Purification and Properties of Thioltransferase from Monkey Small Intestinal Mucosa: Its Role in Protein-s-thiolation

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INTRODUCTION

Maintenance of the redox status of sulfhydryl groups is vital to the physiological function of cells. GSSG formed during oxidative stress is known to form mixed disulfide with proteins, resulting in their altered function. Modification of protein sulhydryl groups by GSH or cysteine has been suggested as one of the post-translational modifications. Glutathione has been identified as being the predominant low molecular weight thiol that is bound to proteins during oxidative stress (Schuppe-Koistinen et al., 1994). Thiolated proteins can regulate the generation of NADPH by altering the rates of the glycolytic and pentose phosphate pathways. Studies have indicated that NADPH and GSH dependent dethiolase can bring about removal of GSH from glutathionylated proteins (Miller et al., 1991). It has been shown that thioltransferase obtained from rat liver could catalyze both formation and decomposition of protein-mixed disulfide. Earlier studies have shown the presence of thioltransferase in various tissues (Larson et al., 1985; Hatakayama et al., 1984, 1985; Hopper et al., 1989; Mieyal et al., 1991).

Although most cells are susceptible to oxidative injury, intestinal epithelium cells seem to be more at risk because of their exposure to oxidants generated both in the mucosa and in the lumen, the latter containing ingested foods and bacterial metabolites. Perfusion of intestinal lumen with oxidants in anesthetized rats has shown oxidation of mucosal GSH to GSSG.
and formation of protein mixed disulfide (Benard and Balasubramanian, 1993). Also in vitro exposure of isolated intestinal mitochondria and brush border membranes (BBM) to GSSG has shown increased pro-s-s-G (protein mixed glutathione) formation (Benard and Balasubramanian, 1995). Increased formation of pro-s-s-G may be catalyzed by the enzyme thioltransferase, and in the present study, this enzyme has been purified from monkey small intestinal mucosa and shown to catalyze thiolation and dethiolation.

MATERIALS AND METHODS

Molecular weight markers, NADPH, glutathione reductase, glutathione disulfide (GSSG), reduced glutathione (GSH), 1-fluoro-2,4-dinitrobenzene (FDNB) and iodoacetic acid were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Hydroxyapatite was obtained from Bio-Rad (Richmond, CA, U.S.A.) and hydroxyethyl disulfide (HEDS) from Aldrich chemical Co. (Milwaukee, WI, U.S.A.). Cibacron blue F3GA was obtained from Ciba-Geigy (Switzerland).

Blue Sepharose was prepared from epichlorohydrin activated Sepharose-4B as described by Ahamed et al. (1977) and protein was determined using BSA as standard (Lowry et al., 1951).

Enzyme assay: The assay was based on the following coupled reaction (Mieyal et al., 1991).

\[
\text{S-CH}_2\text{-CH}_2\text{-OH} \quad + \quad \text{2GSH} \quad \rightarrow \quad \text{GSSG} + \text{2SH-CH}_2\text{-CH}_2\text{-OH}
\]

Thioltransferase

\[
\text{GSSG + NADPH + H}^+ \quad \rightarrow \quad \text{2GSH} + \text{CH}_2\text{-CH}_2\text{-OH}
\]

GSSG reductase

The assay system for thioltransferase activity was as follows. The reaction mixture consisted of 0.5 mM GSH, 0.4 units of glutathione reductase, 2 mM hydroxyethyl disulfide (HEDS), 0.2 mM NADPH, and 0.1 M sodium phosphate buffer, pH 7.5 (all final concentration), in a total volume of 1 ml. The reaction proceeded at 30°C and thioltransferase activity was measured spectrophotometrically at 340 nm. The net enzyme reaction rate was obtained by subtraction of the non-enzymatic reaction rate from the total rate. One unit of thioltransferase was defined as the amount of enzyme that catalyses the formation of 1 μmol of GSSG/min under standard assay conditions.

Purification of thioltransferase

Step 1: Homogenization. Except where indicated, all purification procedures were carried out at 4°C. The whole length of the small intestine from a monkey (Macaca radiata) that was fasted overnight was obtained from adjacent lab experimenting on pancreatic islet cell isolation and washed gently with cold physiological saline. Mucosa was scraped using a glass slide and about 25 g of mucosa was suspended in 250 ml of buffer solution containing 0.25 M sucrose, 10 mM hepes, 25 mM KCl and 5 mM MgCl₂, adjusted to pH 7.5 with Tris. This was homogenized and the homogenate was passed through two layers of cheese cloth. Filtered homogenate was centrifuged at 25,000 g for 40 min. The supernatant obtained was adjusted to pH 6.4 with 5% acetic acid and centrifuged at 22,000 g for 40 min. The resulting supernatant fraction was adjusted to pH 7.0 with 6 N NaOH and is referred to as the high-speed supernatant.

Step 2: Heat treatment. The supernatant was subjected to heat treatment until the temperature reached 62°C. It was cooled immediately in an ice bath and centrifuged at 15,000 g for 30 min.

Step 3: Ammonium sulfate precipitation. The supernatant from the previous step was fractionated with solid ammonium sulfate and the enzyme was collected between 40-65% saturation. This was centrifuged at 15,000 g for 30 min and the precipitate was dissolved in a minimum volume of 20 mM tris–HCl buffer, pH 7.4, and dialysed against the same buffer.

Step 4: Blue Sepharose chromatography. Dialysed enzyme solution was loaded onto a blue Sepharose column equilibrated with 20 mM Tris–HCl buffer, pH 7.4, at a protein concentration of 2 mg/ml gel. The column was washed with 3 bed volumes of the equilibration.
Thioltransferase from monkey intestinal mucosa

buffer and eluted with a salt gradient of 0–0.2 M NaCl in the same buffer. Active fractions were pooled and dialysed against 10 mM sodium phosphate buffer, pH 7.4.

**Step 5: Phenyl Sepharose chromatography.** The sample from the previous step was made up to 1 M with (NH₄)₂SO₄ and applied to a phenyl Sepharose column (1 mg protein/ml gel) equilibrated with 10 mM phosphate buffer, pH 7.4, containing 1 M (NH₄)₂SO₄. The column was washed with 3 bed volumes of the same buffer followed by phosphate buffer containing 0.5 M (NH₄)₂SO₄ and plain phosphate buffer. Finally the enzyme was eluted with phosphate buffer containing 10% ethylene glycol. Active fractions were pooled and dialysed against phosphate buffer.

**Step 6: Hydroxyapatite chromatography.** Dialysed enzyme from the above step was applied to a hydroxyapatite column (0.5 mg protein/ml gel), equilibrated with 10 mM phosphate buffer, pH 7.4. Column was washed with equilibration buffer and bound enzyme was eluted with 50 mM phosphate buffer, pH 7.4. Active fractions were pooled, dialysed against 10 mM phosphate buffer, pH 7.4, and concentrated by lyophilization.

**Determination of kinetic constants**

**pH optimum:** Enzyme assay was carried out in a pH range of 4–10 (for pH 4–6 acetate buffer, pH 6–7.5 phosphate buffer and pH 7.5–10 Tris buffer were used). From the plot of pH versus enzyme activity optimum pH was derived.

**Kₘ value:** To determine the Kₘ value, enzyme assay was carried out at standard conditions with varying concentration of either GSH or hydroxyethyl disulfide. From the Lineweaver–Burk plot of 1/V versus 1/S, the Kₘ value was calculated.

**Molecular weight determination by SDS–PAGE and gel filtration on Sephadex G-200**

Purified enzyme (10 µg) containing 2% (w/v) SDS with 5% (v/v) 2-mercaptoethanol was boiled for 2 min and subjected to gel electrophoresis by the method of Laemmli (1970). The electrophoretic mobility was compared according to Weber and Osborn (1965) with known molecular weight markers phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and lysozyme (12 kDa).

Alternatively 1 mg of the purified enzyme dissolved in equilibration buffer was passed through a Sephadex G-200 column (52.5 cm × 1.2 cm) equilibrated with 20 mM phosphate buffer (pH 7.0)/0.2 M NaCl and 2 ml fractions were collected. The Vₐ/V₀ value of the enzyme was compared with standard marker proteins alcohol dehydrogenase (150 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), trypsinogen (24 kDa) and cytochrome C (12 kDa).

**Demonstration of thioltransferase activity in the absence of glutathione reductase**

To demonstrate transhydrogenase activity, purified thioltransferase (0.06 units) was incubated with 0.5 mM GSH and 2 mM HEDS without glutathione reductase and NADPH for different time intervals. The reaction was terminated with the addition of TCA (5% final concentration) and centrifuged. The supernatant obtained was s-alkylated with iodoacetic acid and derivatized with 1% FDNB. GSSG formed was quantitated by HPLC on an aminopropyl column using a methanol–sodium acetate system (Fariss and Reed, 1987). Control reaction was performed by omitting thioltransferase, NADPH and glutathione reductase. The possible thiol oxidase activity of thioltransferase was checked by incubating 0.5 mM GSH alone with purified thioltransferase for 10 min and GSSG formed was quantitated by HPLC.

**Protein-s-thiolation of mitochondria by thioltransferase**

Mitochondria corresponding to 2 mg protein/ml were incubated with 0.06 units of purified thioltransferase and 1 mM GSSG in a total volume of 1 ml for 15 min. The control incubation had everything except thioltransferase. Following incubation, proteins were precipitated with 5% TCA, centrifuged and the protein pellet was repeatedly washed with 95% ethanol. This was suspended in 50 mM MOPS buffer, pH 8.0, containing 25 mM dithiothreitol, and sonicated for 30 sec. After standing for 1 hr at 37°C, proteins were precipitated with TCA and the supernatant was analysed for GSH by HPLC, as described above (Livesay and Reed, 1984).

**RESULTS**

A summary of purification of thioltransferase from monkey small intestinal mucosa is presented in Table 1. The enzyme was purified 3135-fold with an overall recovery of 20% and the purified enzyme had a specific activity of
Table 1. Purification of thioltransferase from monkey intestinal mucosa

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High speed supernatant</td>
<td>7836</td>
<td>30.1</td>
<td>0.004</td>
<td>100</td>
</tr>
<tr>
<td>Heat treatment and ammonium</td>
<td>726.8</td>
<td>19.12</td>
<td>0.084</td>
<td>63.1</td>
</tr>
<tr>
<td>Sulfate precipitation 40–65%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue Sepharose chromatography</td>
<td>9.26</td>
<td>12.60</td>
<td>1.360</td>
<td>41.8</td>
</tr>
<tr>
<td>Phenyl Sepharose chromatography</td>
<td>7.10</td>
<td>9.10</td>
<td>4.330</td>
<td>30.2</td>
</tr>
<tr>
<td>Hydroxyapatite chromatography</td>
<td>0.48</td>
<td>6.02</td>
<td>12.541</td>
<td>20.1</td>
</tr>
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12.5 units/mg protein. The purified enzyme was homogeneous as judged by SDS–PAGE. As shown in Fig. 1, purified enzyme migrated as a single polypeptide with a molecular mass of 52 kDa. Gel filtration analysis of purified thioltransferase on Sephadex G-200 also revealed that the enzyme has a molecular weight of 52 kDa (Fig. 2).

Table 2 summarizes some of the molecular and kinetic properties of the purified thioltransferase. The enzyme showed a pH optimum of 8.0 under standard assay conditions. Studies using various metal ions showed that only Hg$^{2+}$ and Cu$^{2+}$ inhibited the enzyme whereas metals like Ca$^{2+}$, Mg$^{2+}$ or Mn$^{2+}$ had no significant effect. Some of the anions tested also showed no effect on the enzyme activity (data not shown). Iodoacetic acid at 0.5 mM concentration inhibited the enzyme (data not shown).

**Demonstration of thioltransferase activity in the absence of glutathione reductase**

Attempts to demonstrate the enzymatically catalyzed thiol disulfide interchange in the absence of glutathione reductase and NADPH described under Materials and Methods yielded GSGG in quantities significantly greater than that in the non-enzymatic control. As shown in Fig. 3, presence of thioltransferase in the reaction mixture containing GSH and HEDS alone increased the formation of GSGG significantly in comparison with the non-enzymatic reaction. This indicated that the enzyme catalyzes thiol–disulfide exchange. To rule out the possible involvement of thiol oxidase activity responsible for formation of GSGG, the enzyme was incubated with GSH alone. As shown in the HPLC chromatogram (Fig. 4), the enzyme did not show thiol oxidase activity,
Table 2. Properties of purified thioltransferase from monkey intestinal mucosa

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>Molecular weight</td>
<td>52 kDa</td>
</tr>
<tr>
<td>Number of subunits</td>
<td>1</td>
</tr>
<tr>
<td>pH optimum</td>
<td>8.0</td>
</tr>
<tr>
<td>Apparent $K_m$ for HEDS</td>
<td>2.60 mM</td>
</tr>
<tr>
<td></td>
<td>0.68 mM</td>
</tr>
</tbody>
</table>

HEDS, hydroxyethyl disulphide; GSH, reduced glutathione.

since GSSG was not formed when the enzyme was incubated with GSH alone.

Thioltransferase catalyzed protein-s-thiolation of mitochondria

Protein-s-thiolation of mitochondria was carried out both in the presence and absence of thioltransferase in a medium containing 1 mM GSSG. As shown in Fig. 5, the presence of thioltransferase enhanced the formation of glutathione protein mixed disulfide compared to mitochondria incubated with GSSG alone.

Fig. 3. HPLC chromatogram of thioltransferase catalyzed thiol-disulfide exchange reaction. The experimental details are described in the text. (A) Chromatographic pattern of standard GSH and GSSG. Synthetic substrate HEDS was incubated with GSH in the absence (B) and presence (C) of thioltransferase, omitting glutathione reductase and NADPH. GSSG formed was quantitated by HPLC.

Fig. 4. HPLC demonstration of absence of thiol oxidase activity in purified thioltransferase. Experimental details are described in the text. (A) Chromatographic pattern of standard GSH and GSSG. GSH alone was incubated in the absence (B) and presence (C) of thioltransferase, omitting glutathione reductase and NADPH. GSSG formed was quantitated by HPLC.
With enzyme

GSSG in mM

Protein S-S-G (moles/incubating protein)

0 0.05 0.10 1.0

Fig. 5. Thioltransferase catalyzed protein-s-thiolation of mitochondrial protein. Isolated monkey intestinal mitochondria were incubated with various concentrations of GSSG in presence and absence of thioltransferase, and protein associated GSH was quantitated after protein precipitation and release of GSH from protein. Each value represents mean ± SD of three separate experiments. *Statistically significant from corresponding control experiments (p < 0.05).

DISCUSSION

Although processes involving thiol–thiol-disulfide and thiol–thiosulfate ester reactions are known to take place spontaneously at pH 7.0, an enzyme has been shown to catalyze these interchange reactions (Mannervik et al., 1974; Axellson et al., 1978). Thioltransferase catalyses transfer of the thiol residue from XSSY to GSH, resulting in the formation of GSSG, XSH and YSH. This class of enzymes is found throughout nature, and includes glutaredoxin (thioltransferase), thioredoxin and protein disulfide isomerase. The common features among them are a pair of cysteine residues in the active site and the ability to catalyze reduction of disulfide (Holmgren, 1989).

Thioredoxin and glutaredoxin generally have been linked to the reduction of ribonucleotide reductase and protein disulfide isomerase to protein folding. Protein disulfide isomerase (glutathione : homocysteine transhydrogenase) is distinguished from glutaredoxin and thioltransferase (glutathione : homocysteine transhydrogenase) by coupling to thioredoxin reductase rather than to GSH and GSSG reductase. Although many similarities are observed between glutaredoxin and thioltransferase in terms of subcellular localization and requirement for glutathione reductase and NADPH, reports on amino acid analysis suggest that they may be different proteins (Gan and Wells, 1988). Thioltransferase purified in the present study was found to have a molecular weight of 52 kDa on SDS-PAGE and was monomeric in nature. Gel filtration analysis on Sephadex-200 also revealed that it is a 52 kDa protein. Thioltransferase purified by others from rat small intestine was reported to be 12 kDa and oxidant resistant (Mizoguchi et al., 1994). The same enzyme partially restored the activity of rat small intestinal lactate dehydrogenase, which has been inactivated by ebselen (a selenium containing compound) (Mizoguchi et al., 1995). Although thioltransferase presented here differs from enzyme obtained from other sources in terms of molecular weight, many similar properties were observed. (1) Like enzyme from RBC, monkey intestinal mucosal thioltransferase was heat stable, and can be warmed to 60–65°C for a few minutes without losing significant activity. This suggests that its global conformation may resemble that of proteins that contain extensive β-structure (Holmgren, 1989). (2) Optimum pH of monkey thioltransferase falls within the range of optimum pH reported for the enzyme from other sources. (3) Hydrophobicity of thioltransferase was evident from its interaction with Phenyl Sepharose, which was employed as one of the steps for purification. Amino acid composition of human RBC thioltransferase has revealed the presence of nearly 30% of hydrophobic amino acids (Mieyal et al., 1991). HPLC analysis of thiol–disulfide exchange activity of thioltransferase has substantiated that it differs from glutathione reductase, which requires NADPH for disulfide reduction. Furthermore, glutathione reductase has been shown to be a dimeric protein, having a subunit molecular weight of 50 kDa (Carlberg and Mannervik, 1985). Electrophoretic analysis under both denaturing and non-denaturing
conditions has indicated that monkey intestinal thioltransferase is a monomeric protein. A recent study on thioltransferase from *Trypanosoma cruzi* has reported a molecular weight of 52 kDa (Moutiez et al., 1985).

Selective heat treatment, stability at acidic pH and narrow range ammonium sulfate precipitation enabled recovery of 63% of enzyme activity with 21-fold purification in the first purification step. Using these steps, thioltransferase has been purified from various sources like bovine liver and calf thymus. Purification reported by Axelsson and Mannervik (1978) involved a series of ion exchange, gel permeation and affinity chromatography, which was time consuming and in this case resulted in very low yield (Larson et al., 1985; Mannervik et al., 1981). The different purification steps employed here yielded good results, by using affinity purification on cibacron blue Sepharose, phenyl-Sepharose and hydroxyapatite chromatography.

Thioltransferase assay requires glutathione reductase and NADPH to measure the formation of GSSG, as a result of disulfide exchange between hydroxyethyl disulfide and GSH. In an earlier study, attempts to demonstrate thioltransferase activity in the absence of glutathione reductase and NADPH were not successful (Nagai and Simon, 1968). Since GSSG formed during this reaction easily exchanges with GSH, this results in incomplete measurement of GSSG. In this study, enzyme activity could be demonstrated in the absence of GSSG reductase and NADPH using HPLC. Iodoacetic acid used to s-alkylate free thiol, trapped free GSH, which prevented non-enzymatic exchange of sulfhydryl groups, and the s-alkyl thiols were quantitated by HPLC after derivatization with FDNB. Measurement of GSSG formed during this reaction directly reflected thiol–disulfide exchange activity of thioltransferase, which was significantly different from non-enzymatic reaction. By HPLC analysis the enzyme was found devoid of thiol oxidase activity as incubation of thioltransferase with glutathione alone did not result in the formation of GSSG.

To our knowledge, this is the first report on direct demonstration of thiol–disulfide exchange activity of thioltransferase by HPLC. Thioltransferase purified from rat liver cytosol and other sources, shows broad specificity towards symmetrical or mixed disulfides of glutathione and other thiol molecules. Thiosulfate esters were also shown to be substrate for thioltransferase (Mannervik et al., 1974). Thioltransferase purified from rat liver cytosol and other sources, shows broad specificity towards symmetrical or mixed disulfides of glutathione and other thiol molecules. Thiosulfate esters were also shown to be substrate for thioltransferase (Mannervik et al., 1974).

Mitochondria are known to generate free radicals (Turren and Boveris, 1980; Turrens et al., 1985) and during mitochondrial respiration about 2–3% of oxygen consumed is transformed to reactive oxygen radicals. The presence of GSH and other antioxidants protects mitochondria from oxidative stress. However, under persistent oxidative stress, mitochondria have been shown to accumulate GSSG, which is then reduced back to GSH. A fraction of this GSSG also can form protein mixed disulfide with mitochondrial proteins (Ravindranath and Reed, 1990). Using renal cortical mitochondria, it has been shown that mitochondria can transport externally added GSSG (McKernan et al., 1991). Our earlier study on *in vitro* protein-s-thiolation has shown that mitochondria exposed to GSSG could form protein bound glutathione (Benard and Balasubramanian, 1995). In the present study, the role of thioltransferase in protein-s-thiolation of mitochondria was studied. Thioltransferase catalysed a dose-dependent increase in the formation of mitochondrial protein-bound glutathione when incubated with GSSG. As shown by Mannervik and Axelsson (1975) and Mannervik et al. (1983) thioltransferase can catalyse both thiolation and dethiolation of proteins. Depending on the redox status of the glutathione system, the reaction may be driven either to dethiolation or thiolation. Oxidized glutathione generated during oxidative stress may facilitate the reaction towards the formation of protein mixed glutathione. When reduced glutathione is restored to normal levels by glutathione reductase, the reaction will be directed to the decomposition of already formed protein mixed disulfide. Although this protein-s-thiolation and dethiolation occur spontaneously, presence of thioltransferase considerably enhances these reactions. Modification of cysteine residue of proteins by protein-s-thiolation is known to alter the activity of enzymes and also determines the intracellular stability of modified proteins. Especially, enzymes of glucose metabolism such as phosphofructokinase and pyruvate kinase were shown to be modified by protein mixed disulfide formation and thioltransferase has been shown to catalyse this reaction (Ziegler, 1985). The role of thioltransferase has also been implicated in thiol homeostasis, as it removes glutathione from oxidatively modified haemoglobin, i.e Hb-s-s-G (Mieyal et al., 1991). Disulfide-inactivated glutathione-s-transferase has also been shown...
to be reactivated by thioltransferase and GSH, implicating its indirect role in detoxification (Terada et al., 1993). Mitochondrial protein-s-thiolation reported in this study may have physiological significance, as the presence of thioltransferase will facilitate temporary storage of glutathione in the form of protein mixed disulfide. These studies have shown that thioltransferase isolated from monkey intestinal mucosa can catalyse thiol–disulfide exchange between disulfide molecules and GSH, and can also enhance the formation of protein bound glutathione in the cell.

SUMMARY

Thioltransferase catalyzing thiol–disulfide exchange was purified from monkey intestinal mucosa and was homogeneous, as judged by SDS–PAGE. In certain properties the enzyme showed similarities to thioltransferase from other tissues, but it differed in others. Thiol–disulfide exchange activity of the purified enzyme was shown by HPLC. Purified enzyme was found to catalyze protein-s-thiolation of proteins.

CONCLUSION

The studies presented here have shown that intestinal mucosa contains thioltransferase which resembles thioltransferase from other tissues in certain aspects. This enzyme can catalyse thiolation and dethiolation of cellular proteins.

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