# Reversible *S*-glutathionylation of human 6-pyruvoyl tetrahydropterin synthase protects its enzymatic activity

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**6-Pyruvoyl tetrahydropterin synthase (PTS) converts 7,8-dihydroneopterin triphosphate into 6-pyruvoyltetrahydropterin and is a critical enzyme for the *de novo* synthesis of tetrahydrobiopterin, an essential cofactor for aromatic amino acid hydroxylases and nitric-oxide synthases. Neopterin derived from 7,8-dihydroneopterin triphosphate is secreted by monocytes/macrophages, and is a well-known biomarker for cellular immunity. Because PTS activity in the cell can be a determinant of neopterin production, here we used recombinant human PTS protein to investigate how its activity is regulated, especially depending on redox conditions. Human PTS has two cysteines: Cys-43 at the catalytic site and Cys-10 at the N terminus. PTS can be oxidized and consequently inactivated by $H_2O_2$ treatment, oxidized GSH, or *S*-nitrosoglutathione, and determining the oxidized modifications of PTS induced by each oxidant by MALDI–TOF MS, we show that PTS is *S*-glutathionylated in the presence of GSH and $H_2O_2$. *S*-Glutathionylation at Cys-43 protected PTS from $H_2O_2$-induced irreversible sulfinylation and sulfonylation. We also found that PTS expressed in HeLa and THP-1 cells is reversibly modified under oxidative stress conditions. Our findings suggest that PTS activity and *S*-glutathionylation is regulated by the cellular redox environment and that reversible *S*-glutathionylation protects PTS against oxidative stress.**

6-Pyruvoyl tetrahydropterin synthase (PTS)[2] catalyzes the second step of the *de novo* biosynthesis of tetrahydrobiopterin (BH4), an essential cofactor of aromatic amino acid hydroxylases, nitric oxide synthases, and alkylglycerol monooxygenase (1). PTS converts 7,8-dihydroneopterin triphosphate ($NH_2TP$), a product of GTP cyclohydrolase I (GCH), into 6-pyruvoyl tetrahydropterin, which is further converted into BH4 by sepiapterin reductase. In addition to the BH4 synthetic pathway, $NH_2TP$ is converted into 7,8-dihydroneopterin by the elimination of inorganic triphosphate followed by oxidation, resulting in neopterin. Neopterin is thus considered a byproduct of BH4 *de novo* synthesis (2).

The physiological significance of BH4, such as its role in the synthesis of monoamine neurotransmitter and nitric oxide (NO) and in phenylalanine metabolism, and its deficiency are well-documented (1, 3). In contrast, the physiological role of neopterin is still controversial, although there have been reports indicating that it may enhance hydrogen peroxide activity (4), activate NF-κB (5, 6), promote apoptosis (7), and exert antioxidative and anti-inflammatory effects by activating Nrf2 (8) or affect cognition-related functions (9). Furthermore, 7,8-dihydroneopterin, which is derived from $NH_2TP$ by the removal of a triphosphate, may act as a scavenger of reactive oxygen species (ROS) (10). The plasma neopterin concentration is often used as a marker for cell-mediated immune activation because activated monocytes/macrophages secrete neopterin (11, 12). When cellular-immunity is activated, GCH in macrophages is induced up to 50-fold, which may exceed endogenous PTS activity, making PTS a rate-limiting step in the BH4 synthesis pathway (13). Consequently, $NH_2TP$ is preferably converted to 7,8-dihydroneopterin and neopterin rather than synthesized to BH4. Thus, the enzymatic activity of PTS seems to be critical for controlling the levels and ratio of BH4 and neopterin.

Cysteinyl thiol is readily subjected to various post-translational modifications because of its high reactivity. Reversible oxidative modifications and the reduction have an impact on the physiological roles of the proteins and are referred to as a redox regulation (14–16). Depending on the cellular conditions and the natures of the proteins, redox active cysteine is subjected to disulfide formation with vicinal inter- and intramolecular cysteine. In addition, cysteine reversibly binds to GSH (*S*-glutathionylation) and nitric oxide (*S*-nitrosylation) and is oxidized mildly by reactive species (sulfenylation) (17–21). These oxidative modifications can be regenerated by reduced glutathione (GSH), an abundant antioxidant tripeptide that is present at millimolar concentrations in the cell, and reducing proteins such as thioredoxin (Trx) and glutaredoxin (Grx) (22–24). In addition to the reversible oxidations, the sulfenylated cysteine is further oxidized irreversibly to sulfinic ($Cys\text{-}SO_2H$) and sulfonic ($Cys\text{-}SO_3H$) acids. PTS requires thiol reductants such as DTT and 2-mercaptoethanol for activation (25) and is inhibited by thiol-alkylating reagents (26), suggesting the involvement of redox regulation.

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[2] The abbreviations used are: PTS, 6-pyruvoyl tetrahydropterin synthase; AsA, ascorbic acid; BH4, tetrahydrobiopterin; GCH, GTP cyclohydrolase I; Grx, glutaredoxin; GSNO, *S*-nitrosoglutathione; $NH_2TP$, 7,8-dihydroneopterin triphosphate; NO, nitric oxide; PTS-SG, *S*-glutathionylated PTS; ROS, reactive oxygen species; Trx, thioredoxin; DMEM, Dulbecco's modified Eagle's medium; MWCO, molecular mass cut-off.

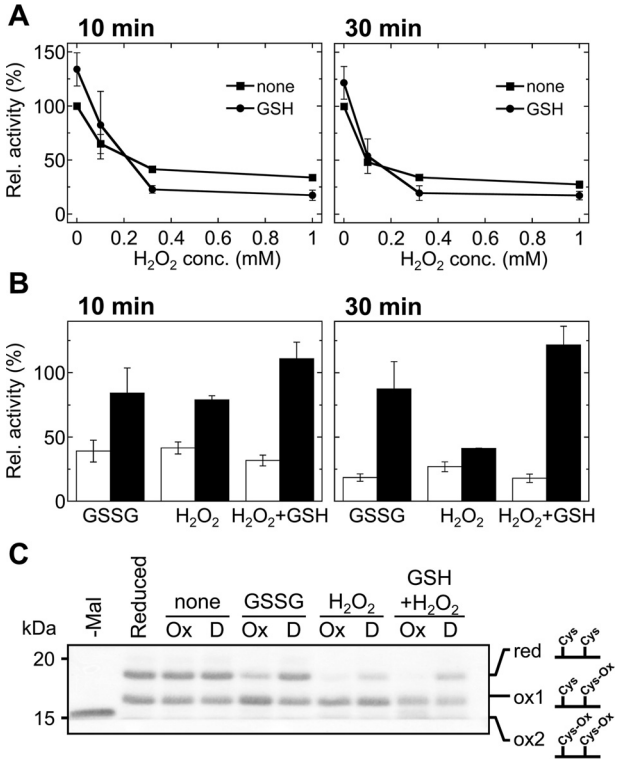


**Figure 1. Oxidative inactivation and reductive reactivation of PTS.** *A*, PTS was incubated with the indicated concentrations of $H_2O_2$ in the presence or absence of 0.5 mM GSH at 37 °C for the indicated times, and the activity was measured. *B*, PTS was incubated with 1 mM GSSG, 1 mM $H_2O_2$, and 0.5 mM GSH + 1 mM $H_2O_2$ at 37 °C for the indicated times. PTS was further incubated with 5 mM DTT for 10 min. The activity of oxidized (*open column*) and reduced (*closed column*) PTS was measured. The activity of PTS treated without oxidants was set as 100%. *C*, PTS oxidized for 30 min (*Ox*) and then reduced with DTT (*D*) were separated by the maleimide labeling method. −*Mal*, without labeling; *Reduced*, without removal of DTT in a storage solution. Each value represents the mean ± S.D. ($n = 3$).

Here, we report that PTS is a target protein of *S*-glutathionylation at Cys-43, protecting this residue from irreversible oxidation. GSH can reduce the modification and activate PTS at physiological concentrations. Moreover, we show that the cysteine residue of PTS is modified in HeLa and THP-1 cells under oxidative stress conditions.

## Results

### *Oxidative inactivation and reductive reactivation of PTS*

First, we purified recombinant PTS protein using the buffer without DTT as a thiol-protective reagent and found that PTS enzymatic activity was increased by the treatment with DTT as previously reported (25), suggesting that the PTS protein could be subject to oxidative modification. To determine the modifications, we purified the reduced form of PTS in the presence of DTT, which was removed just before the analyses.

We first examined whether the enzymatic activity of PTS is regulated by the redox status. The reduced PTS protein was incubated with $H_2O_2$ and GSH plus $H_2O_2$ for 10 and 30 min, resulting in loss of PTS activity (Fig. 1*A*). PTS activity was decreased to about 50% even at 100 $\mu$M $H_2O_2$ with and without GSH. The inactivation was slightly enhanced at 0.32 and 1 mM $H_2O_2$ in the presence of GSH, implying *S*-glutathionylation. To reveal reversibility of the oxidative inactivation, excess DTT was added for the reduction of PTS after oxidation with 1 mM GSSG to promote *S*-glutathionylation, 1 mM $H_2O_2$ and GSH + 1 mM $H_2O_2$ (Fig. 1*B*). GSSG- and GSH + $H_2O_2$-oxidized PTS restored PTS activity, indicating reversible modifications of PTS. In contrast, PTS oxidized with $H_2O_2$ alone for 30 min showed no reversible activation, whereas PTS oxidized for 10 min demonstrated reductive restoration, suggesting that the oxidative states of the cysteine(s) proceeded from reversible sulfenylation to irreversible sulfinylation and sulfonylation.

### *Redox states of the cysteine residue*

To investigate whether the inactivation and activation that were dependent on the redox conditions were due to the cysteinyl modification, we employed the maleimide labeling method for the separation of the reduced and oxidized forms of the proteins. The thiol-modifying maleimide-$PEG_{11}$-biotin specifically binds to thiol and lowers protein mobility on SDS-PAGE, allowing titration of the number of oxidized cysteine residues. As shown in Fig. 1*C*, labeled and separated PTS, which has 2 cysteine residues, was found with three distinct mobilities, indicating that the number of oxidized cysteines can be determined by this method (the red, ox1, and ox2 bands correspond to 0, 1, and 2 oxidized cysteines, respectively). Approximately one-half of PTS was found in the ox1 band, corresponding to a single cysteine oxidized form, even in the presence of 10 mM DTT. The fully reduced form (red band) was decreased and shifted to the ox1 band by oxidizing treatments. Reduction by 5 mM DTT recovered the proportion of reduced and oxidized forms in GSSG- and GSH + $H_2O_2$-oxidized PTS. In contrast, DTT had little effect on $H_2O_2$-oxidized PTS. The proportions of fully reduced PTS and its reversibility corresponded to the redox-dependent changes in activity, indicating that modification of a cysteine regulates the enzymatic activity of PTS.

### *S-Nitrosylation of PTS*

We next assessed the possibility of *S*-nitrosylation, the other reversible modification of cysteine residues. The incubation of PTS with 1 mM *S*-nitrosoglutathione (GSNO), which is a major physiological donor of NO, resulted in the inactivation of PTS. Because *S*-nitrosothiol is specifically reduced by ascorbic acid (AsA), GSNO-oxidized PTS was further incubated with AsA. Although DTT could fully restore PTS activity, AsA reduction showed partial recovery (Fig. 2*A*). In contrast to GSH- and $H_2O_2$-induced oxidation (Fig. 1), two cysteine residues were oxidized by GSNO as shown in Fig. 2*B* (ox2 band). Consistent with the activity, DTT could recover the redox state in the reduced form. However, the ox2 band was reduced partially by reduction with AsA, whereas the fully reduced form (red band) was not observed. The difference between the reduction patterns with DTT and AsA suggested that GSNO treatment induced different oxidative modifications; one was AsA reducible, and the other was not. To further clarify the *S*-nitrosylation of PTS, the GSNO-oxidized PTS was analyzed by biotin switch assay (27). The ox1 bands were found in both apply and elution

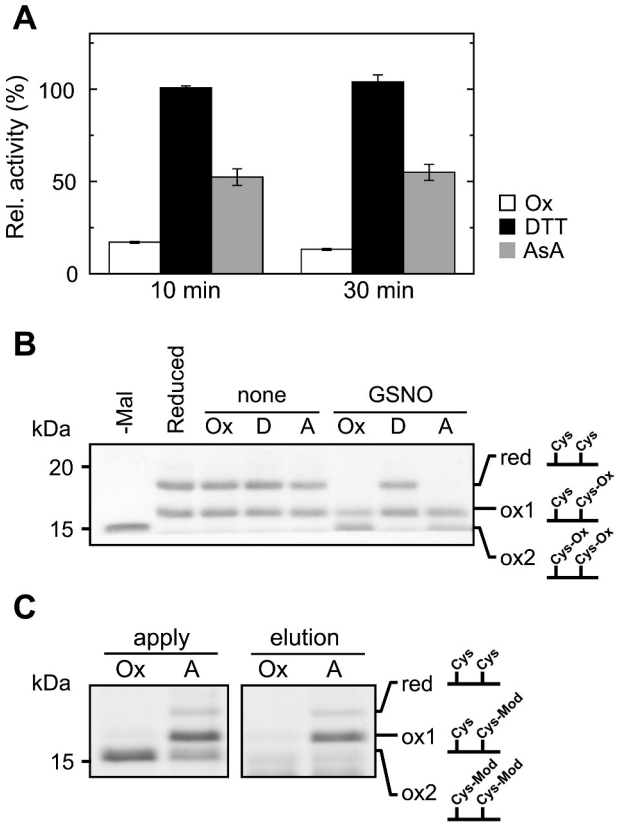


**Figure 2. GSNO-mediated reversible oxidation.** PTS was incubated with 1 mM GSNO at 37 °C for the indicated time. The oxidized PTS was reduced with 5 mM DTT and 5 mM AsA for 10 min. *A*, the PTS activity of oxidized (*open column*) and reduced with DTT (*closed column*) and with AsA (*shaded column*) was measured. The activity of PTS treated without oxidants was set as 100%. Each value represents the mean ± S.D. ($n = 3$). *B*, PTS oxidized for 30 min (*Ox*) and then reduced with DTT (*D*) and AsA (*A*) were separated by maleimide labeling method. −*Mal*, without labeling; *Reduced*, without removal of DTT in a storage solution. *C*, GSNO-oxidized PTS was analyzed by modified biotin switch assay. *Cys-Mod*, cysteine was modified with oxidative modifications or *N*-ethylmaleimide.

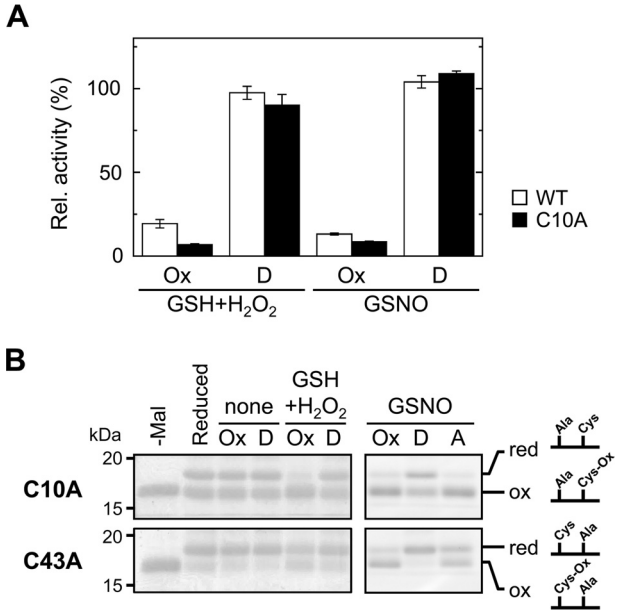


**Figure 3. Redox sensitivity of the Cys mutant PTS.** Wild-type (*WT*) and mutant PTS were oxidized with 0.5 mM GSH + 1 mM $H_2O_2$ and 1 mM GSNO at 37 °C for 30 min, and then reduced with 5 mM DTT or 5 mM AsA for 10 min. The activity of PTS treated without oxidants was set as 100%. *A*, the activities of WT (*open column*) and C10A (*closed column*) oxidized (*Ox*) and reduced with DTT (*D*) was measured. Each value represents the mean ± S.D. ($n = 3$). *B*, PTS oxidized (*Ox*) and reduced with DTT (*D*) and with AsA (*A*) were separated by the maleimide labeling method. −*Mal,* without labeling; *Reduced*, without removal of DTT in a storage solution.

by AsA reduction, showing that one cysteine was subjected to *S*-nitrosylation (Fig. 2*C*).

### *Cys-43 is the main target of oxidative modification*

PTS protein contains 2 cysteine residues at positions 10 and 43. Cys-43 is a catalytic residue and is therefore conserved in almost all PTSs (1, 28). To identify the critical cysteine(s) for redox regulation, we prepared Cys-to-Ala substitution mutants (C10A and C43A). Because the C43A mutant showed no activity due to the loss of a catalytic residue, we compared the changes in the activity of the C10A mutant protein with the WT protein. The C10A mutant showed the same inactivation and reactivation as the WT protein when oxidized by GSH + $H_2O_2$ and GSNO (Fig. 3). The reversible modification by GSH + $H_2O_2$ on the C10A mutant was shown by maleimide labeling. The GSNO-treated C10A mutant was not reduced by AsA, suggesting that the modification of Cys-43 was not *S*-nitrosylation. The results indicate that Cys-43 is a major target of the oxidative modifications and is therefore a regulatory cysteine. In the case of the C43A mutant, Cys-10 was modified by GSNO and partly reduced by AsA, indicating *S*-nitrosylation of Cys-10.

### *Determination of the oxidative modification by MALDI–TOF MS*

The different oxidative modifications of PTS at different cysteine positions are indicated in Figs. 2 and 3. Therefore, we analyzed the oxidized mutants by MALDI–TOF MS to determine the modifications directly. Because *S*-nitrosothiol is destroyed during laser-induced ionization, the *S*-nitrosylated protein cannot be distinguished from the unmodified one (29). No clear difference was observed between the GSNO-oxidized and nontreated C43A mutant in the mass spectra (Fig. 4*A*). Considering the modification of Cys-10 and AsA reducibility (Fig. 3), the modification of Cys-10 was identified as *S*-nitrosylation. The increased mass of C10A mutant after $H_2O_2$ oxidation of 32 and 48 Da indicates that Cys-43 was subjected to irreversible sulfinylation and sulfonylation, respectively. Upon GSH + $H_2O_2$ oxidation, the obvious shift in the mass of 307 Da of the C10A mutant indicated *S*-glutathionylation of Cys-43, as expected. These results are consistent with the data shown in Fig. 3. The spectrum obtained from the GSNO-treated C10A mutant was identical to that obtained from the *S*-glutathionylated form, indicating that *S*-glutathionylation but not *S*-nitrosylation occurred at Cys-43 in addition to *S*-nitrosylation at Cys-10.

### *GSH is a potential intracellular reducer of PTS*

GSH is the most abundant (~10 mM) low-molecular-weight thiol in the cell and has an important role for the maintenance of cellular redox states. The cellular redox state is also coupled with the Trx and Grx systems. We investigated the intracellular reducing system for *S*-glutathionylated PTS (PTS-SG). DTT

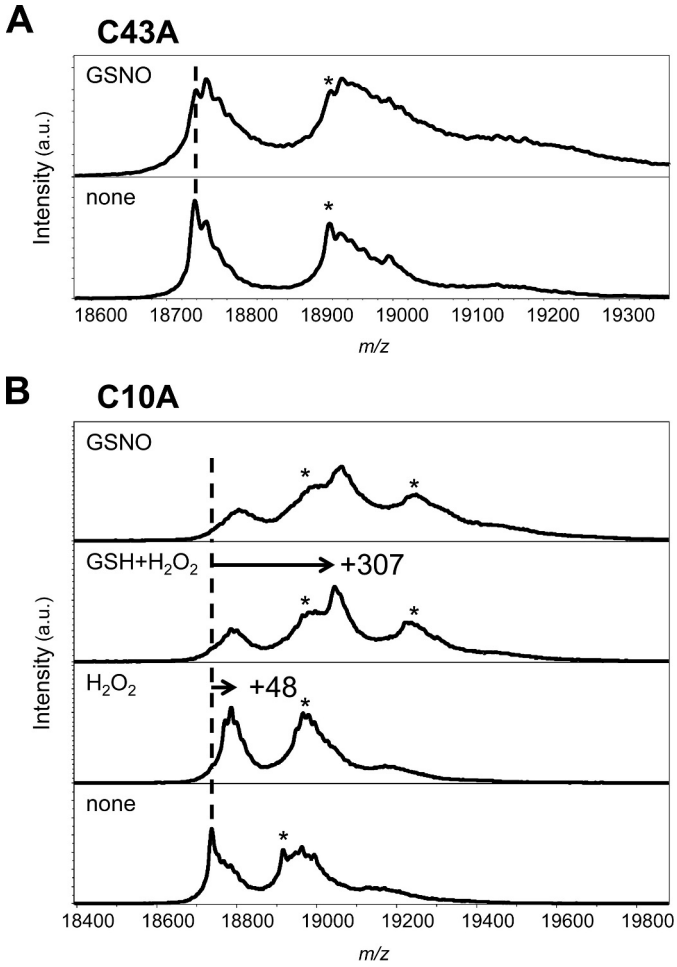


**Figure 4. MALDI–TOF MS.** C43A (*A*) and C10A (*B*) PTS oxidized with the indicated oxidants were analyzed by MS. *Asterisks* show matrix adducts and their oxidative derivatives.

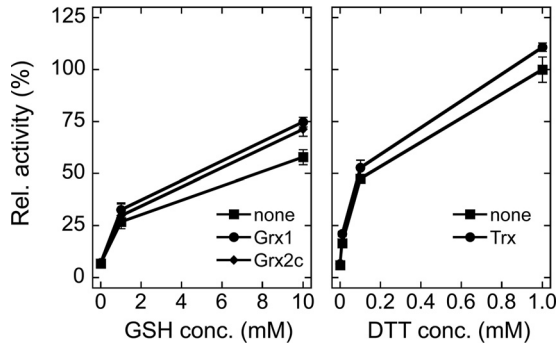


**Figure 5. Effects of reductant and redoxins on PTS-SG reactivation.** *Left panel*, indicated concentrations of GSH and 1 $\mu$M cytosolic Grx were incubated with PTS-SG for 10 min at 37 °C. *Right panel*, indicated concentrations of DTT and 1 $\mu$M Trx were incubated with PTS-SG for 10 min at 37 °C. Then, the activity was measured. The activity of PTS treated with 1 mM DTT was set as 100%. Each value represents the mean ± S.D. ($n = 3$).

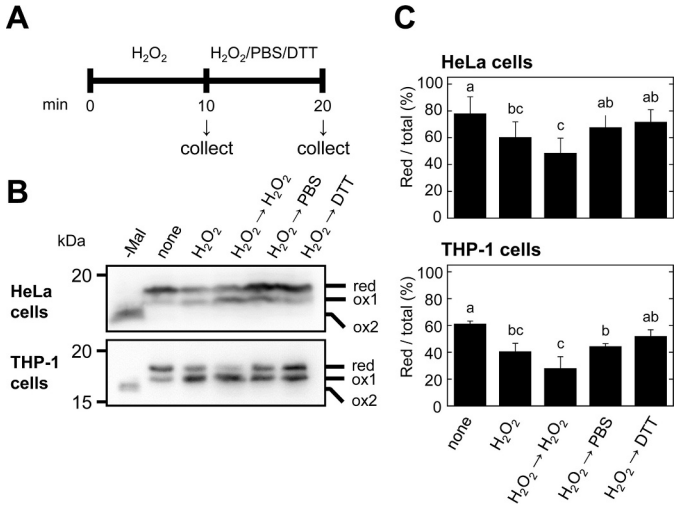


**Figure 6. Redox states of FLAG-PTS in HeLa and THP-1 cells.** FLAG-PTS expressing HeLa and THP-1 cells were incubated in DMEM medium containing 1 mM $H_2O_2$ for 10 min. Then, the medium was exchanged with medium containing 1 mM $H_2O_2$, PBS, and 1 mM DTT, and further incubated for 10 min (*A*). The proteins were extracted with 10% TCA, labeled with maleimide-$PEG_{11}$-biotin, and FLAG-PTS was detected by Western blot analysis (*B*). The reduction level was quantified as the ratio of the reduced form to the total (*C*). Each value represents the mean ± S.D. ($n = 7$ for HeLa cells, $n = 3$ for THP-1 cells). *Different letters* denote significant differences among treatment ($p < 0.05$, One-way analysis of variance post hoc Tukey's HSD test).

and GSH were used as sources of reducing equivalents for the Trx and Grx systems, respectively. If PTS is a target of Trx or Grx, the reduction of PTS must occur at lower concentrations of reducing equivalents in the presence of Trx or Grx. However, no enhancement of the restored activity was observed with any concentration of DTT in the presence of Trx, suggesting that Trx is not a reducer of PTS-SG (Fig. 5, *left panel*). Although Grx1 and Grx2c slightly enhanced the reactivation of PTS-SG, GSH alone could restore sufficient PTS activity (Fig. 5, *right panel*), suggesting that the redox state of PTS depends on the intracellular GSH redox potential.

### *Redox states of PTS in HeLa and THP-1 cells*

Finally, we investigated whether PTS altered the redox states depending on the cellular environment. Because the level of endogenous PTS was too low to be detected in HeLa and THP-1 cells, we transfected a FLAG-PTS-expressing plasmid into cells. THP-1 cells were differentiated into macrophage-like cells with phorbol 12-myristate 13-acetate. The oxidative stress condition was generated by the addition of 1 mM $H_2O_2$ to the culture medium. The redox states were then detected by Western blotting following the maleimide labeling. Approximately 20 and 40% of FLAG-PTS was found in the ox1 band in HeLa and THP-1 cells without oxidative treatment, respectively (Fig. 6, *B* and *C*), suggesting that one of the cysteine residues (probably Cys-43) is highly reactive and subject to the oxidative modification. The addition of $H_2O_2$ led PTS to become more oxidized within 10 min. After the addition of 1 mM DTT to the medium or the removal of $H_2O_2$ after the oxidative stress, the redox states of PTS tended to be reduced slightly (Fig. 6), indicating that the reversible modification of the PTS protein occurred in the cells.

## Discussion

The redox state of a protein strongly impacts its physiological role by changing the stability, function, and enzymatic activity of the protein, depending on the cellular conditions. Although the requirement of thiol reductants such as dithioerythritol, 2-mercaptoethanol, and DTT for *in vitro* PTS activity and inhibition by alkylation reagents are known, the redox regulation of

PTS remains unclear (25, 26). Here, we revealed that *S*-glutathionylation of PTS reversibly regulates its enzymatic activity.

All of the oxidants that we employed inactivated PTS through modifications of cysteine residues (Figs. 1 and 2). Oxidation with $H_2O_2$ showed a time-dependent loss of the reversible activation via the irreversible sulfenylation and sulfonylation (Fig. 4). In the presence of GSH during $H_2O_2$ oxidation (GSH + $H_2O_2$), PTS was protected from this irreversible modification by *S*-glutathionylation (Figs. 1 and 4). Therefore, *S*-glutathionylation of PTS is suggested to be a protective mechanism against irreversible oxidation. This finding further implies that PTS protein is maintained as a recoverable form to be able to respond more rapidly to environmental changes than protein expression.

The regulatory and protective *S*-glutathionylation occurred at Cys-43 (Figs. 3 and 4). Because the Cys-43 of PTS is considered to be ionized and attract a proton from the substrate during the catalytic reaction (28, 30), Cys-43 must be acidic and have a low p$K_a$ compared with free cysteine. The ionized (deprotonated) Cys is both a good catalyst for enzymatic reaction and a good target for oxidative modifications. In agreement with this, approximately one-half of WT and C10A PTS protein, expressed in *Escherichia coli* and purified with DTT, was found in the irreversibly oxidized form.

In addition to catalytic, and thus conserved Cys-43, human and ape PTS have one additional cysteine residue at position 10. This cysteine was specifically modified with GSNO and confirmed as an *S*-nitrosylation (Figs. 2–4). It is difficult to distinguish how the *S*-nitrosylation at Cys-10 contributes to the regulation of PTS activity, because GSNO treatment simultaneously induced *S*-glutathionylation of Cys-43. Protein–protein interactions, stability, and sensitivity to oxidative modification at Cys-43 of PTS might be regulated by the modification at Cys-10 in the cell. Considering the requirement of BH4 for NO synthesis, *S*-nitrosylation could be involved in a feedback mechanism to prevent the excess production of BH4 and/or NO. Further investigations will elucidate the significance of the *S*-nitrosylation of human and ape Cys-10.

GSH, Trx, and Grx are major antioxidant systems in the cells and maintain the protein thiol as a reduced form. Although cytosolic Grx1 and Grx2c slightly enhanced the reduction of PTS-SG, the activation of PTS-SG was mainly dependent on GSH (Fig. 5). Most GSH is found in the cytosol at 1–10 mM, and the ratio of GSH to GSSG is 30–100:1 in unstressed cells (31, 32). When cells were exposed to oxidative stress, GSH was immediately oxidized (32, 33). Therefore, the observed $H_2O_2$-induced oxidation of FLAG-PTS must be caused by the decrease in the GSH/GSSG ratio and subsequent binding of GSH, as shown in Fig. 6. The slight recovery of the redox states of FLAG-PTS by the removal of $H_2O_2$ from the culture medium might be due to enhanced production of NADPH by glucose-6-phosphate dehydrogenase, and subsequent recovery of intracellular GSH potential by degradation of $H_2O_2$ and reduction of GSSG into GSH by thioredoxin and GSH reductase systems (34). Furthermore, FLAG-PTS in THP-1 cells were found as more oxidized states than in HeLa cells after each treatment. This might reflect the more oxidizing conditions such as protein carbonylation (35), methionine sulfoxidation (36), and GSH potential (33) by stimulation with phorbol 12-myristate 13-acetate in THP-1 cells (Fig. 6).

Activated phagocytic cells generate ROS mainly by NADPH oxidase, xanthine oxidase, and myeloperoxidase as a part of an inflammatory response. The generated ROS not only changes phagocytic cells to the oxidative condition but are also released into the extracellular environment, causing damage to the cells around inflammation sites (37). At the same time, macrophages synthesize and secrete neopterin and 7,8-dihydroneopterin at the expense of BH4 synthesis, although the physiological role of pteridines is not fully understood. The significant increase in GCH expression up to 50-fold in macrophages (13, 38) is thought to be a cause of neopterin secretion. Along with this, the inactive *S*-glutathionylation of PTS must be involved in the BH4 and neopterin synthetic pathways. A recent study revealed that lipopolysaccharide injection into the abdominal cavity induced a rapid increase in neopterin in the serum and hippocampus within 15 min (8), suggesting the post-translational regulation of neopterin production and secretion prior to the increased expression of GCH. Considering the immediate response of ROS production from phagocytic cells (39–41), the redox states of PTS must be affected by *S*-glutathionylation and thus regulate the amount and/or ratio of BH4 and neopterin for anti- or pro-inflammatory effects to macrophages and surrounding cells at the time of inflammation.

In conclusion, PTS was a target of reversible *S*-glutathionylation at Cys-43 to prevent irreversible modification with $H_2O_2$. *S*-Glutathionylation and deglutathionylation are dependent on the oxidized and reduced forms of GSH, suggesting that the intracellular GSH redox condition is the determinant of the extent of modification and thus the activity of PTS. Further investigation of the association of the intracellular redox states with the synthesis of BH4 and neopterin should elucidate the role of redox regulation of PTS.

## Experimental procedures

### Proteins

A gene encoding *E. coli* GCH (*Ec*GCH) was amplified with a $His_6$ tag at the N terminus and cloned into pET23a expression vector (Novagen, Madison, WI). *E. coli* strain BL21 (DE3) harboring the obtained plasmid was cultured at 37 °C in LB medium supplemented with 50 $\mu$g/ml of ampicillin. When $A_{600}$ reached 0.4, protein expression was induced by the addition of 0.4 mM isopropyl $\beta$-D-1-thiogalactopyranoside followed by further culture at 25 °C overnight. *Ec*GCH protein was purified using a HisTrap HP column (GE Healthcare, UK) in a solution containing 25 mM Tris-HCl (pH 7.5), 100 mM NaCl. The column was washed with 100 mM imidazole, and the protein was eluted with 200 mM imidazole. The obtained protein was dialyzed against 25 mM Tris-HCl (pH 7.5), 100 mM NaCl twice, concentrated with Amicon Ultra-15 (molecular weight cut-off (MWCO) 30 kDa, Millipore, Bedford, MA), and stored at −80 °C in the presence of 15% (v/v) glycerol.

Human PTS gene was fused with $His_{10}$ tag at the N terminus and cloned into pET16b (Novagen). Site-directed mutagenesis for cysteine substitution was performed using the PrimeSTAR Mutagenesis Basal Kit (TAKARA, Kyoto, Japan) according to

the manufacturer's instructions. Based on the numbering of the amino acids according to UniProt, $His_{10}$ tag and linker upstream of the first methionine of PTS are excluded from the numbering. WT and mutated PTS proteins were expressed in the same way as *Ec*GCH. The protein was purified using HisTrap HP column in a solution containing 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM DTT. The column was washed with 300 mM imidazole, and the protein was eluted with 500 mM imidazole. The obtained protein was dialyzed against 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM DTT twice, concentrated with Amicon Ultra-15 (MWCO 30 kDa), and stored at −80 °C in the presence of 15% (v/v) glycerol. DTT was removed with NAP-5 desalting column (GE Healthcare) before analyses. To prepare PTS-SG, after the elution in the absence of DTT from the HisTrap column, PTS was incubated with 1 mM GSSG for 30 min at 37 °C. The PTS-SG was separated from GSSG and concentrated with Amicon Ultra-15 (MWCO 30 kDa).

Human Trx and human cytosolic Grxs (Grx1 and Grx2c) were fused with $His_6$ tag at the C terminus and cloned into pET23a. The sequence of Grx2c is in accordance with a previous article (42). The expressions and purifications were performed in the same way as *Ec*GCH.

All purification procedures were performed at 4 °C. Protein concentration was determined by a Bradford assay using bovine γ-globulin as a standard.

### Oxidation and reduction treatments

PTS protein was incubated with indicated concentrations of $H_2O_2$, GSSG, and $H_2O_2$ in the presence of 0.5 mM GSH (GSH + $H_2O_2$), and GSNO at 37 °C for 10 and 30 min. After the incubation, 5 mM DTT or 5 mM AsA were added to the oxidized protein solutions and incubated for 10 min at 37 °C. The activity and redox state of the protein were assessed.

### Measurements of PTS activity

PTS was incubated in a reaction mixture containing 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 8 mM $MgCl_2$, 58 μM $NH_2TP$, and 0.5–0.7 μM PTS at 37 °C for 30 min, and the reaction was quenched by the addition of 1 M perchloric acid and 75 mM EDTA. The resulting 6-pyruvoyl tetrahydropterin was converted into a stable pterin by the oxidation with 1% (w/v) $I_2$, 2% (w/v) KI in 1 M HCl for 30 min at room temperature. Excess iodine was removed by the addition of 2% (w/v) AsA for 10 min at 4 °C. Insoluble protein was removed by centrifugation at 20,000 × *g* for 10 min, and the pterin contents were analyzed by HPLC with fluorescence detection (43). All procedures were performed in the dark until AsA was added.

$NH_2TP$ was prepared by incubating *Ec*GCH with 1 mM GTP, 25 mM Tris-HCl (pH 7.5), and 100 mM NaCl for 1 h in the dark at 37 °C. *Ec*GCH protein was removed with Amicon Ultra-15 (MWCO 10 kDa), and the flow-through was stored at −80 °C until use. The $NH_2TP$ concentration was determined by conversion to neopterin by alkaline phosphatase (Sigma) and iodine oxidation, and quantified by HPLC with fluorescence detection.

### Visualization of the redox states of PTS

The redox states of cysteines of PTS protein were determined by the shift of electrophoretic mobility by labeling with maleimide compound (44). After the incubation with oxidants and reductants, PTS was precipitated with 5% TCA, labeled with maleimide-$PEG_{11}$-biotin (Thermo Fisher Scientific, Waltham, MA), and resolved by nonreducing SDS-PAGE.

### Biotin switch assay

*S*-Nitrosylation of PTS was determined based on a biotin switch assay with slight modifications (27). Reduced thiols of GSNO-oxidized PTS were blocked with 10 mM *N*-ethylmaleimide in the presence of 1% SDS for 30 min at room temperature. Following removal of unreacted *N*-ethylmaleimide by TCA/acetone precipitation, PTS was reduced with 5 mM AsA and simultaneously labeled with maleimide-$PEG_{11}$-biotin for 30 min at room temperature. Unreacted maleimide-$PEG_{11}$-biotin was removed by TCA/acetone precipitation. The labeled PTS was solubilized with 100 mM Tris-HCl (pH 7.5) and 0.1% SDS and purified with neutravidin-agarose (Thermo Fisher Scientific). The applied and eluted PTS were separated by nonreducing SDS-PAGE.

### MALDI–TOF MS

The oxidative modifications of PTS protein were determined by MALDI–TOF MS immediately after the oxidant treatment. MALDI–TOF MS was performed using an UltrafleXtreme (Bruker, Billerica, MA). A sinapinic acid was used as a matrix.

### Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France) at 37 °C in a 5% $CO_2$ incubator. FLAG-PTS protein was expressed in the cells using pFLAG-CMV2 vector (Sigma) and CalPhos mammalian transfection kit (Clontech, Mountain View, CA) as a transfection reagent. THP-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1× antibiotic-antimycotic (Thermo Fisher Scientific) at 37 °C in a 5% $CO_2$ incubator. THP-1 cells were transfected with FLAG-PTS plasmid and X-tremeGENE HP DNA (Roche, Basel, Switzerland). After transfection for 24 h, the cells were differentiated by the addition of 100 nM phorbol 12-myristate 13-acetate, and cultured for 3 more days with the replacement of culture medium with fresh medium every 24 h.

### Western blot analysis

The HeLa and THP-1 cells were exposed to 1 mM $H_2O_2$ containing medium for 10 min. The medium was replaced with fresh medium in the presence or absence of 1 mM DTT, or further incubated for 10 min (Fig. 6*A*). The cells were washed with ice-cold PBS three times, and ice-cold 10% TCA was added directly for an extraction and fixation of the redox states of proteins in the cells. The denatured proteins were collected by centrifugation and washed with ice-cold acetone. The obtained proteins were dissolved and labeled in the nonreducing sample buffer containing 3 mM maleimide-$PEG_{11}$-biotin, and sub-

jected to Western blot analysis according to a conventional procedure. FLAG-PTS protein was detected using a primary anti-FLAG antibody (Clontech) and an anti-mouse secondary antibody (GE Healthcare). Band intensities from at least three individual experiments were determined using ImageJ.

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