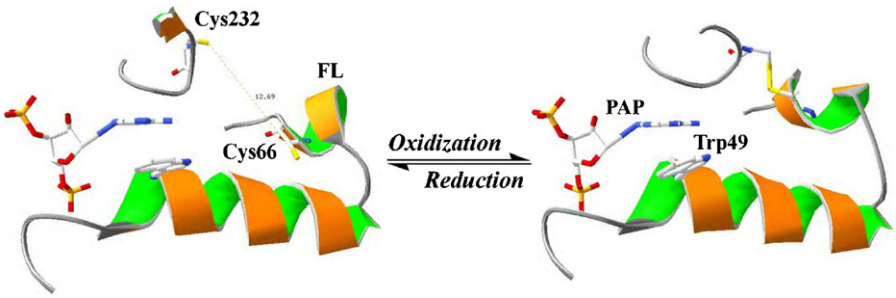

Fig. 4. Proposed regulation mechanism of SULT by a redox switch.

sulfotransferase. However, it is unlikely that S-glutathiolation, which requires only a single cysteine, affects the activity of sulfotransferase because both targeted cysteines were required to produce redox regulation on this enzyme. Therefore, we proposed a mechanism sketched in Fig. 4 to explain how SULT could be regulated in various redox states based on kinetic and structural data. The experimental conditions used in this research were comparable with the *in vivo* environment. The concentrations of PAP and glutathione were in μM [31] and mM range [38], respectively, in rat liver. The glutathione redox couple was present in mammalian cell in concentrations between 1 and 10 mM, with the reduced (GSH) predominating over the oxidized (GSSG) form [39]. In the resting cell, the ratio of GSH to GSSG exceeded 100, whereas in various models of oxidative stress this ratio was reported to decrease to values between 10 and 1 [40]. Thus, the data reported here should be useful to describe what could have appeared *in vivo*.

There are five cysteines in *r*SULT1A1 (Fig. S1). Titration with DTNB (Table 1) indicated that only Cys66 and Cys232 could be effectively oxidized by GSSG. This was consistent with previous finding that, after incubation with GSSG, the formation of the intramolecular disulfide between Cys66 and Cys232 of *r*SULT1A1 was identified [8]. However, whether or not the other three cysteines involve in the redox process is not clear. Two redox-insensitive SULTs, *h*SUL1A1 and *h*SULT2A1, were engineered to show that similar redox regulation can be created in the absence of the other three cysteines (Tables 1–4). DNTB titration (Table 1) indicated that the co-presence of the two targeted cysteines in *h*SULT1A1 and *h*SULT2A1 significantly increased their oxidative potential. In addition, the removal of cysteines in *r*SULT1A1 by site-directed mutagenesis gave similar results that only Cys66 and Cys232 were sensitive to redox treatments. Finally, the mutants of *r*SULT1A1 that contain only two of the five cysteines were also prepared and their dissociation constants with PAP were examined (Table S4). Only the dissociation constant of C82S/C283S/C289S (the triple cysteine mutant that contains Cys 66 and Cys232) was drastically altered in different redox environment.

PAP binding was intimately responsive to the redox treatment (Table 2 and Table S4) in the presence of the two targeted cysteines. This observation explains what has been observed earlier that the removal of tight binding PAP requires redox treatments [9]. It indicated that redox treatments induced protein structural change in nucleotide binding site and then in turn regulated enzyme activity. This function was introduced through site-directed mutagenesis to other SULTs that were redox insensitive (Tables 1 and 2). The structure of SULTs (Fig. 2) shows that the relative locations of these two cysteines (about 12 Å) and nucleotide binding site (around 15 Å) are some distance away from each other. This raises an interesting question as how Cys66 and Cys232 interacts and how the nucleotide binding site of SULT is affected. CD spectral analyses showed that SULTs maintained their secondary structures in different redox states (Fig. S2). The change of CD spectra at 285 nm (Fig. 3) indicated conformational change around aromatic acid upon redox treatment only when both Cys66 and Cys232 were present. This change was accompanied by a nucleotide binding confirmed by the absorption at 260 nm in CD spectra. The aromatic amino acid responsible for the change of the CD spectra was proposed to be Trp49 and was confirmed by site-directed mutagenesis. The change of the CD spectra was absence with a single mutation (W49A mutant of *r*SULT1A1, Fig. 3D). This observation was consistent with previous finding that Trp49 directly affect PAP binding [30].

Modeled structure shown in Fig. 2 could represent structure of all SULT enzymes according to their high identity in sequences and known crystal structures (1EFH and 1LS6). According to this structure, the distances between two the thiols, Cys66 and Cys232 of *r*SULT1A1, Cys70 and Cys236 of *h*SULT1A1 mutant P236C, and Cys66 and Cys227 of *h*SULT2A1 mutant I66C/K227C, were 12.7 Å, 12.8 Å and 11.8 Å, respectively. To form a disulfide bond (bond length is 2.05 Å), flexibility of the enzyme structure is required. According to computational analysis of known cytosolic SULT protein sequences and structures [41], the region, from Gln59 to Pro70 of *r*SULT1A1, is a flexible loop (FL). The average B-factor of the FL was significantly higher than that of the holoenzyme for most SULTs except *h*SULT1B1 (2Z5F) and 2B1b (1Q20 and 1Q22) as shown in Table S2. B-factor, the atomic mean-square displacement, which is also known as temperature factor or Debby–Waller factor, is an important structural flexibility index of the ground-state protein conformation [42]. This calculations were consistent with the crystal structures (1AQU, 1AQY, 1BO6, 1CJM, 1ZD1, 1Z29, 2ETG, and 2H8K) that there are several unresolved residues (shadow area of FL sequence in Table S2) on the FL of known SULT structures. The FL described above gave a plausible explanation why the two targeted cysteines, even though 12 Å apart, can form disulfide bond. The existence of this FL can also explain why Lys 65 and Cys66 of *r*SULT1A1 were labeled by a PAP analogue, ATP-dialdehyde, intended to react with neighboring amino acids around PAP binding site, [32]. Cys66 is part of the FL and this makes the long distance interaction possible. The next question is how this redox event between the two targeted cysteines affects PAP binding and PST activity.

How the formation of Cys66 and Cys232 disulfide bond may affect the environment of Trp 49 could be apparent as shown in Fig. 4 for *r*SULT1A1 and in Fig. 4S for other SULTs. Trp49 of *r*SULT1A1 is part of a helix that directly connects to the Cys66 containing FL. Following the formation of disulfide bond, the Trp49 containing helix could be forced to dislocate and the coordination between Trp49 and adenine of PAP was affected. It has been shown that Trp49 is important for PAP binding [33]. The tryptophan provided the hydrophobic stack for the adenine ring of PAP in all SULTs except *h*SULT4A1 [41]. It would be expected that conformational change of Trp49 significantly affected sulfonation activity for the affinity of PAP and PAPS are critical to SULT catalyzed reactions [8,23]. Mutation at the conserved Trp49 of *r*SULT1A1 (W49A) gave the K_d of PAP to the mutant enzyme similar to that of GSSG treated wild-type SULTs (Table 2). In addition, the spectral differences between TCEP and GSSG-treated W49A mutant of *r*SULT1A1 (Fig. 3A) could no longer be observed (Fig. 3D). These two observations were both consistent with our proposed mechanism that conformational change of Trp49 following redox treatments affected the affinity of PAP/PAPS to *r*SULT1A1.

In conclusion, the proposed mechanism for redox regulation of cytosolic sulfotransferase is illustrated in Fig. 4. The formation of a C66–C232 disulfide bond was facilitated by the Cys66 containing FL that was flexible enough to allow the interaction between two distanced cysteines. Formation of disulfide bond produced strain and induced the local structural change of the enzyme, which affected the Trp49 containing helix that directly links to the FL. This event caused the disturbance of the conformation of Trp49 that was an important coordinator of PAP/PAPS. Thus, the binding affinity of PAP/PAPS was impaired in oxidative state and exhibited significant change in the enzyme kinetics. Finally, this mechanism also stated that the redox regulation was reversible in a redox environment. It appeared that only wild-type *r*SULT1A1 but not the human SULT was subjected to the redox regulation. This should be an important information for the studies in drug metabolism when rat is used as a model animal.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bcp.2012.04.003.

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