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S-glutathionylation of human glyceraldehyde-3-phosphate dehydrogenase and possible role of Cys152-Cys156 disulfide bridge in the active site of the protein

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ABSTRACT

Background: We previously showed that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is S-glutathionylated in the presence of H_2O_2 and GSH. S-glutathionylation was shown to result in the formation of a disulfide bridge in the active site of the protein. In the present work, the possible biological significance of the disulfide bridge was investigated.

Methods: Human recombinant GAPDH with the mutation C156S (hGAPDH_C156S) was obtained to prevent the formation of the disulfide bridge. Properties of S-glutathionylated hGAPDH_C156S were studied in comparison with those of the wild-type protein hGAPDH.

Results: S-glutathionylation of hGAPDH and hGAPDH_C156S results in the reversible inactivation of the proteins. In both cases, the modification results in corresponding mixed disulfides between the catalytic Cys152 and GSH. In the case of hGAPDH, the mixed disulfide breaks down yielding Cys152-Cys156 disulfide bridge in the active site. In hGAPDH_C156S, the mixed disulfide is stable. Differential scanning calorimetry method showed that S-glutathionylation leads to destabilization of hGAPDH molecule, but does not affect significantly hGAPDH_C156S. Reactivation of S-glutathionylated hGAPDH in the presence of GSH and glutaredoxin 1 is approximately two-fold more efficient compared to that of hGAPDH_C156S.

Conclusions: S-glutathionylation induces the formation of Cys152-Cys156 disulfide bond in the active site of hGAPDH, which results in structural changes of the protein molecule. Cys156 is important for reactivation of S-glutathionylated GAPDH by glutaredoxin 1.

General significance: The described mechanism may be important for interaction between GAPDH and other proteins and ligands, involved in cell signaling.

1. Introduction

S-glutathionylation is a posttranslational modification of proteins at cysteine residues, resulting in the formation of a mixed disulfide (Protein-S-SG) between a cysteine residue of a protein and the cysteine residue of glutathione. Currently, it has been demonstrated that many proteins are capable of being S-glutathionylated [1–3]. This reaction proceeds spontaneously, as well as it can be catalyzed by enzymes [4]. Spontaneous modification with reduced glutathione (GSH) has been demonstrated for proteins containing cysteine residues subjected to oxidation (hemoglobin, beta-actin, tyrosine phosphatase, peroxiredoxin 2, and glyceraldehyde-3-phosphate dehydrogenase) [5–10]. In this case, GSH reacts with sulfenic acid derivatives of the cysteine residues resulting in the mixed disulfide, which prevents the formation of irreversible oxidation products (sulfinates and sulfonates). Consequently, one of the function of this modification is the protection of proteins

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hGAPDH, human recombinant glyceraldehyde-3-phosphate dehydrogenase; hGAPDH_C156S, human recombinant glyceraldehyde-3-phosphate dehydrogenase with C156S mutation; GSH, reduced glutathione; Grx1, glutaredoxin 1; Trx1, thioredoxin 1; TR, thioredoxin reductase

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from the irreversible oxidation. At the same time, S-glutathionylation of various proteins and enzymes (carbonic anhydrase III, PTP1B, STAT3, c-jun, NF-kB, caspase 3, and others) can change their properties [11]. The redox sensing and ROS forming properties of pyruvate dehydrogenase, oxoglutarate dehydrogenase, and complex I were shown to be modulated by S-glutathionylation [12–14]. Deglutathionylation of proteins can take place spontaneously at a high GSH/GSSG ratio, or catalyzed by glutaredoxins [15–17] as well as by thioredoxins [18–20]. Diversity of proteins undergoing S-glutathionylation and the reversibility of this modification suggest that it can be involved in regulation of different cell functions depending on the redox state of the cell. For this reason, S-glutathionylation attracts the attention of researchers.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme composed of four identical subunits, each containing an active site with two cysteine residues. One of them (Cys150 in rabbit or Cys152 in human GAPDH) takes part in catalysis, being involved in the formation of the intermediate covalent product with the substrate (glyceraldehyde-3-phosphate). The second cysteine (Cys154 in rabbit or Cys156 in human GAPDH) is not involved in catalysis, but it is highly conserved [21]. However, its function is still unclear. The catalytic cysteine residue easily oxidizes in the presence of relatively low H_2O_2 concentrations, which results in the inactivation of the enzyme [22,23]. As was mentioned above, proteins containing cysteine residues that are sensitive to oxidation are readily subjected to S-glutathionylation. This is confirmed by the fact that S-glutathionylated GAPDH was found in plant and animal tissues [1,24]. In our previous work, we demonstrated that rabbit muscle GAPDH is S-glutathionylated in the presence of H_2O_2 and reduced glutathione (GSH). As was shown by MALDI-TOF MS analysis, together with the mixed disulfide GAPDH-SSG, S-glutathionylation resulted in GAPDH species with the intrasubunit disulfide bridge between the catalytic Cys150 and the adjacent Cys154 [10]. The modification resulted in the inactivation of the enzyme. In contrast to the oxidized GAPDH, S-glutathionylated enzyme could be completely reactivated in the presence of dithiothreitol, or partially reactivated in the presence of GSH or thioredoxin/thioreductase system. The conclusion was made that the formation of the mixed disulfide with GSH at the catalytic cysteine residue together with the intramolecular disulfide bridge protect GAPDH from irreversible inactivation by H_2O_2, which allows a reversible inhibition of glycolysis and suggests the activation of pentose-phosphate pathway in response to oxidation stress. However, given that the formation of the mixed disulfide with GSH is sufficient to protect the protein against irreversible oxidation, the physiological relevance of the disulfide bridge in the active site of GAPDH during S-glutathionylation remained unclear. It should be noted that the CTTNC motif in the active site of GAPDH is conserved in the vast majority of species, and it is very likely that the formation of the disulfide bridge between two active site cysteines during S-glutathionylation should have some biological sense. The following assumptions are possible: 1) the disulfide bridge provides more efficient protection of GAPDH from the irreversible inactivation than the mixed disulfide GAPDH-SSG; 2) formation of the disulfide bridge changes the geometry of the active site, which facilitates the access of GSH or specific proteins (glutaredoxin or thioredoxin) to the active site, providing more efficient reduction of the enzyme; 3) since GAPDH in the animal cell is known to be involved in a number of non-glycolytic functions [25], the formation of the disulfide bridge in the active site of GAPDH may change the tertiary structure of the protein and influence the interaction of GAPDH with other proteins and ligands (for example, nucleic acids), which could be of importance for redox signaling. To understand the role of the disulfide bridge in the active site of GAPDH, it was necessary to compare the properties of two S-glutathionylated enzymes: wild-type GAPDH and GAPDH with the mutation C156S excluding the formation of the intrasubunit disulfide bridge. The goal of the present work was to obtain human recombinant wild-type GAPDH (hGAPDH) and GAPDH with C156S substitution (hGAPDH_C156S), to compare structural changes caused by S-glutathionylation of these proteins, and to study the ability of the modified proteins to reactivate in the presence of GSH, glutaredoxin and thioredoxin.

2. Materials and methods

2.1. Chemicals and enzymes

In the work we used the following chemicals: dithiothreitol (Amresco); EDTA, and glycine (MP Biomedicals); glyceraldehyde-3-phosphate diethyl acetal (barium salt), glutathione reduced, NAD^+, and thioredoxin reductase from rat liver (Sigma-Aldrich); phenylmethylsulfonyl fluoride (PMSF) (BioChemica).

Recombinant human thioredoxin 1 was from Abcam: specific activity of > 150 A650/cm/min/mg (by measuring the increase of insulin precipitation (A650) resulting from the reduction of insulin); recombinant human glutaredoxin 1 (ACROBiosystems).

Untagged human recombinant wild-type GAPDH (hGAPDH) was obtained as described previously [26].

2.2. Construction of pEThGAPDH_C156S plasmid

The pEThGAPDH plasmid encoding untagged human GAPDH [26] was used as DNA template. The mutation C156S was generated using Quick-Change site directed mutagenesis kit (Agilent, USA, Cat # 200518) according to the instructions of the manufacturer. Two complementary synthetic oligonucleotides – mC156SUp (5′-GCCTCCTGCA CCACCAACAGCTTAGCACCCCTGGCCAAG) and mC156SDn (5′CTTGG CCAGGGGTGCTAAGCTGTTGGTGGTGCAGGAGGC) – were used as mutagenic primers. The resulting pEThGAPDH_C156S plasmid was used to transform electrocompetent DH10B cells prepared according to a rapid protocol [27]. Plasmid DNA was purified from Amp-resistant transformants and sequenced with T7 prom (5′-AATTAATACGACTCA CTATAGGG) and T7 term (5′-ATGCTAGTTATTGCTCAGCGGTGG) primers. Plasmid DNA from selected mutant clone without extraneous mutations was used to transform BL21 (DE3) *E. coli* cells (Novagen, USA).

2.3. Cultivation of the producer strains

The cells were cultivated according to the procedure described for autoinduction [28]. One clone of the transformed cells was transferred from a Petri dish to a flask with 2 mL of ZY medium containing 0.8% glucose, ampicillin (100 μg/mL) and chloramphenicol (30 μg/mL), and the cells were grown overnight at 37 °C. The night culture was inoculated (by 200 μL) into 1-L flasks containing 200 mL of ZYP-5052 medium and ampicillin (100 μg/mL). The culture was grown for 20 h at 28 °C under shaking. 1.2 L of the culture resulted in 12–14 g of cell biomass.

2.4. Purification of hGAPDH_C156S

The untagged protein hGAPDH_C156S was purified from *E. coli* cells by ammonium sulfate fractionation, as it was described earlier for untagged hGAPDH [26]. Briefly, the cell pellet (7 g) was suspended in 35 mL of working buffer (10 mM phosphate-buffered saline, 2 mM dithiothreitol, pH 7.4) containing 0.5 mM NAD^+ and 0.5 mM PMSF. The cells were broken by sonication on ice (8 times for 15 s with 30 s interval at 30% amplitude) using a Branson ultrasonic digital sonifier. The suspension was centrifuged (17,000 *g*, 15 min, 4 °C) to remove broken cells. The resulting cell extract was gradually saturated with ammonium sulfate, collecting the fractions of 0–50%, 50–70%, and 70–80% saturation by centrifugation. The fraction of 70–80% saturation was used for crystallization of hGAPDH_C156S. For this purpose, the protein pellet was dissolved in working buffer so that the protein concentration constituted approximately 2 mg/mL (by Bradford's method) and centrifuged (15,000 *g*, 10 min, 4 °C) to remove

undissolved particles (denatured proteins). A beaker with the solution was placed in an ice bath, and finely ground ammonium sulfate was added by small portions to the solution with constant gentle stirring until turbidity develops (approximately 0.4 g ammonium sulfate per mL of the solution). The pH value of the solution was adjusted to 8.0 with 10% ammonia solution, and it was left at 4 °C for 1–2 days. The resulting suspension was centrifuged, and the crystallization procedure was repeated as described above. In contrast to wild-type hGAPDH, the step of G-100 Sephadex chromatography was not necessary in the case of hGAPDH_C156S, since two crystallizations resulted in pure protein.

The protein was stored as ammonium sulfate suspension at 4 °C. To reduce the oxidized sulfhydryl groups, before each experiment, an aliquot of the suspension was centrifuged, and the protein pellet was dissolved in 10 mM potassium phosphate, 2 mM dithiothreitol, pH 7.4. After 1-h incubation at room temperature, the protein solution was transferred to a required buffer solution using dialysis or gel-filtration on a Sephadex G-50.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The purity of the enzyme preparations was analyzed by SDS-PAGE in 12% polyacrylamide gel according to the standard procedure.

2.6. Protein concentration

Protein concentration in extracts, protein fractions, and in solutions of purified recombinant hGAPDH and hGAPDH_C156S was determined by the Bradford's assay: the Bradford reagent (1 mL) was mixed with 1–2 μL of a tested solution, and after 1 min, the absorption of the sample was determined at 595 nm. The calibration plot was created using a solution of rabbit muscle GAPDH, whose concentration was determined spectrophotometrically at 280 nm ($A_{280}^{0.1\%}$ = 1.07).

2.7. Assay for the dehydrogenase activity of GAPDH

The dehydrogenase activity of GAPDH was monitored using a Shimadzu UV-1800 spectrophotometer as the initial rate of NADH accumulation (340 nm) during the oxidation of glyceraldehyde-3-phosphate (GA-3-P) at 22 °C. Assay mixture (1 mL) contained 50 mM glycine, 50 mM potassium phosphate, pH 8.9, 1 mM EDTA, 0.5 mM NAD^+, 1 mM GA-3-P, and 1–4 μg of the native or S-glutathionylated GAPDH. The reaction was started by the addition of GA-3-P to the assay mixture, and the increase in the absorption was monitored for 1 min. The activity was calculated from the linear part of the curve, which corresponded to the first 15 s after the addition of GA-3-P. The specific activity of the enzyme was determined as the value of the reaction rate (μmol NADH/min) divided by the amount (mg) of the enzyme in the sample.

2.8. Concentration of hydrogen peroxide

Concentration of hydrogen peroxide was determined spectrophotometrically at 230 nm using molar absorption coefficient of 72.7 M^{-1} cm^{-1}.

2.9. Oxidation of hGAPDH and hGAPDH_C156S in the presence of H_2O_2

A protein solution (0.5 mg/mL in 10 mM potassium-phosphate buffer, pH 7.5) was divided into three samples, and hydrogen peroxide was added to the samples to final concentrations of 0.2, 0.4, 0.8 mM or 1.2 mM. To monitor the protein oxidation, aliquots (2–10 μL) were taken from the samples during the incubation to determine the dehydrogenase activity as described in section 2.7. The experimental points of each inactivation curve were fitted to the exponential decay equation:

$$A = A_0 \cdot e^{-kt}$$

where A_0 is the original enzyme activity, k is the pseudo first order oxidation rate constant, and t is the incubation time. The k values were determined by the non-linear regression method using SigmaPlot 12.5 software. Then the k values were plotted against corresponding H_2O_2 concentrations yielding a straight line with a slope that corresponded to the value of the second order rate oxidation constant K ($M^{-1} \cdot s^{-1}$).

2.10. S-glutathionylation of hGAPDH and hGAPDH_C156S

To a solution of hGAPDH (0.5 mg/mL in 10 mM potassium-phosphate buffer, 1 mM EDTA, pH 7.5), reduced glutathione was added to a final concentration of 0.1 mM, and then hydrogen peroxide was added (final concentration, 0.1 mM). In the case of hGAPDH_C156S, since the mutant protein was more resistant to oxidation, the concentration of GSH and H_2O_2 was 0.2 mM to ensure the same level of inactivation of both proteins. During the incubation, aliquots of 2–5 μL were taken from the solution to determine the dehydrogenase activity of GAPDH as described in section 2.7.

2.11. MALDI-TOF MS analysis

MALDI-TOF MS analysis was performed on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with an Nd laser by detection of MH+ molecular ions. Before experiments, protein samples (native and S-glutathionylated) were dialyzed against bidistilled water, and the resulting solutions (0.2 mg/mL) or their tryptic digests were analyzed. To get a tryptic map, 50 μL of a protein sample were mixed with 10 μL of modified trypsin (15 μg/mL, Promega) in 0.05 M NH_4HCO_3, and the solution was incubated for 50 min at 37 °C. The proteolysis was stopped by addition of 10 μL of 0.5% trifluoroacetic acid (TFA). An aliquot of 0.5 μL was mixed with 1 μL of 2,5-dihydroxybenzoic acid solution (20 mg/mL in 30% acetonitrile, 0.5% TFA). The intact proteins were measured in the linear mode; the accuracy of average mass peak measurement was within 20 Da. The spectra of tryptic digests were recorded in reflector mode, and the accuracy of monoisotopic mass peak measurement was within 30 ppm. Mass-spectra were processed with the use of FlexAnalysis 3.3 software (Bruker Daltonics, Germany). Proteins were identified using the NCBI database and Mascot peptide mass fingerprint search program (www.matrixscience.com).

2.12. Reactivation of oxidized and S-glutathionylated hGAPDH and hGAPDH_C156S

For oxidation, solutions of hGAPDH and hGAPDH_C156S (0.5 mg/mL) in 10 mM potassium-phosphate buffer, 1 mM EDTA, pH 7.5, were incubated in the presence of 0.1 mM or 0.2 mM H_2O_2, respectively, to achieve the similar level of residual enzymatic activity (2–4%).

For S-glutathionylation, the proteins were treated as described in section 2.10. When the enzymatic activity reached 2–4% of the original value, each sample was divided into 4 portions of 30 μL to assay the reactivation in the presence of 5 mM GSH (1), 5 mM GSH and 0.8 μg (2.1 μM) Grx1 (2), 0.24 mM NADPH, 1 μg (2.8 μM) Trx1, and 0.74 μg (1 μM) TR (3), and without additions (4). To monitor the reactivation, the dehydrogenase activity in the samples 1–4 was determined before reactivation and after 24-h incubation in the presence of indicated components at 22 °C.

2.13. Denaturation of proteins in guanidine hydrochloride

Unfolding of the proteins in guanidine hydrochloride (GdnHCl) was monitored by the changes in the tryptophan fluorescence. Protein samples (0.1 mg/mL in 10 mM potassium phosphate, pH 7.0) were incubated in the presence of 0–3 M GdnHCl at 22 °C for 1 h. After the

incubation, the emission spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Scientific) in a 0.3 cm path length cuvette (excitation wavelength was 295 nm, emission was recorded in the range of 300–400 nm). The unfolding of proteins resulted in a shift of the emission peak from 335 to 355 nm due to the change in the microenvironment of tryptophan residues, making the ratio of fluorescence intensities at these wavelengths a good indication of the protein state [29]. The obtained unfolding curves (ratio of 355/335 nm fluorescence intensities vs GdnHCl concentration) were fitted to a four parameters logistic function to derive the concentration of GdnHCl at the curve midpoint. Fitting was performed with the aid of SigmaPlot software (version 11, Systat Software).

2.14. Differential scanning calorimetry (DSC)

Microcalorimetric measurements were carried out on a VP-DSC microcalorimeter (MicroCal, USA) at a heating rate of 1 K/min for 0.25 mg/mL protein solutions as described elsewhere [30]. Before experiments, native and S-glutathionylated proteins were dialyzed against PBS, pH 7.0. The protein solutions were diluted with the same buffer to reach the concentration of 0.25 mg/mL (1.7 μM). Curves were corrected for the instrumental baseline obtained by heating the buffer solution used for protein dilution. To analyze functions of excess heat capacity, the Origin-DSC software package was used. Computer deconvolution of melting curves was performed under the assumption that a melting curve is an algebraic sum of peaks corresponding to cooperative one-step transitions. The accuracy of the values of calorimetric enthalpy (ΔH) and thermal transition midpoint (T_m) was ± 10% and ± 0.2 °C, respectively.

2.15. Statistical analysis

The non-parametric Mann–Whitney–Wilcoxon test (*U* test) was used to identify the differences between two sets with quantitative variables. The difference between the groups was considered as statistically significant at $p < .05$. All statistics were performed with the aid of SigmaPlot software (version 11, Systat Software).

3. Results

3.1. Isolation of hGAPDH and hGAPDH_C156S

We intentionally used tag-free constructs, since the presence of tags hampers protein folding and promotes isolation of misfolded proteins with low enzymatic activity. Human recombinant proteins hGAPDH and hGAPDH_C156S were isolated from *E. coli* producer strains using ammonium sulfate fractionation (Fig. 1, A and B). In the case of hGAPDH (Fig. 1 A), an additional step of G-100 Sephadex chromatography was necessary. 1 L of the cell culture resulted in 3–4 mg of hGAPDH and 5–7 mg of hGAPDH_C156S. The specific activity of the freshly purified hGAPDH and hGAPDH_C156S constituted 118 ± 5 and 102 ± 5 μmol NADH/min per mg protein at 25 °C, respectively.

3.2. Oxidation and S-glutathionylation of hGAPDH and hGAPDH_C156S

Previously, we demonstrated that S-glutathionylation of rabbit muscle GAPDH in the presence of GSH and H_2O_2 was induced by oxidation of the catalytic cysteine residue to cysteine sulfenic acid [10]. Consequently, sensitivity of GAPDH to oxidation is important for the efficiency of S-glutathionylation. For this reason, we investigated the sensitivity of hGAPDH and hGAPDH_C156S towards H_2O_2.

In the active site of GAPDH, the catalytic cysteine residue (Cys152 in human) forms an ion pair with His179, which lowers the pK value of the SH-group and facilitates the nucleophilic attack of glyceraldehyde-3-phosphate by the thiolate-anion [31]. For this reason, Cys152 possesses enhanced reactivity compared to other cysteine residues and readily oxidizes in the presence of relatively low H_2O_2 concentrations at physiological pH values. Besides, recently it has been suggested that the adjacent Cys156 additionally increases the reactivity of Cys152 towards H_2O_2 due to the formation of a net of hydrogen bonds (proton relay) that also involves conserved Thr153 and Tyr314 [32]. These factors explain an enhanced sensitivity of Cys152 to sulfhydryl reagents and oxidants.

Enzymatic activity of GAPDH is proportional to the content of the catalytic cysteines in the reduced (-SH) form. Consequently, inactivation curves reflect the decrease in the content of the catalytic SH-groups due to their oxidation with the formation of SOH-groups, which then oxidize further to form SO_2H and SO_3H derivatives (Scheme 1, reactions 1–3). It is important that the active site cysteines tend to form a disulfide bond (Scheme 1, reaction 4). Redox proteomic analysis demonstrated the formation of the disulfide bond between two cysteine residues in the active site of GAPDH in exponentially growing *E. coli* [33]. The intrasubunit disulfide bond was also detected in rabbit muscle GAPDH by ESI-MS analysis after the treatment of the enzyme with Andgeli's salt ($Na_2N_2O_3$) that is known as HNO donor [34]. Presumably, oxidation of the catalytic cysteine residue to cysteine sulfenic acid (spontaneous or in the presence of oxidants) promotes the formation of the disulfide bond in some part of the active sites of GAPDH according to mechanisms of cysteine oxidation in proteins [23,35,36].

Fig. 2 (A and B) shows inactivation of hGAPDH and hGAPDH_C156S in the presence of different concentrations of H_2O_2. As seen from Fig. 2, the wild-type protein hGAPDH is more sensitive to oxidation by H_2O_2 (A) compared to hGAPDH_C156S (B).

The pseudo first order oxidation rate constants (k, min^{-1}) observed at different H_2O_2 concentrations were calculated from the inactivation

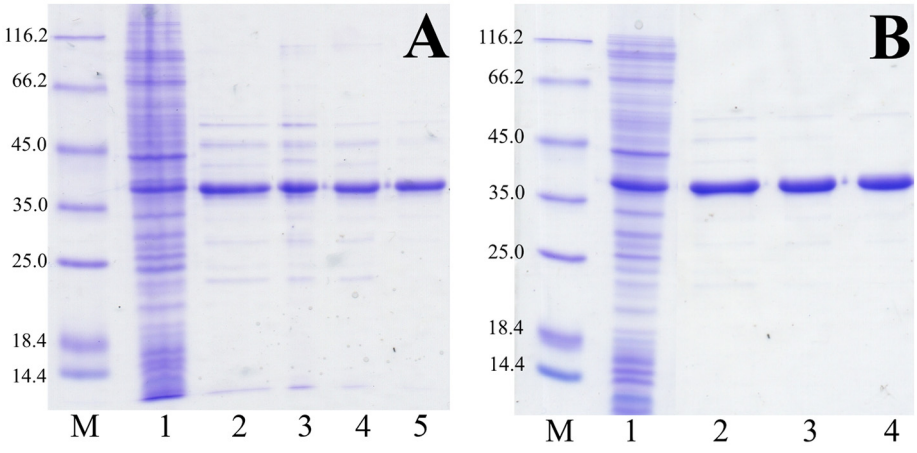
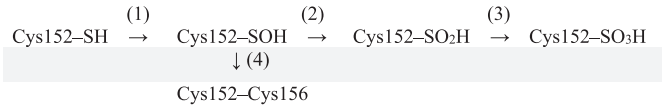


Fig. 1. Purification of hGAPDH (A) and hGAPDH_C156S (B) from *E. coli* producer strains.
A) 1, original extract; 2, fraction of 70–80% saturation with ammonium sulfate; 3 and 4, crystallization and recrystallization; 5, after gel-filtration on a G-100 Sephadex column.
B) 1, original extract; 2, fraction of 70–80% saturation with ammonium sulfate; 3 and 4, crystallization and recrystallization.



Scheme 1. Oxidation of cysteine residues in the active site of human GAPDH in the presence of H_2O_2.

curves as described in section 2.9. The value of the second order rate constant for the oxidation of hGAPDH by H_2O_2 was determined as the slope of the straight line plotted as the dependence of k versus H_2O_2 concentration (Fig. 2C, *1*) and constituted 11.0 ± 0.4 $M^{-1}{\cdot}s^{-1}$. This agrees with the value obtained previously for rabbit muscle GAPDH (10 ± 1 $M^{-1}{\cdot}s^{-1}$) under similar conditions (potassium phosphate buffer, pH 7.5, 22 °C) [37]. For hGAPDH_C156S, the second order rate constant was found to be 4.9 ± 0.1 $M^{-1}{\cdot}s^{-1}$ (Fig. 2C, *2*). Consequently, the mutation C156S decreases the rate of hGAPDH oxidation by H_2O_2 by approximately twofold. These results agree with the idea that Cys156 increases the sensitivity of Cys152 towards H_2O_2 [32].

Since hGAPDH_C156S was more resistant to oxidation, to obtain S-glutathionylated hGAPDH_C156S with the same residual activity as in the case of S-glutathionylated hGAPDH (2–4% of the original value), we had to increase concentration of H_2O_2 by twofold compared to that used for S-glutathionylation of hGAPDH (Fig. 3, A and B, respectively). The concentration of GSH was also increased so that the GSH/H_2O_2 ratio was the same as in the case of hGAPDH.

As seen from Fig. 3 A and B, the presence of GSH does not affect significantly the rate of inactivation of the proteins hGAPDH and hGAPDH_C156S by H_2O_2 (curves *2* and *3*). To evaluate the content of reversibly oxidized Cys152, the enzymes oxidized by H_2O_2 in the absence and in the presence of GSH (residual activity of 2–4%) were incubated with 5 mM dithiothreitol (DTT) for 1 h, and then the enzymatic activity was determined. As seen in Fig. 3C (left bars), both proteins oxidized by H_2O_2 contained 13–17% of reversibly oxidized catalytic cysteines. In the case of oxidation in the presence of GSH (Fig. 3C, right bars), the content of reversible products was significantly higher and constituted 68% in both hGAPDH and hGAPDH_C156S. These data point to a formation of S-glutathionylated proteins in the case of their oxidation in the presence of GSH.

3.3. MALDI-TOF MS analysis

Previously, we showed that S-glutathionylation of rabbit muscle GAPDH resulted in the formation of the mixed disulfide between the catalytic Cys150 and GSH, but the content of GAPDH-SSG was relatively low to explain the inactivation of the protein. Moreover, after trypsinolysis of the protein, GSH moiety was not revealed in the peptide containing the active site of GAPDH. Instead, Cys150-Cys154 disulfide bond was observed. These data allowed us to assume that the presence of Cys154 in the proximity to the mixed disulfide Cys150-SSG leads to its breakdown with the formation of the C150-C154 disulfide bridge [10]:

$$Cys150 - S - SG + Cys154 - SH \rightarrow Cys150 - S - S - Cys154 + GSH$$

If this assumption is true, a mutation of the non-catalytic cysteine residue (Cys156 in human GAPDH) must increase the stability of GAPDH-SSG. To test this, the products of S-glutathionylation of hGAPDH and hGAPDH_C156S were investigated.

Fig. 4 A shows the spectra of hGAPDH without additions (top panel) and after the oxidation in the presence of GSH (bottom panel). The major peak with m/z = 35,951 (Fig. 4A, top panel) corresponds to a subunit of hGAPDH (calculated molecular mass, 36,053 Da). Incubation of hGAPDH in the presence of H_2O_2 and GSH results in the appearance of a new peak with m/z = 36,257, corresponding to the addition of one GSH moiety (Fig. 4A, bottom panel). Fig. 4B shows the spectra of the mutant protein hGAPDH_C156S, without modifications (top panel) and after the incubation of the protein in the presence of H_2O_2 and GSH (bottom panel). The major peak with m/z = 35,924 in Fig. 4B, top panel corresponds to a subunit of native hGAPDH_C156S (calculated molecular mass, 36,037 Da). Incubation of hGAPDH_C156S in the presence of H_2O_2 and GSH results in a significant decrease in the peak of the native subunit and emergence of a new peak with m/z = 36,235 (Fig. 4B, bottom panel) that corresponds to a subunit containing one GSH moiety.

Since oxidation in the presence of GSH resulted in the inactivation of both wild-type and mutant GAPDH, and the activity can be restored by addition of DTT, we expected that the mixed disulfide is formed at the catalytic Cys152 residue. However, analysis of the trypsin hydrolysate of S-glutathionylated hGAPDH did not reveal incorporation of GSH into the peptide of the active site (Fig. 5A).

As seen from Fig. 5A (bottom panel), we do not observe any peaks in the spectrum that could correspond to modification of the peptide 146–162 (m/z = 1719) with GSH. However, GSH incorporates into the peptide 235–260 (m/z = 2902). This points to the modification of Cys247 that is outside the active site and is not involved in catalysis. Therefore, this modification cannot be associated with either inactivation of hGAPDH or its protection against inactivation. As seen from the presented spectra (Fig. 4 A and B, bottom panels), most part of the modified proteins hGAPDH and hGAPDH_C156S contains one glutathione residue per subunit (peaks with m/z of 36,257 and 36,235, respectively). The protection of the proteins hGAPDH and hGAPDH_156S from irreversible oxidation observed in the presence of GSH (Fig. 3C) suggests that the catalytic Cys152 undergoes S-glutathionylation primarily as the most reactive residue, but the corresponding peptide with GSH was not detected in the tryptic hydrolysate.

In contrast, analysis of the tryptic hydrolysate of the S-glutathionylated hGAPDH_C156S revealed the presence of GSH moiety in the

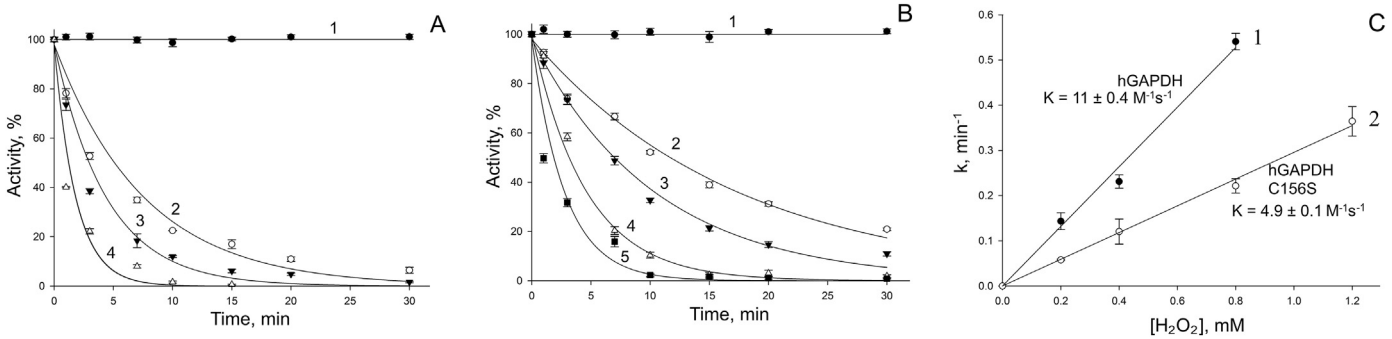


Fig. 2. Inactivation of hGAPDH (A) and hGAPDH_C156S (B) in the presence of H_2O_2. The samples contained 0.5 mg/mL of hGAPDH or hGAPDH_C156S in 10 mM potassium phosphate buffer, pH 7.5, without additions (1) or in the presence of 0.2, 0.4, 0.8, or 1.2 mM H_2O_2 (2–5, respectively). During the incubation, aliquots were taken from the samples to determine the dehydrogenase activity as described in section 2.7. C) Determination of second order rate constants for the oxidation of hGAPDH and hGAPDH_C156S by H_2O_2.

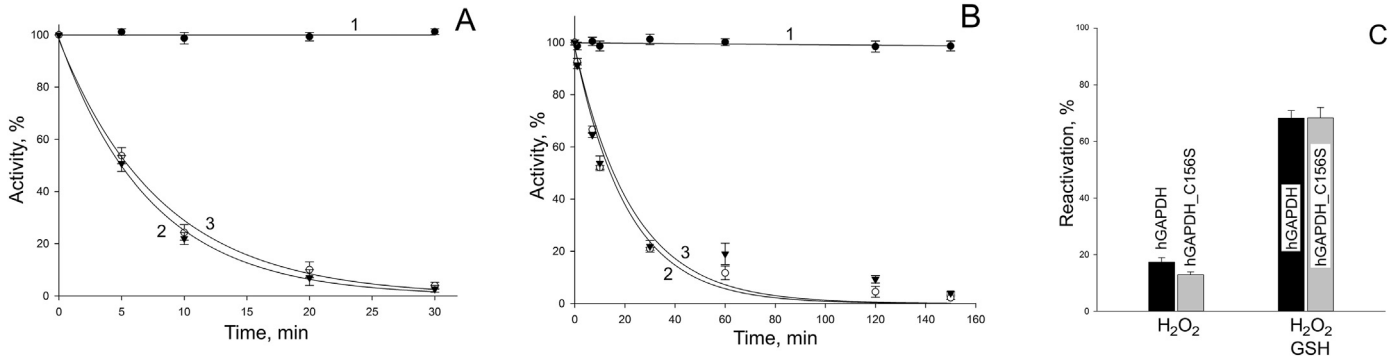


Fig. 3. Inactivation of hGAPDH and hGAPDH_C156S by H_2O_2 in the absence and in the presence of GSH. A) hGAPDH was incubated without additions (1); with 0.1 mM H_2O_2 (2) or with 0.1 mM GSH together with 0.1 mM H_2O_2 (3); B) hGAPDH_C156S was incubated without additions (1); with 0.2 mM H_2O_2 (2) or with 0.2 mM GSH together with 0.2 mM H_2O_2 (3). C) Reactivation of the proteins treated with H_2O_2 (left bars) and H_2O_2 with GSH (right bars) in the presence of 5 mM DTT: after inactivation, hGAPDH and hGAPDH_C156S were incubated in the presence of 5 mM DTT for 1 h at 22 °C, and then the enzymatic activity was determined (percentage of the original activity).

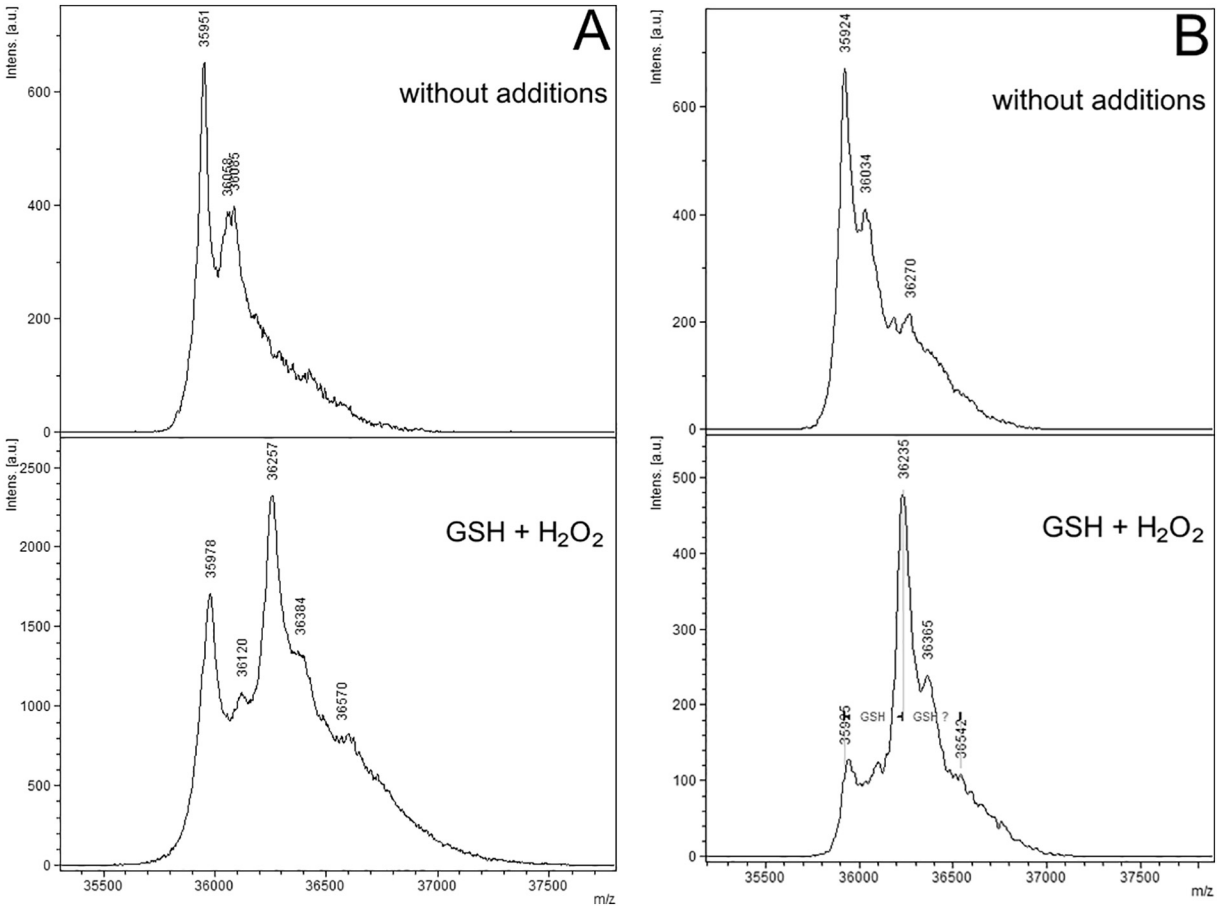


Fig. 4. MALDI-TOF MS analysis of hGAPDH (A) and hGAPDH_C156S (B). Solutions of hGAPDH and hGAPDH_C156S (1 mg/mL in 10 mM potassium phosphate, 1 mM EDTA, pH 7.5) were incubated for 1 h at 22 °C without additions (top panel) or in the presence of H_2O_2 and GSH (0.1 mM H_2O_2 + 0.1 mM GSH for hGAPDH and 0.2 mM H_2O_2 + 0.2 mM GSH for hGAPDH_C156S, bottom panel). Before analysis, the samples were dialyzed against bidistilled water for 4 h.

peptides 146–162 and 235–260 (Fig. 5B).

The peptide 146–162 includes catalytic cysteine residue Cys152, and the peptide 235–260 includes Cys247 that is not involved in catalysis (Fig. 6). Consequently, the mutation C156S prevents the breakdown of the mixed disulfide between the catalytic Cys152 and GSH.

Taken together, the obtained results suggest that the incubation of hGAPDH and hGAPDH_C156S in the presence of H_2O_2 with GSH results in the formation of the corresponding mixed disulfides with GSH (S-glutathionylation). The modification mostly affects the catalytic Cys152 residue. In hGAPDH, the mixed disulfide at Cys152 is unstable, since it cannot be revealed after hydrolysis of the S-glutathionylated protein with trypsine. Presumably, this may be due to the presence of nearby Cys156, which reacts with the mixed disulfide to form an intrasubunit disulfide bridge. This is confirmed by the fact that in hGAPDH_C156S, the mixed disulfide is stable and can be detected in the tryptic hydrolysate of the S-glutathionylated protein.

We cannot exclude that the formation of the disulfide bridge is promoted by the conditions of the experiment (37 °C and trypsin

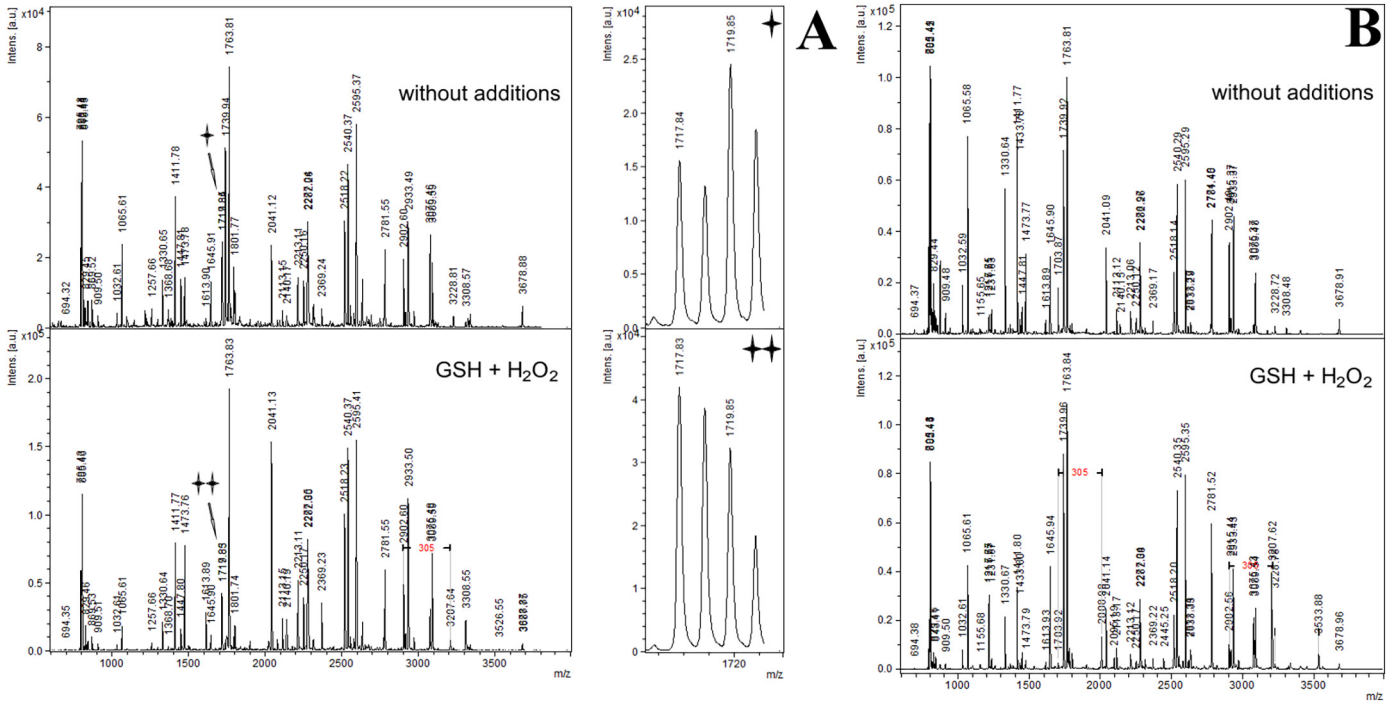


Fig. 5. A) MALDI-TOF MS spectra of tryptic hydrolysates of S-glutathionylated hGAPDH (A) and hGAPDH_C156S (B): proteins without additions (top panel) or after incubation in the presence of 0.1 mM H_2O_2 and 0.1 mM GSH (bottom panel).
A) Left panel: the peak with m/z = 2902 corresponds to the peptide 235–260. The peaks with m/z = 1717/1719 (indicated by arrow) correspond to the peptide 146–162 with disulfide bond or in the reduced form, respectively. Right panel: enlarged fragments of the spectra.
B) In hGAPDH_C156S, GSH is incorporated into the peptides 146–162 (m/z = 1703.87) and 235–260 (m/z = 2902.56) yielding the peptides with m/z of 2008.08 and 3207.62, respectively.

MGKVKVGVNG FGRIGRLVTR AAFNSGKVDI VAINDPFIDL NYMVYMFQYD 50
STHGKFHGTV KAENGKLVIN GNPITIFQER DPSKIKWGDA GAEYVVESTG 100
VFTTMEKAGA HLQGGAKRVI ISAPSADAPM FVMGVNHEKY DNSLKIISNA 150
SCTTNSLAPL AKVIHDNFGI VEGLMTTVHA ITATQKTVDG PSGKLWRDGR 200
GALQNIIPAS TGAAKAVGKV IPELNGKLTG MAFRVPTANV SVVDLTCRLE 250
KPAKYDDIKK VVKQASEGPL KGILGYTEHQ VVSSDFNSDT HSSTFDAGAG 300
IALNDHFVKL ISWYDNEFGY SNRVVDLMAH MASKE 330

Fig. 6. Amino acid sequence of hGAPDH_C156S. The catalytic cysteine (Cys152) is shown by arrow. Peptides 146–162 and 235–260 containing GSH moiety are highlighted.

cleavage), but we assume that the same process could occur in the S-glutathionylated hGAPDH without trypsine treatment at a lower rate. Considering that GAPDH is a tetrameric protein, different subunits may contain GAPDH-SSG or the intrasubunit disulfide bridge, and the relative content of these products may depend on the temperature and time of incubation without reducing agents.

3.4. Comparison of the properties of S-glutathionylated proteins hGAPDH and hGAPDH_C156S

To reveal the involvement of Cys156 in structural changes caused by S-glutathionylation, we compared the behavior of S-glutathionylated enzymes hGAPDH and hGAPDH_C156S using different methods.

3.4.1. Reactivation of S-glutathionylated hGAPDH and hGAPDH_C156S in the presence of GSH, glutaredoxin 1 and thioredoxin 1

Reactivation of oxidized and S-glutathionylated proteins was investigated in the presence of GSH and recombinant human proteins glutaredoxin 1 (Grx1) and thioredoxin 1 (Trx1). Results of the experiment are presented in Fig. 7.

In contrast to the reactivation by DTT, the efficiency of reactivation of hGAPDH and hGAPDH_C156S in the presence of GSH, Grx1 and Trx1 was different. The percentage of the reactivation of hGAPDH after oxidation as well as after S-glutathionylation was higher compared to that of hGAPDH_C156S (Fig. 7 A and B).

Fig. 7A shows reactivation of hGAPDH and hGAPDH_C156S after oxidation by H_2O_2. The content of the reversibly oxidized SH-groups in hGAPDH and hGAPDH_C156S was evaluated after the incubation with DTT and constituted 20 and 16%, respectively (Fig. 7A, bars 5). As seen in Fig. 7A, the percentage of reactivation of the oxidized hGAPDH in the presence of GSH and GSH/Grx1 (bars 2 and 4) more than 2-fold exceeds that of the oxidized hGAPDH_C156S. These experiments indicate that the presence of Cys156 is of importance for Grx1-catalyzed reactivation of the oxidized hGAPDH and for its spontaneous reactivation in the presence of GSH. In hGAPDH, the reversible oxidation products include Cys152–SOH (cysteine sulfenic acid) and Cys152-Cys156 disulfide bridge (see Scheme 1). In the case of hGAPDH_C156S, the only reversible product is Cys152–SOH, because Cys156 is replaced with a serine. Hence, a possible role of Cys156 may be the participation in the formation of Cys152-Cys156 disulfide bond according to Scheme 1.

According to the presented results, the oxidized hGAPDH containing Cys152-Cys156 disulfide bridge is better reactivated in the presence of GSH and GSH/Grx1 mixture compared to the oxidized hGAPDH_C156S containing Cys152–SOH.

As follows from the results described in sections 3.2 and 3.3, GSH protects hGAPDH from irreversible oxidation according to the Scheme 2:

According to Scheme 2, in hGAPDH, the expected products of S-glutathionylation include the mixed disulfide with GSH and the intrasubunit Cys152-Cys156 disulfide bridge. Since GAPDH is a tetrameric protein, different subunits may contain different products (mixed disulfide with GSH or intramolecular disulfide bond). In the mutant protein hGAPDH_C156S, the mixed disulfide is the only possible product.

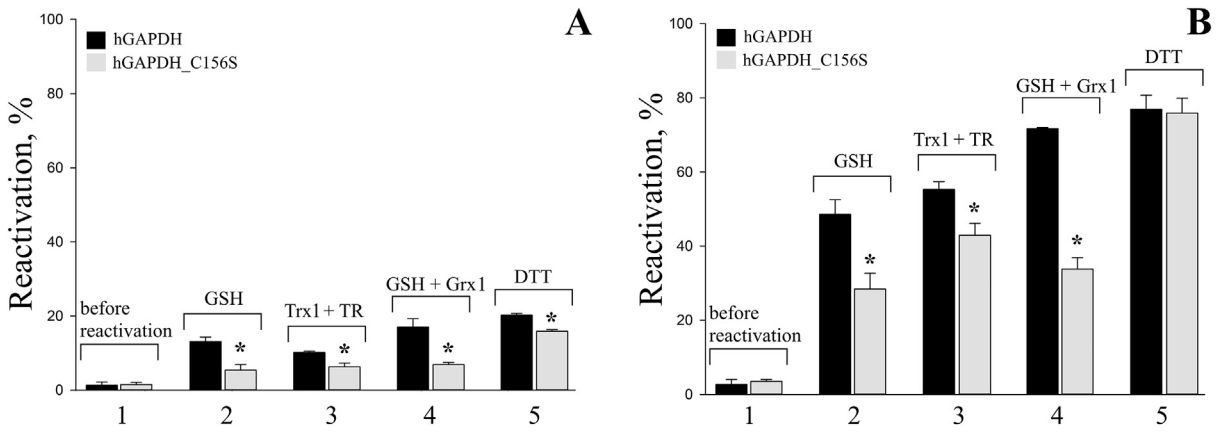


Fig. 7. Reactivation of hGAPDH and hGAPDH_C156S after oxidation (A) and after S-glutathionylation (B). hGAPDH and hGAPDH_C156S were inactivated in the presence of H_2O_2 alone (A) or in the presence of H_2O_2 with GSH (B) until the enzymatic activity reached 2–4% of the original value (A and B, bars 1). The inactivated proteins were reactivated by the incubation (24 h) in the presence of 5 mM GSH (A and B, bars 2), thioredoxin1/thioredoxin reductase mixture (A and B, bars 3), or 5 mM GSH and glutaredoxin 1 (A and B, bars 4). The content of reversibly modified catalytic cysteines was evaluated by the incubation (24 h) with 5 mM DTT (A and B, bars 5).
The data are presented as the mean values of three independent experiments ± SD.
*) Statistically significant difference ($p < .05$) according to Mann–Whitney–Wilcoxon test (U test).

Fig. 7B shows reactivation of hGAPDH and hGAPDH_C156S after S-glutathionylation in the presence of H_2O_2 and GSH. After incubation with DTT, both proteins are reactivated by 76% (Fig. 7B, bars 5). This suggests that both proteins contain 76% of reversibly oxidized cysteines. However, the efficiency of reactivation in the presence of GSH, GSH/Grx1 and Trx1/TR is different for hGAPDH and hGAPDH_C156S. The most pronounced difference is observed in the presence of GSH/Grx1: the percentage of reactivation constitutes 72 and 34% for hGAPDH and hGAPDH_C156S, respectively (Fig. 7B, bars 4). The less pronounced difference in the reactivation was observed in the presence of GSH alone (48 and 28%, respectively) (Fig. 7B, bars 2) and in the presence of Trx1/TR (55 and 43% for hGAPDH and hGAPDH_C156S, respectively) (Fig. 7B, bars 3). Consequently, according to Scheme 2, the mutant protein containing mixed disulfide with GSH is reduced by Grx1 or by GSH with lower efficiency compared to the wild-type protein containing C152–C156 disulfide bridge. Besides, in the case of hGAPDH_C156S, the efficiency of the reactivation in the presence of GSH is close to that in the presence of GSH/Grx1 (28 ± 4 and 34 ± 3%, respectively) (Fig. 7B, gray bars 2 and 4). Therefore, Grx1 is inefficient in the case of hGAPDH_C156S. In contrast, in the case of hGAPDH, the extent of reactivation constitutes 48% in the presence of GSH alone and 72% in the presence of glutaredoxin together with GSH (Fig. 7B, black bars 2 and 4), indicating that Grx1 increases the percentage of reactivation by 1.5-fold. Therefore, Grx1 predominantly reactivates hGAPDH and has a low efficiency in the case of hGAPDH_C156S. These data show that Cys156 is important for the reactivation of S-glutathionylated GAPDH. As a possible explanation, we may assume that the formation of C152-C156 disulfide bridge results in structural changes in the active site of hGAPDH that provide an efficient access of GSH and Grx1. However, we cannot exclude other mechanisms. For example, Cys156 may provide correct orientation of GSH or Grx in the active site of hGAPDH.

3.4.2. Unfolding of S-glutathionylated hGAPDH and hGAPDH_C156S in the presence of guanidine hydrochloride

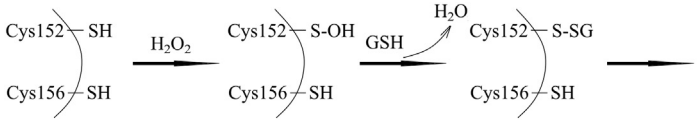
To evaluate the stability of the investigated proteins towards guanidine hydrochloride (GdnHCl), we determined the parameter $[GdnHCl]_{50}$, which corresponds to the GdnHCl concentration that is necessary to reach denaturation of a half of a protein. The content of the denatured protein after incubation with GdnHCl was estimated by the shift of the fluorescence emission maximum from 335 to 355 nm (see Section 2.13 for details).

Fig. 8 presents unfolding curves obtained for hGAPDH (A) and hGAPDH_C156S (B). The original spectra are given in the supplements (Supplementary Fig 1 and Supplementary Fig 2). As can be seen from Table 1, S-glutathionylation does not affect the stability of hGAPDH and hGAPDH_C156S, since the values of $[GdnHCl]_{50}$ do not change significantly after S-glutathionylation of the proteins. Thus, this method did not reveal significant difference between two S-glutathionylated proteins. However, these experiments show that S-glutathionylation does not lead to unfolding of the proteins, since the starting points (in the absence of GdnHCl) are close in the case of the native and modified proteins (Fig. 8, A and B).

3.4.3. Investigation of thermal stability of proteins hGAPDH and hGAPDH_C156S by differential scanning calorimetry

Differential scanning calorimetry (DSC) method was used to reveal structural changes of the proteins caused by S-glutathionylation. Fig. 9 shows heat absorption curves for the native hGAPDH and hGAPDH_C156S (1 and 3, respectively). Heat absorption curves with peaks are typical for globular proteins and reflect the process of protein unfolding with growing temperature. The T_m value (thermal transition midpoint) corresponds to the position of the maximum of the curve and reflects the temperature at which the content of a denatured protein is equal to the content of non-denatured protein. The ΔH value (calorimetric enthalpy) corresponds to the area under the peak. It is the energy required to denature a protein [38].

It is seen that S-glutathionylation of hGAPDH leads to a significant decrease in the ΔH value (Fig. 9, curves 1 and 2, Table 2). This suggests that the S-glutathionylated hGAPDH requires less energy for its unfolding compared to the native protein. Consequently, S-glutathionylation of hGAPDH weakens the interactions between amino acid



Scheme 2. Oxidation of cysteine residues in the active site of human GAPDH in the presence of H_2O_2 and GSH. One of four GAPDH subunits is shown.

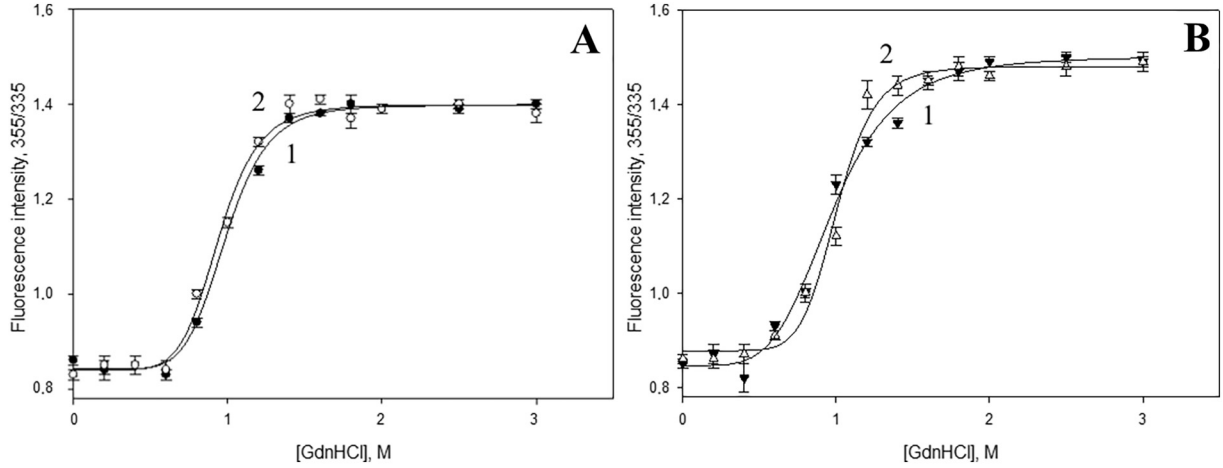


Fig. 8. Unfolding of hGAPDH (A) and hGAPDH_C156S (B) in the presence of GdnHCl: 1) native protein; 2) S-glutathionylated protein. Protein solutions (0.1 mg/mL) were incubated in the presence of different concentration of GdnHCl, and then the fluorescence emission spectra were recorded in the range of 300–400 nm (excitation at 295 nm).

Table 1
Resistance of proteins hGAPDH and hGAPDH_C156S towards denaturation in GdnHCl.

	$[GdnHCl]_{50}$, M	
	Native protein	S-glutathionylated protein
hGAPDH	0.99 ± 0.02	0.94 ± 0.02
hGAPDH_C156S	0.98 ± 0.03	1.0 ± 0.02

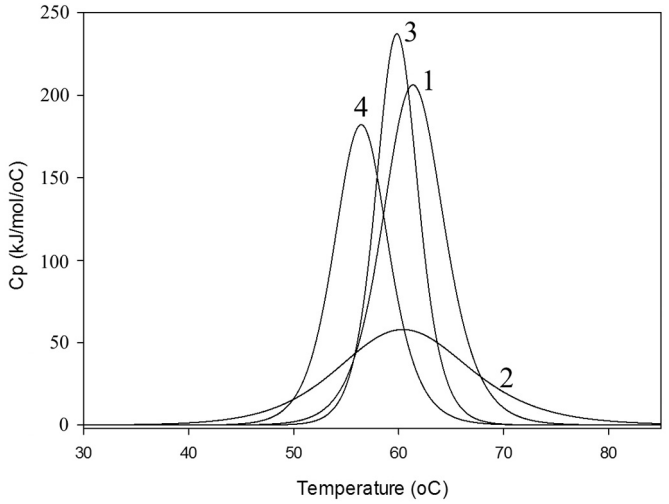


Fig. 9. Heat absorption curves for hGAPDH and hGAPDH_C156S: 1) native hGAPDH; 2) S-glutathionylated hGAPDH; 3) native hGAPDH_C156S; 4) S-glutathionylated hGAPDH_C156S.

Table 2
Parameters calculated from heat absorption curves (Fig. 9) for the native and S-glutathionylated proteins hGAPDH and hGAPDH_C156S.

Protein sample		Tm, °C	ΔH, kJ/mol
1	hGAPDH, native	61.4 ± 0.2	1612 ± 160
2	hGAPDH (S-glut.)	60.6 ± 0.2	971 ± 97
3	hGAPDH_C156S, native	59.8 ± 0.2	1280 ± 128
4	hGAPDH_C156S (S-glut.)	56.4 ± 0.2	1261 ± 126

residues within the protein globule that are important for maintaining the globular structure. In contrast, S-glutathionylation of hGAPDH_C156S affects ΔH value to a much lesser extent (Fig. 9, curves 3 and 4, Table 2).

Significant changes in the heat absorption profile of hGAPDH after its S-glutathionylation (Fig. 9, curves 1 and 2) indicate that the modification results in pronounced structural rearrangements in the hGAPDH molecule. The formation of a stable mixed disulfide (GAPDH-SSG) as in the case of S-glutathionylation of hGAPDH_C156S affects the character of the heat absorption profile to a much less extent (Fig. 9, curves 3 and 4). This suggests that the changes observed in the case of hGAPDH must be a consequence of the formation of some alternative product. Since in S-glutathionylated hGAPDH we did not detect any other products except for the mixed disulfide and the intramolecular disulfide bridge, we assume that the observed changes could be due to the disulfide bridge.

4. Discussion

The active site of GAPDH contains two cysteine residues (Cys152 and Cys156 in human). Cys152 is essential for catalysis, taking part in the formation of the covalent intermediate product with glyceraldehyde-3-phosphate. The second cysteine residue (Cys156) does not take part in catalysis. In some microorganisms (for example, bacteria of the genus *Thermus*), this cysteine residue is replaced with serine. However, in eukaryotic GAPDH, Cys156 is highly conserved, and its role has remained unclear until the present time. Recently, it has been suggested that Cys156 selectively increases the reactivity of the catalytic Cys152 towards H_2O_2 due to the formation of a net of hydrogen bonds [32]. The authors suppose that the enhanced sensitivity of GAPDH to H_2O_2 indicates its importance for regulatory processes and may serve for the regulation of the cell response to the increase in hydrogen peroxide concentration. In the present work, we isolated untagged recombinant proteins hGAPDH and hGAPDH_C156S with a high specific activity (118 ± 5 and 102 ± 5 μmol NADH/min per mg protein at 25 °C) that is comparable to the activity of GAPDH isolated from natural sources. The constants of their oxidation by H_2O_2 determined by the direct method constituted 11.0 ± 0.4 and 4.9 ± 0.1 $M^{-1}{\cdot}s^{-1}$, respectively. Therefore, the C156S mutation affects the dehydrogenase reaction in a less degree (15%) than the ability of Cys152 for oxidation (56%). This could be related to the fact that the reactivity of Cys152 is not the single factor determining the overall rate of the dehydrogenase reaction. As has been known, the reaction of glyceraldehyde-3-phosphate oxidation includes three stages: 1)

formation of the covalent intermediate product (phosphoglyceroyl-enzyme) with the catalytic cysteine residue; 2) release of the formed NADH and binding of NAD^+; and 3) phosphorolysis of the phosphoglyceroyl-enzyme. The rate-limiting stage of the overall reaction is the release of NADH at high pH values and phosphorolysis of the phosphoglyceroyl-enzyme at physiological pH values [39]. Consequently, a mutation that affects the reactivity of Cys152 may not affect the rate of the dehydrogenase reaction, since the formation of the phosphoglyceroyl-enzyme is not a limiting stage.

In our previous study, we demonstrated that S-glutathionylation of rabbit muscle GAPDH results in the formation of the mixed disulfide GAPDH-SSG that is unstable and breaks down yielding an intrasubunit disulfide bridge in the active site [10]. It was assumed that the breakdown of the GAPDH-SSG is promoted by the non-catalytic active site cysteine residue (Cys156 in human GAPDH). In the present work, we obtained recombinant human protein hGAPDH_C156S and demonstrated that S-glutathionylation of hGAPDH_C156S results in the formation of a stable mixed disulfide GAPDH-SSG. Therefore, a possible role of Cys156 is destabilization of the mixed disulfide with GSH resulting in the formation of Cys152-Cys156 disulfide bond. MALDI-TOF MS analysis does not allow evaluation of the relative content of the GAPDH-SSG and the C152-C156 disulfide bond. However, DSC analysis revealed significant changes in the heat absorption profiles of S-glutathionylated hGAPDH compared to hGAPDH_C156S, which points to a noticeable difference in the spatial structure of the S-glutathionylated proteins. This suggests that the products of S-glutathionylation of the wild-type and mutant hGAPDH are different. Presumably, different subunits of the tetrameric hGAPDH may contain the mixed disulfide GAPDH-SSG or the disulfide bond. The relative content of both products may depend on the conditions (time of incubation and temperature).

We demonstrated that S-glutathionylated hGAPDH is more efficiently reactivated in the presence of GSH and GSH/Grx1 compared to hGAPDH_C156S. As a possible explanation of more efficient reactivation of hGAPDH, we assume that loosening of the protein globule caused by the formation of the Cys152-Cys156 disulfide bridge may facilitate the access of GSH and glutaredoxin 1 to the active site. However, we cannot exclude other mechanisms of the involvement of Cys156 in the reactivation of S-glutathionylated hGAPDH.

Since S-glutathionylation results in reversible GAPDH inactivation, this modification must reversibly inhibit the glycolytic pathway in response to increased level of H_2O_2. This will redirect the metabolic flux through the pentose-phosphate pathway, thus increasing production of NADPH, which is important to protect the cells from oxidative stress [40,41]. In particular, NADPH is a coenzyme of glutathione reductase, an enzyme that is necessary for GSH recycling. Thus, S-glutathionylation of GAPDH could be a trigger for the activation of the antioxidant defense system. After the restoration of the normal GSH/GSSG level, GAPDH can be reactivated by Grx1.

The changes in the spatial structure of hGAPDH in response to S-glutathionylation may be involved in regulation of numerous processes. There are many reports on the ability of GAPDH to interact with nucleic acids [42–45], heme [46], proteins such as transferrin [47,48], tubulin [49], alpha-synuclein [50], Siah1 and GOSPEL [51,52] and on the possible role of these interactions in the regulation of various cellular processes (transcription, iron uptake, apoptosis, etc.). The interaction of GAPDH with Siah1 (an E3-ubiquitinligase) is of particular interest in connection with the induction of apoptosis [51]. The suggested mechanism of apoptosis involves S-nitrosylation of GAPDH at the catalytic cysteine residue yielding GAPDH-SNO and its subsequent binding to Siah1. The formed GAPDH-SNO/Siah1 complex is transferred into the nucleus and initiates apoptosis [51,53]. Possibly, S-glutathionylation of GAPDH may affect the interaction of GAPDH with some proteins and ligands.

There is another aspect that needs to be taken into account. In the case of S-glutathionylation of GAPDH, the formation of the Cys152-Cys156 disulfide bridge results in the release of GSH molecule (Scheme 2). Consequently, the described mechanism not only protects the protein from irreversible inactivation, but also provides GSH recycling, which is important for the cell under conditions of oxidative stress. Considering a high concentration of GAPDH in the cell and its sensitivity to oxidation, we can assume that S-glutathionylation of GAPDH is a GSH-dependent mechanism of neutralization of excessive H_2O_2, which includes reversible oxidation of GAPDH and GSH recycling. In this case, GAPDH plays the role of a buffer, which is able to protect the cell from local fluctuations of H_2O_2.

5. Conclusion

Incubation of the recombinant proteins hGAPDH and hGAPDH_C156S in the presence of H_2O_2 and GSH results in the formation of the corresponding mixed disulfides between the catalytic Cys152 and GSH (S-glutathionylation). In the wild-type hGAPDH, the mixed disulfide is unstable and reacts with neighboring Cys156 resulting in the formation of Cys152-Cys156 intrasubunit disulfide bridge. S-glutathionylation of hGAPDH_C156S results in the formation of a stable mixed disulfide between Cys152 and GSH.

S-glutathionylation of hGAPDH leads to conformational alterations of the protein that can be detected by changes in the parameters of its thermal denaturation. The 1.6-fold decrease in the value of calorimetric enthalpy of thermal unfolding observed after S-glutathionylation of hGAPDH indicates a weakening of the interactions between amino acid residues involved in maintaining the globular structure of the protein. In contrast, S-glutathionylation of hGAPDH_C156S does not affect significantly the parameters of thermal unfolding. These data indicate the involvement of Cys156 in structural rearrangements caused by S-glutathionylation.

The reactivation of S-glutathionylated hGAPDH in the presence of GSH and GSH/Grx1 is more efficient (by 1.7- and 2.1-fold, respectively) compared to the reactivation of hGAPDH_C156S, suggesting that Cys156 is important for both spontaneous and enzymatic reactivation of S-glutathionylated GAPDH.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2020.129560.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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