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Effect of the glutathione/glutathione disulfide redox couple on thiopurine methyltransferase

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Abstract

The susceptibility of recombinant human thiopurine methyltransferase (hTPMT) to thiol–disulfide exchange was investigated. The enzyme was incubated in buffers of the redox couple GSH and GSSG. The values of the chosen concentrations and concentration ratios of the redox couple equaled those expected to occur *in vivo*. Activity measurements of the enzyme over time in these buffers at 30°C indicated that thiol–disulfide exchange may be a part of the posttranslational modulation of hTPMT activity. Activity varied between 5% and 100%, with the lowest activities in buffers of low [GSH]/[GSSG] concentration ratios and of low total concentration of the redox couple. A thiol–disulfide exchange mechanism involving a mixed disulfide was proposed. Titration of the protein thiol groups with Ellmann's reagent (5,5′-dithiobis[2-nitrobenzoic acid]) revealed that at least two protein thiols were readily accessible for conjugation with the reagent, while others were conjugated more slowly. The previous model of hTPMT constructed by our group was in accordance with the experimental results. Inspection of the model indicated that one of the protein thiols subject to slow thiol–disulfide exchange may be situated at the binding site of the co-substrate of the enzyme and thus be responsible for the glutathione/glutathione disulfide modulation of the activity of hTPMT.

Keywords: Thiopurine methyltransferase; Glutathione; Redox; Modulation; Thiol–disulfide exchange; Three-dimensional model

1. Introduction

hTPMT (EC 2.1.1.67) is a small (28.2 kDa) [1,2] cytosolic enzyme that catalyzes the *S*-methylation of aromatic and heterocyclic sulfhydryl compounds, utilizing AdoMet as methyl donor [3]. The enzyme is clinically important, as it metabolizes the cytostatic drugs 6-MP and 6-thioguanine as well as some of their nucleotide derivatives by this mechanism [2,4]. It is well documented that individual TPMT activity is regulated by autosomal genetic polymorphisms due to point mutations in the TPMT alleles [5,6]. Inhibitory interactions with benzoic acid derivatives [3] and drugs such as sulfazalazine [7] and furosemide [8] have also been found. However, studies on posttranslational endogenous regulatory factors have not been described.

Protein thiols, provided by the cysteine side chains, are generally the most reactive functional groups on polypeptides. *In vivo*, the thiols of intracellular enzymes may be subject to non-enzymatic reversible oxidation, reduction and conjugation by endogenous disulfide and sulfhydryl compounds, respectively [9]. This may subsequently change the activity of the enzyme. Certain enzymes, some of which are important in glucose metabolism, are modulated in this manner [10] by physiological variations of the redox couple GSSG and GSH. It has been shown that the activity of such enzymes mainly depends on the [GSH]/[GSSG] ratio, which is correlated to the metabolic state of the organism [9]. In light of this concept and since no other enzyme or allosteric effector is known to modulate hTPMT, the current study attempted to determine whether: (a) thiol–disulfide exchange might play a part in the biological control of hTPMT; (b) a general protein thiol–disulfide exchange mechanism applies to hTPMT; and (c) some aspects of the preliminary structural model for hTPMT [11] could be ver-

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Abbreviations: hTPMT, human thiopurine methyltransferase; AdoMet, *S*-adenosylmethionine; 6-MP, 6-mercaptopurine; DTT, dithiothreitol; DTNB, 5,5′-dithiobis[2-nitrobenzoic acid]; P-SH, protein containing a sulfhydryl group; P-SSG, protein oxidized by GSSG to a mixed disulfide; and PSS, protein with internal disulfide bridge.

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ified by enzyme activity recordings in relation to protein thiol–disulfide exchange.

2. Materials and methods

2.1. Materials

pBluescript was from Stratagene. Cells, viruses, and pVL1392 vector (MaxBac 2.0 ® kit) were from Invitrogen. Q-Sepharose and Superdex-75 were from Pharmacia. GSH (SigmaUltra grade), GSSG (SigmaUltra grade), Triton X-100 (both regular and peroxide-free), DTT, AdoMet, 6-MP, DMSO, guanidine, and DTNB were from Sigma-Aldrich. *S*-Adenosyl-L-[methyl-^{14}C]methionine was from Amersham. UltimaGold XR scintillation fluid was from Packard.

2.2. Recombinant hTPMT

The open reading frame for human liver TPMT was subcloned from pBluescript as a *Pst*1/*Bam*H1 fragment into the baculovirus transfer vector pVL1392. According to the MaxBac 2.0 ® kit instructions, this construct was co-transfected into the *Spodoptera frugiperda* Sf9 cell line together with wild-type *Autographa california* nuclear polyhedrosis virus DNA. The resultant recombinant baculovirus particles were cloned by plaque assay and a single clone (c5) expanded by infection of Sf9 cells in bulk culture. Recombinant hTPMT was produced by infection of 1-L suspension cultures (10^6 cells/mL) of Sf9 cells with c5 baculovirus at a multiplicity of infection of five. Cells were harvested 60 hr postinfection, and the lysate subjected to ion-exchange chromatography using Q-Sepharose. The recombinant hTPMT was resolved using a linear NaCl gradient (0–200 mM) in 25 mM Tris–HCl, pH = 7.8, containing 1 mM DTT. Active fractions were pooled, concentrated by ultrafiltration, and subjected to gel filtration chromatography using a Superdex-75 fast-performance liquid chromatographic column and PBS/1 mM DTT as eluant. The resultant product was homogenous when examined by SDS–PAGE with Coomassie brilliant blue staining. The enzyme was stored for later use at −70°.

All procedures described below that included incubation or dialysis of hTPMT were carried out under an inert oxygen-free Ar or N_2 atmosphere. Solutions used for this purpose were depleted of dissolved oxygen by N_2 gassing for at least one hour before use.

2.3. Reactivation and dialysis

Recombinant hTPMT which had been exposed to air by storage in the freezer for 2–3 years was only 60–70% active and was reactivated. This was undertaken by incubating the enzyme in dialysis buffer, 480 μg/mL, with 1 mM DTT for 2 hr at 30° followed by dialysis of the incubation mixture at room temperature by continuous exchange of dialysis buffer to reduce [DTT] rapidly. A dialysis chamber with a high membrane surface to volume ratio was constructed for this purpose. The time necessary to reduce small molecule concentrations by half was measured to be 0.77 hr. Measurement was done by monitoring the decrease in [DTT] by addition of excess DTNB (Ellmann's reagent) [12] to small samples collected from the interior of the chamber over time with subsequent readings of optical absorbance (λ = 412 nm) on a photospectrometer. Cutoff for the membrane was 12 kDa and dialysis time was 24 hr. Dialysis buffer consisted of 0.1 M potassium phosphate, 1 M glycerol, 1% (w/w) Triton X-100, pH = 7.2.

2.4. TPMT activity assay

Generally, the activity of hTPMT in samples was measured by the method of Weinshilboum *et al*. [13], which is based on the TPMT-mediated conversion of 6-MP to 6-[^{14}C]methyl-mercaptopurine with *S*-adenosyl-[methyl-^{14}C]methionine as methyl donor. Some modifications of this method were made to minimize alterations to the hTPMT redox buffer system during the assay incubation period and to increase the signal/background ratio of the scintillation measurements. In detail, 4 μL 0.2 M 6-MP in DMSO was added to 100 μL sample, and the reaction was started by adding 7 μL of a mixture of 250 μM each AdoMet and *S*-adenosyl-L-[methyl-^{14}C]methionine in 7 mM sulfuric acid, pH = 2.2. The reaction mixture (pH = 7.2) was then incubated for 15 min at 30° and stopped by the addition of 200 μL 0.1 M sodium tetraborate, pH = 10.0. Methylmercaptopurine was extracted from the reaction mixture with 5.0 mL 20% (v/v) isoamyl alcohol in toluene. Four milliliters of the organic extract was added to 8 mL scintillation fluid and measured in a scintillation counter. Blanks contained no 6-MP. Each sample was assayed in duplicate and results reported as the average.

2.5. 6-MP–GSSG reactivity

The reactivity between 6-MP and GSSG was tested by incubating 7.2 mM 6-MP in a redox buffer for 15 min. The buffer consisted of 1.67 mM GSSG, 8.33 mM GSH, and 25 mM potassium phosphate, pH = 7.2. This mixture was used to mimic the most oxidative buffer of the redox buffers described below, [GSH]/[GSSG] = 5. Controls did not contain GSH or GSSG. Reversed-phase high performance liquid chromatography (HPLC with C-18 column, 25 mM potassium phosphate pH = 7.2 as mobile phase) including a diode array detector was used to detect any decrease in 6-MP concentration (λ = 320 nm). An absorption spectrum was recorded (λ = 260–350 nm) to detect any overlapping peaks within the peak of 6-MP on the retention chromatogram.

2.6. hTPMT in redox buffers

Combinations of redox buffers were made by employing a total [GSH] + [GSSG] concentration = 3, 5, or 10 mM,

and a [GSH]/[GSSG] ratio = 5, 10, 20, 40, 80, 160, 320, or ≫ 300 (GSH only, no GSSG). In addition, the buffers contained 0.1 M potassium phosphate, 1 M glycerol, 0.5% (w/w) peroxide-free Triton X-100, pH = 7.2. Reactivated and dialyzed hTPMT from the reactivation/dialysis procedure above was diluted to 1/200 in each redox buffer and incubated at 30° in the dark. For this purpose, polypropylene (low protein interaction) vials that had been stored in nitrogen atmosphere for one week before use to diffuse out trapped oxygen from the plastic were used. Samples were assayed for TPMT activity at 6, 15, 30, 66, and 122 hr by application of the TPMT activity assay described above.

2.7. Computer simulation

General equations for protein thiol–disulfide exchange described in the literature [9] were simulated in an attempt to find a mechanism for hTPMT thiol–disulfide exchange. Some of the equations did not involve a mixed disulfide and some involved irreversible steps. Differential equations describing the reaction rates between each step were integrated and solved with respect to enzyme concentration. The concentrations of GSH and GSSG were regarded as high and not influenced by enzyme concentration. An attempt was made to duplicate the results from the hTPMT redox procedure described above based on arbitrary chosen rate constants. The integration procedure and testing of different rate constants, i.e the simulation procedure, were time-consuming and therefore done with the aid of a suitable computer program, Maple®.

2.8. Titration of protein thiols

Sulfhydryl groups in hTPMT were titrated by conjugation reaction with DTNB [12] after reactivation/dialysis of the protein. The following three forms of hTPMT were titrated: (a) native hTPMT complexed with its ligand AdoMet; (b) native hTPMT, uncomplexed; and (c) denatured hTPMT. Titration was done by adding 10 μL 0.68 mg/mL of native hTPMT to 70 μL of a buffer (pH = 7.5) which for each case (a–c) consisted of: (a) 333 μM AdoMet, 0.1 M potassium phosphate, 0.1% (w/w) Triton X-100; (b) 0.1 M potassium phosphate, 0.1% (w/w) Triton X-100; and (c) 6 M guanidine. Absorbance was monitored for 14 min on a photospectrometer (λ = 412 nm) after the addition of 1.6 μL 25 mM DTNB in 0.1 M potassium phosphate, pH = 7.5. Blanks did not contain hTPMT.

3. Results

3.1. 6-MP–GSSG reactivity

As measured by HPLC, the 6-MP concentration decreased from 7.2 to 6.8 ± 0.2 mM (N = 5) during the 15-min incubation period in the presence of both GSSG and GSH. Without GSH the 6-MP concentration decreased to 6.5 ± 0.1 mM (N = 5). Analysis of samples stored for up to 2 hr at room temperature showed no further decrease in 6-MP. Inspection of spectra indicated no overlapping peaks due to reaction products of 6-MP within the peak of 6-MP on the chromatogram.

3.2. hTPMT in redox buffers

hTPMT was incubated in buffers of different combinations of the [GSH]/[GSSG] ratio and the total [GSH] + [GSSG] concentration. The influence of the redox couple on TPMT activity was observed over time by assaying the activity of the enzyme. The results are illustrated in Fig. 1, a–c. For a total redox couple concentration of 10 mM (Fig. 1a), the activity remained constant during the 122-hr period for [GSH]/[GSSG] ratios above 160. This activity was therefore regarded as 100% activity. All other activities were reported relative to this activity (Fig. 1, B and C). The figure shows that activity decreased to almost zero during the incubation period when relative high levels of GSSG were present. The activity varied between 5% and 100% in buffers representing levels and ratios of the redox couple expected to exist *in vivo* [9], as illustrated by the shaded area. For total [GSH] + [GSSG] concentrations of 5 and 3 mM, there was a drop in activity even for the highest [GSH]/[GSSG] ratios during the incubation period.

3.3. Computer simulation

An attempt was made to duplicate the results depicted in Fig. 1, A–C with the aid of computer simulation of general equations for protein thiol–disulfide exchange described in the literature. A mechanism for hTPMT thiol–disulfide exchange based on best fit is proposed in Fig. 3.

3.4. Titration of protein thiols

Sulfhydryl groups in hTPMT complexed with AdoMet, uncomplexed hTPMT, and denatured hTPMT were conjugated by reaction with DTNB. Absorbance was recorded on a photospectrometer, as seen in Fig. 2. There was no increase in absorbance after 10 min for denatured and complexed hTPMT. Thus, absorbance recorded after 10 min was chosen to represent the number of sulfhydryl groups exposed for conjugation. Denatured hTPMT with all sulfhydryl groups exposed gave the highest absorbance. Apparently about one-third of the total number of sulfhydryl groups seemed to be exposed for titration in complexed hTPMT, as measured from the absorbance.

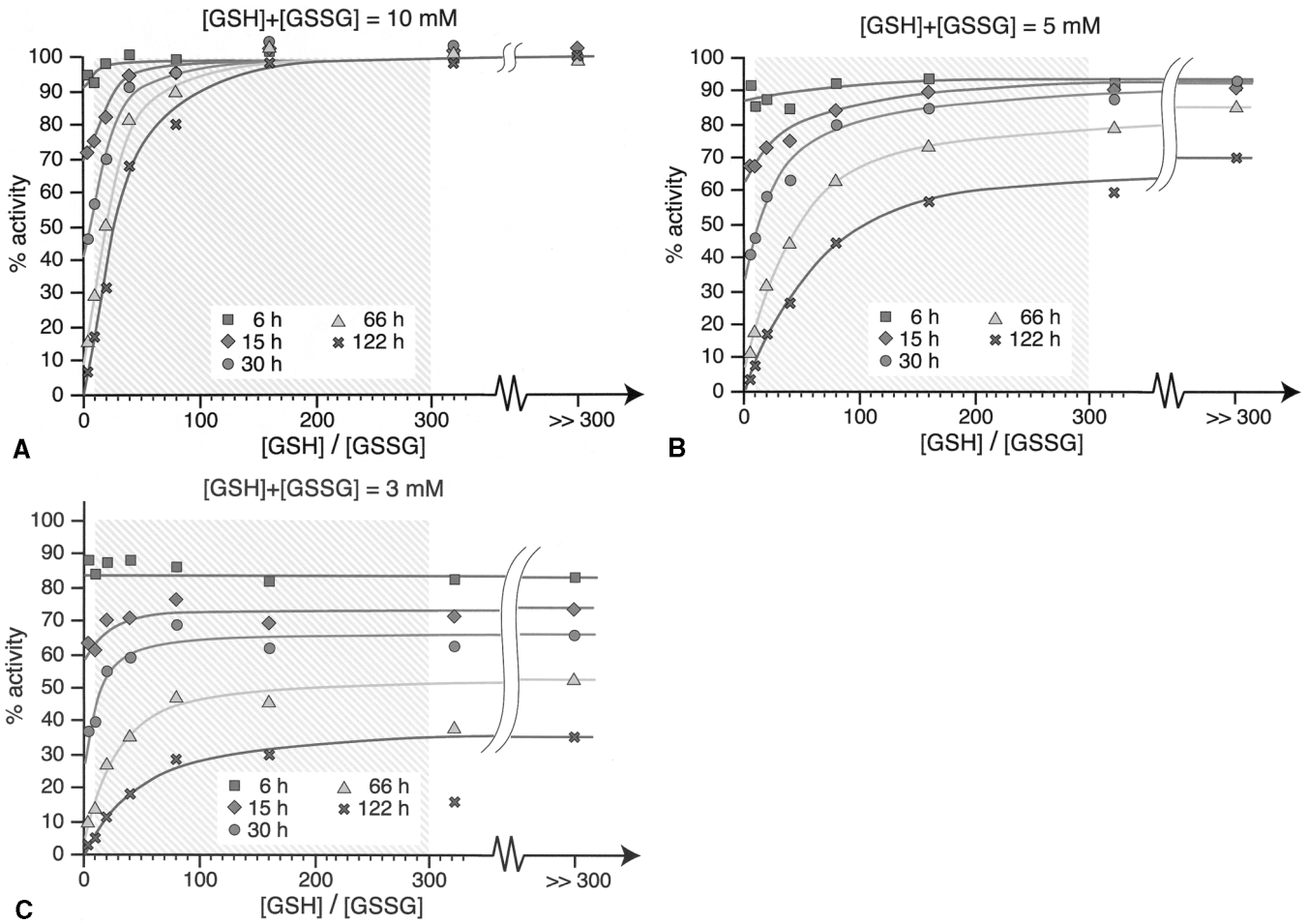

Fig. 1. hTPMT was incubated in different GSH/GSSG redox buffers at 30° and enzyme activity was measured over time. The redox buffers consisted of combinations of different [GSH] + [GSSG] total concentrations and [GSH]/[GSSG] ratios. Shaded area indicates ratios expected to occur *in vivo*.

4. Discussion

4.1. 6-MP–GSSG reactivity

The basic principle of the hTPMT assay is to measure the turnover rate of the enzyme under conditions of substrate saturation. It was thus crucial for the reliability of the hTPMT activity assay that the substrate 6-MP, possessing potential reducing capability as a sulfhydryl compound, not be depleted by oxidation by GSSG during the assay incubation period. The results showed that 6-MP concentration decreased from 7.2 to 6.8 ± 0.2 mM during the 15-min period of incubation with both GSSG and GSH present, but with no further decrease, which indicated that 6-MP was oxidized and that redox equilibrium between 6-MP and GSSG was reached within this period. The 6-MP concentration of 6.8 mM towards the end of the assay incubation period was sufficient to ensure substrate saturation, as the saturating concentration is ≥4 mM [13]. In the absence of GSH, the 6-MP concentration decreased further, to 6.5 ± 0.1 mM during the 15-min period. This indicated that 6-MP participates in a redox equilibrium with GSH and is partially protected by GSH from oxidation by GSSG. The interaction between 6-MP and GSSG might also potentially distort the [GSH]/[GSSG]) ratio and subsequently the hTPMT activity, depending on the rate of the thiol–disulfide exchange between the protein and the surrounding redox compounds. Fig. 1, A–C shows almost no change in enzyme activity during the first 6 hr of thiol–disulfide exchange. The change in redox status of the enzyme within the relatively short assay incubation period was therefore regarded as negligible.

4.2. TPMT in redox buffers

As seen in Fig. 1A, there was no decrease in hTPMT activity during the 122-hr period for high [GSH]/[GSSG] ratios. The high stability of the protein during this long period at a constant temperature of 30° was a result of the preceding effort to optimize buffer conditions so as to extend protein half-life. The purpose was to isolate thiol–disulfide exchange as the only factor influencing activity by reducing destabilizing factors as much as possible. In this context, different recommended solutes with buffering ca-

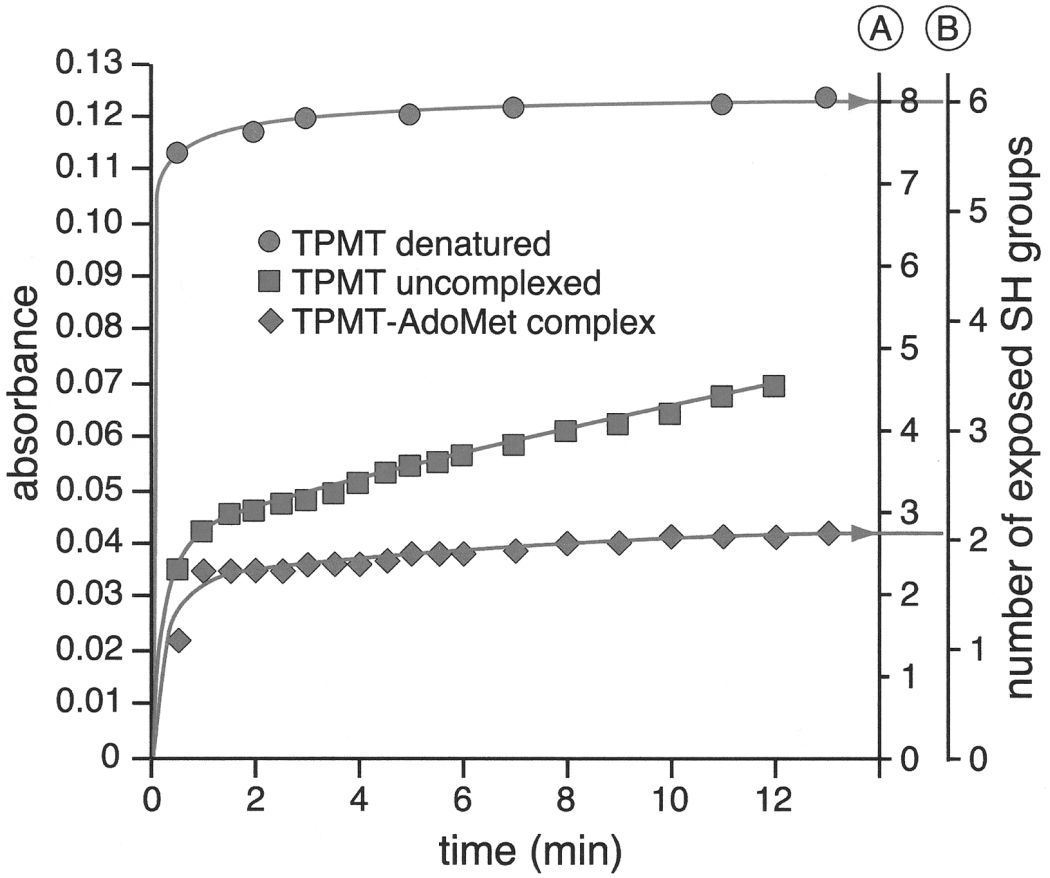

Fig. 2. Three different forms of hTPMT were titrated with DTNB and absorbance was measured on a photospectrometer. The absorbance is proportional to the number of protein thiols exposed. There are eight cysteines in hTPMT (A). Two of the thiols are adjacent in the denatured protein and may easily form a disulfide bridge which makes them unreactive to DTNB, leaving six exposed thiols for DTNB titration (B).

pacity, osmolytes, surfactants, and combinations of these [14,15] were tried (data not shown). The zwitterionic buffers MOPS (3-[*N*-morpholino]propanesulfonic acid) and HEPES inhibited/deactivated hTPMT significantly in spite of the fact that these compounds are generally held to be enzyme stabilizers. It has been proposed that small, in part negatively charged organic compounds generally possess affinity to the substrate (6-MP) binding site of hTPMT [11]. Use of such compounds, including MOPS and HEPES, was therefore omitted. Osmolytes stabilized hTPMT, of which glycerol gave the most pronounced effect. The resulting activity-preserving buffer system, which was used in this study, increased the half-life of the protein several orders of magnitude compared to buffers described for hTPMT in the literature.

The major cytoplasmic thiol–disulfide redox buffer in most cells is glutathione in its reduced (GSH) and oxidized (GSSG) state. In the liver, glutathione is the most dominant sulfhydryl compound, and one of its major roles in the organism is to reduce and detoxify oxidative metabolites such as peroxides and other oxygen radical species. Glutathione levels vary between tissues, e.g. 11 mM in normal rat liver and 2–3 mM in heart. Based on measurements on rat liver, most glutathione exists as GSH and the [GSH]/[GSSG] ratio is indicated to be approximately 300–400 in a normal fed liver. In a fasted rat liver, this ratio may decrease to 100. If mild oxidative stress is induced in the liver by perfusion of an oxidative agent the ratio may decrease to levels as low as 10, or lower if the stress is severe. Many cytoplasmic sulfhydryl group containing proteins (P-SH) have been shown to lose their activity upon oxidation by GSSG to a mixed disulfide (P-SSG) or by further oxidation to a protein disulfide (PSS, i.e internal disulfide bridge). It is regarded as unlikely that significant amounts of enzymes should exist in oxidized forms under normal conditions where the [GSH]/[GSSG] ratio $\geq$ 300 (for review, see [9]). Fig. 1, A–C shows that the activity of hTPMT is modulated *in vitro* by the glutathione levels and ratios that are reported to occur *in vivo* (shaded area). The left half of the curve in Fig. 1A (122 hr) resembles that of a protein that reaches redox equilibrium with its environment. But during the 122-hr period, no obvious equilibrium between protein thiols and the redox buffer was apparent, as this would be associated with overlapping/superimposed curves. The lack of equilibrium is in accordance with a partly irreversible inactivation mechanism. Computer simulation performed to bring best accordance between the data basis of Fig. 1 and general protein thiol–disulfide exchange

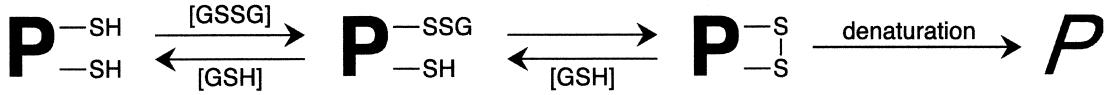

Fig. 3. The proposed mechanism for the protein thiol–disulfide exchange of hTPMT. The derivatization of the mechanism was based on computer simulation of different equations for protein thiol–disulfide exchange to find best fit for the results depicted in Fig. 1, A–C. $P(SH)_2$ is the active form of hTPMT.

mechanisms gave best fit for the mechanism depicted in Fig. 3, which involves an intermediate mixed disulfide. This mechanism has some characteristic features which are reflected by Fig. 1. When not regarding the irreversible step, the amount of active enzyme or $[P(SH)_2]$ in this type of equation is dependent on both [GSH] and the [GSH]/[GSSG] ratio [9]. This implies that at a fixed [GSH]/[GSSG] ratio, the enzyme activity or $[P(SH)_2]$ will increase as [GSH] or the total [GSH] + [GSSG] concentration increases. Moreover, at a fixed [GSSG], the probability of the protein to denaturing decreases as [GSH] increases, since this clearly causes a leftward transfer of protein from the P(SS) state to the P(SH)(SSG) state and from the P(SH)(SSG) state to the $P(SH)_2$ state (Fig. 3). This is illustrated by comparison of the activity that corresponds to [GSH]/[GSSG] = 100 in Fig. 1B and the activity that corresponds to [GSH]/[GSSG] = 200 in Fig. 1A, both of which represent a [GSSG] $\approx$ 0.05. The computer simulation indicated this phenomenon, but failed to explain the steady level of enzyme activity for [GSH]/[GSSG] ratios $>$ 200 over time in Fig. 1a. However, it is very likely that TPMT activity is influenced by biological variations in glutathione levels.

4.3. Titration of protein thiols

There are a total of eight cysteines in the peptide chain of hTPMT [1] contributing to an equal number of sulfhydryl groups in a fully reduced state. Denaturation of the protein leads to an unfolding of the chain, exposing all the sulfhydryl groups. In its native active form, only sulfhydryl groups on the surface of the protein are exposed, as the remaining groups are buried inside the structure. Sulfhydryl groups participating in disulfide bridges before titration begins are unlikely to leave the oxidized state to be oxidized/conjugated by DTNB, since only the thiolate ion is reactive in the thiol–disulfide exchange reaction [9]. Only sulfhydryl groups that are closely spaced have the ability to make stable disulfide bridges. These groups can be distant in the primary structure, but may be in close vicinity to each other in the tertiary structure. Two of the cysteines in hTPMT are sequentially adjacent (Cys133, Cys134; Fig. 4). Adjacent cysteines are likely to form disulfide bridges in an oxidative environment but require a *cis* peptide bond between them, which in normal situations induces local strain in the peptide backbone and therefore is uncommon. Nonetheless, disulfide bridges are found between sequentially close cysteines in naturally occurring proteins, as the unfavorability of the formation of a *cis* peptide bond is more than compensated for by the easy formation of a favorable disulfide bridge [16]. If this is the case for hTPMT, the number of sulfhydryl groups exposed for conjugation after denaturation would be six, since two sulfhydryl groups might already be oxidized by traces of oxygen or oxidizing contaminants. The eventuality of eight and six exposed thiols in the denaturated state is illustrated in Fig. 2 by alternatives (A) and (B), respectively. For the two cases, the number of exposed sulfhydryl groups for hTPMT complexed with AdoMet was (A) slightly less than three and (B) two.

The absorbance for uncomplexed hTPMT never stabilized, but continued to rise in contrast to complexed hTPMT, as seen in Fig. 2. A probable cause is that the structure of uncomplexed hTPMT is relatively unstable and slowly unfolds over time, continuously exposing new sulfhydryl groups ready for conjugation. This instability may be induced by the conjugated TNB molecule, which may impose stress on the protein structure due to steric interactions. AdoMet seems to contribute to stability to the protein as seen from the stable absorbance after 10 min; indeed, it has been reported to stabilize hTPMT [17] and to bind with very high affinity to a related methyltransferase [18]. It appears from the model of hTPMT [11] that the two cysteines closest to AdoMet on Fig. 4 (Cys71, Cys183) are situated at an exposed position at the bottom of the cleft that functions as the binding site for this ligand. In the hTPMT–AdoMet complex, these cysteines seem to be shielded by AdoMet. One or both of these cysteines might be responsible for the modulation of the activity of the enzyme, as conjugation of one of them would probably alter the binding site of AdoMet.

The model seems to be in fair agreement with the experimental results (Fig. 2), since the hTPMT–AdoMet complex exposed the lowest number of conjugatable cysteines. Furthermore, the model predicts that two cysteines (Cys212, Cys216) at the surface of the protein will be easily conjugated, in agreement with Fig. 2. The enzyme might even be active in the conjugated form, since such a loose surface conjugation does not necessarily disturb the structure of the protein, but this was not investigated. The cysteines at the bottom of the AdoMet binding cleft should be reachable only from the entrance of the cleft. They are therefore likely to be conjugated at a slower rate due to a relatively low number of favorable collisions with DTNB. Fig. 2 illustrates this hypothesis for uncomplexed hTPMT. However, there is uncertainty concerning two of the cysteines, Cys165 and Cys234. In the model, Cys165 is partly buried in the structure and pointing inwards, which makes it apparently un-

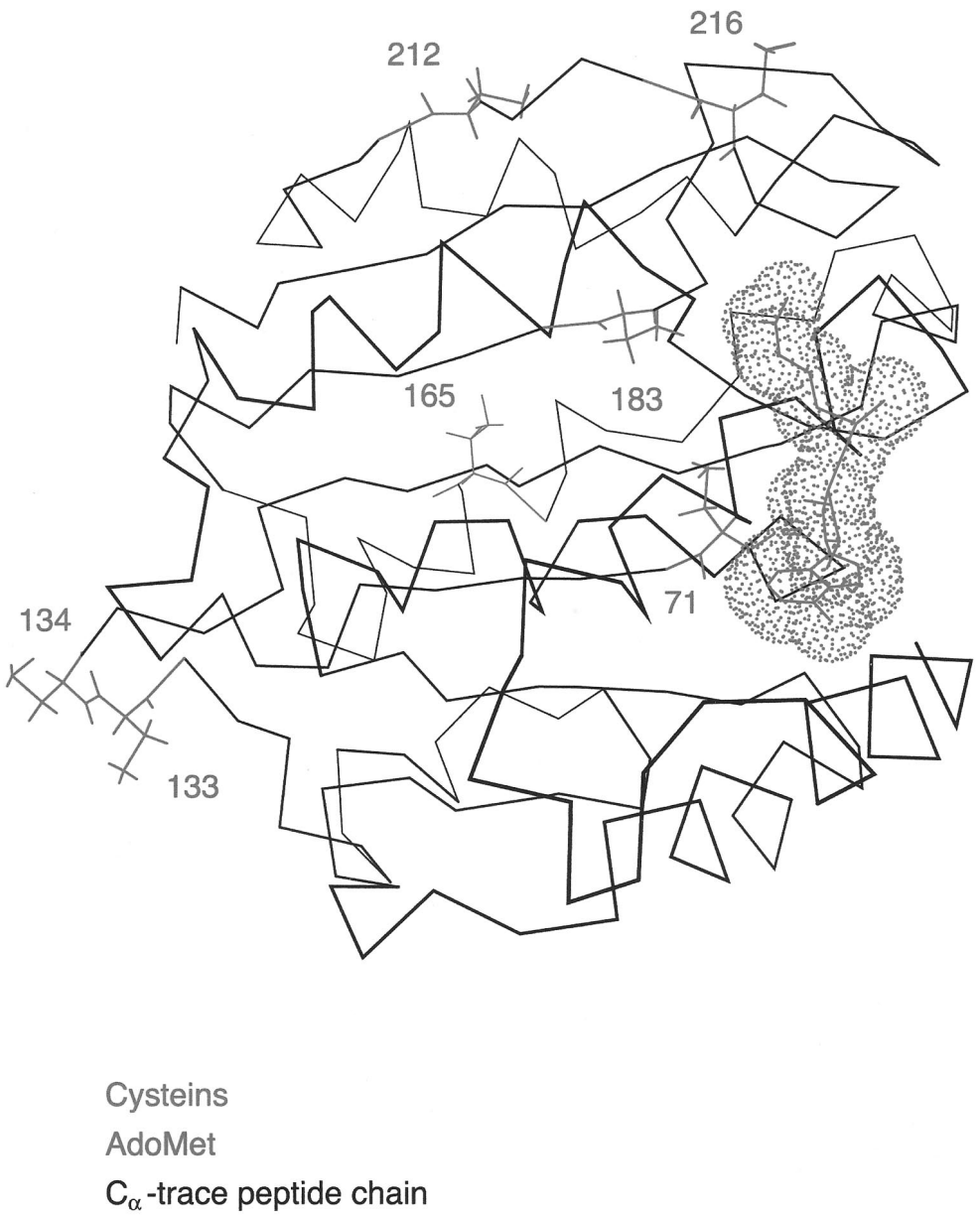

Fig. 4. The model of hTPMT [11] contains seven of the eight cysteines. Cys234 belongs to the carboxy-terminal part of the peptide that was not included when the model was constructed. Cys71 and Cys183 are believed to be shielded from DTNB titration by AdoMet and might have a role in the thiol–disulfide exchange mechanism that modulates the activity of the enzyme.

reachable for modification by DTNB. A shift in the structure alignment for hTPMT [11] would contribute to the opposite situation. Cys165 is located at the C-terminal end of α-helix 7 in the model, which was constructed by homology with methyltransferases of known three-dimensional structure. Changing the position of Cys165 to the position of Tyr166 in the structure alignment would give a side-chain position highly available for modification by DTNB. Cys234 belongs to the carboxy-terminal part of the peptide chain which was not included in the previous model construct, thus making it impossible to predict the availability of this cysteine. The current study predicts it to be buried.

4.4. Conclusion

This study has indicated that the activity of hTPMT is influenced by naturally occurring levels of GSH and GSSG. The molecular modulation mechanism for hTPMT by the glutathione redox couple is of common type protein thiol–disulfide exchange and involves a mixed disulfide. There seem to be at least two sulfhydryl groups on the protein readily accessible for thiol–disulfide exchange. Some cysteines seem to be situated in the AdoMet binding cleft and are shielded by AdoMet from modification in the hTPMT–AdoMet complex. Conjugation of one of these might alter

the AdoMet binding site and thereby modulate the activity of the enzyme. The previous model of hTPMT [11] is in agreement with the experimental results of this study.

Acknowledgments

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