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Ферменти редокс-циклу глутатіону як потенційні мішені гем-опосередкованого окислення при гемолізі: аналіз *in silico*

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Редокс-гомеостаз глутатіону (γ-глутамілцистеїнілгліцину) в еритроцитах людини залежіть від активності глутатіонпероксидази (GPX1, КФ 1.11.1.9), глутатіонредуктази (GSR, КФ 1.8.1.7), глутаредоксину 1 (GRX1) і NADPH-генеруючих ферментів пентозофосфатного шляху, глюкозо-6-фосфатдегідрогенази (G6PD, КФ 1.1.1.49) і 6-фосфоглюконатдегідрогенази (PGD, КФ 1.1.1.44). Накопичення вільного гему при гемолізі може вплинути на активність протеїнів, у зв'язку з чим був проведений *in silico* аналіз структури ферментів редокс-циклу глутатіону з метою виявлення можливих гем-зв'язуючих сайтів та залишків цистеїну, здатних до окислення. Анотації протеїнів були взяті з UniProt. Докінг гему проведений у PatchDock з RMSD кластерування 1,5 Å і з використанням PDB структур протеїнів та гему. Потенціал окислення цистеїнів оцінювався за допомогою Cy-Preds. Для мономерів GSR (1DNC, 3DJJ, 3DK9, 2GRT) та димерів (3SQP, 2GH5) передбачено зв'язування гему через His81 біля дисульфідного зв'язку між ланцюгами та через Cys59 біля сайтів зв'язування FAD і GSSG. Гем-зв'язуючі сайти у GPX1 (2F8A) і GPX3 (2R37) також виявлені у ділянці між ланцюгами та у активному центрі (His80). Зв'язування гему з GLRX1 (4RQR) передбачено майже виключно поблизу N-кінця, незважаючи на доступність всіх цистеїнів разом з CPYC мотивом у активному центрі. В мономері G6PD (2BH9, 5UKW) гем-зв'язуючі ділянки виявлені у сайті зв'язування NADP+ та в α-спиралі 437–447, розташованій у димері 2BHL на поверхні між ланцюгами. Гем стикувався до PGD (4GWG, 4GWK) у ділянці зв'язування субстрату біля His187. Таким чином, активні центри ферментів та ділянки взаємодії ланцюгів були виявлені в більшості варіантів докінгу гему. У кожному протеїні виявлено від одного (у PGD) до трьох (у GSR) схильних до окислення цистеїнів, в тому числі серед потенційних сайтів зв'язування гему. Опосередкований гемом окислювальний ефект на ферменти редокс-циклу глутатіону у еритроцитах і плазмі крові може явитись важливим механізмом посилення гемолізу при стресі та патології.

Ключові слова: *редокс-цикл глутатіону, NADPH, зв'язування гему, гемоліз, молекулярний докінг.*

Glutathione redox cycle enzymes as potential targets for heme-mediated oxidation under hemolysis: *in silico* analysis

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Glutathione (γ-glutamylcysteinylglycine) redox homeostasis in human erythrocytes is dependent on the activities of glutathione peroxidase (GPX1, EC 1.11.1.9), glutathione reductase (GSR, EC 1.8.1.7), glutaredoxin 1 (GRX1) and NADPH-generating enzymes of pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44). Free heme accumulation under hemolysis can affect proteins activity thereby *in silico* analysis of glutathione redox cycle enzymes structure was performed in order to reveal putative heme-binding sites and oxidizable cysteine residues. Protein annotations were taken from UniProt. Heme docking was performed by PatchDock with clustering RMSD 1,5 Å using PDB structures of proteins and heme. Cysteines oxidation potential was estimated by Cy-Preds. Heme binding GSR monomers (1DNC, 3DJJ, 3DK9, 2GRT) and dimers (3SQP, 2GH5) was predicted through His81 close to interchain disulfide bond and through Cys59 near FAD and GSSG binding sites. Heme-binding areas in GPX1 (2F8A) and GPX3 (2R37) also were revealed in the interchain region and in active centre (His80). GLRX1 (4RQR) was predicted to bind heme almost exclusively near the N-end in spite of accessibility of all cysteines including CPYC motif in active centre. G6PD monomer (2BH9, 5UKW) revealed heme-docking areas in NADP+ binding region and α-helix 437–447 located in dimer 2BHL at the interchain surface. Heme docking to PGD (4GWG, 4GWK) was in substrate binding region near His187. So enzymes active centres and chain interaction regions were revealed in the most of heme docking variants. From one (in PGD) to three (GSR) cysteines susceptible to oxidation were found in each protein including cysteines that were predicted to bind heme. Heme-mediated oxidative effect on glutathione redox cycle enzymes in erythrocytes and blood plasma could be an important mechanism of hemolysis progression under stress and pathologies.

Key words: *glutathione redox cycle, NADPH, heme binding, hemolysis, molecular docking.*

Ферменты редокс-цикла глутатиона как потенциальные мишени гем-опосредованного окисления при гемолизе: анализ *in silico*

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Редокс-гомеостаз глутатиона (γ-глутамилцистеинилглицина) в эритроцитах человека зависит от активности глутатионпероксидазы (GPX1, КФ 1.11.1.9), глутатионредуктазы (GSR, КФ 1.8.1.7), глутаредоксина 1 (GRX1) и NADPH-генерирующих ферментов пентозофосфатного пути, глюкозо-6-фосфатдегидрогеназы (G6PD, КФ 1.1.1.49) и 6-фосфоглюконатдегидрогеназы (PGD, КФ 1.1.1.44). Накопление свободного гема при гемолизе может повлиять на белковую активность, в связи с чем был проведен *in silico* анализ структуры ферментов редокс-цикла глутатиона с целью выявления возможных гем-связывающих сайтов и остатков цистеина, способных к окислению. Анотации белков были взяты с UniProt. Докинг гема проведен с помощью PatchDock с RMSD кластерирования 1,5 Å и с использованием PDB структур белков и гема. Потенциал окисления цистеинов оценивался с помощью Cy-Preds. Для мономеров GSR (1DNC, 3DJJ, 3DK9, 2GRT) и димерных форм (3SQP, 2GH5) предсказано связывание гема через His81 возле дисульфидной святи между цепями и через Cys59 в активном центре возле сайтов связывания FAD и GSSG. GPX1 (2F8A) и GPX3 (2R37) также выявили гем-связывающие сайты преимущественно в участке между цепями и в активном центре (His80). Связывание гема с GLRX1 предсказано практически исключительно вблизи N-конца (4RQR), несмотря на доступность всех цистеинов, включая CPYC мотив в активном центре. В мономере G6PD (2BH9, 5UKW) гем-связывающие участки выявлены преимущественно в сайте связывания NADP+ и в α-спиралі 437–447, расположенной в димере 2BHL на поверхности между цепями. Гем стыковался к PGD (4GWG, 4GWK) в участке связывания субстрата возле His187. Таким образом, активные центры ферментов и участки взаимодействия цепей были выявлены в большинстве вариантов докинга гема. От одного (у PGD) до трех (у GSR) склонных к окислению цистеинов выявлено в каждом белке, в том числе цистеины, для которых предсказано связывание гема. Опосредованный гемом окислительный эффект на ферменты редокс-цикла глутатиона в эритроцитах и плазме крови может явиться важным механизмом усиления гемолиза при стрессе и патологии.

Ключевые слова: *редокс-цикл глутатиона, NADPH, связывание гема, гемолиз, молекулярный докинг.*

Introduction

Glutathione is tripeptide (γ-glutamylcysteinylglycine) involved in the oxidative stress response and adaptation as water-soluble antioxidant and co-substrate of glutathione-dependent enzymes. Imbalances of glutathione redox homeostasis followed by the accumulation of glutathione disulfide (GSSG) are observed under cardiovascular and neurological diseases, diabetes and other pathologies (Luschak, 2012).

Defense function of glutathione in blood is mostly linked to reduced glutathione (GSH) oxidation under direct interaction with oxidants or in glutathione peroxidase reaction with consequent reduction by NADPH-dependent glutathione reductase (Andersen et al., 1997). The only source of NADPH in erythrocytes is pentose-phosphate pathway (PPP) thereby the deficiency of PPP key enzyme glucose-6-phosphate dehydrogenase causes hemolytic anemia (OMIM #300908). GSH also may be attached to proteins cysteine residues and be removed by glutaredoxin having a glutathione-disulfide oxidoreductase activity in the presence of NADPH and glutathione reductase. Glutathionylation contributes to thiol groups defense from oxidation and provides the important reserve of red blood cell glutathione under the oxidative stress (Hanschmann et al., 2013). Glutathione redox cycling in human erythrocytes is, therefore, dependent on the activities of glutathione peroxidase (GPX, EC 1.11.1.9), glutathione reductase (GSR, EC 1.8.1.7), glutaredoxin 1 (GRX1) and NADPH-generating enzymes of PPP: glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44).

Hemolysis occurring under various stresses and pathological conditions in mammals leads to hemoglobin release with its further degradation and free heme accumulation in blood plasma (Chiabrando et al., 2014; Immenschuh et al., 2017). Heme can directly damage cell structures through its prooxidant and detergent action (Chiabrando et al., 2014), furthermore, its attachment to heme regulatory motifs (HRM), such as Cys-Pro, described in various proteins, is considered to be a signaling event (Mense, Zhang, 2006). Short-term heme binding to proteins is performed mostly through lipophilic amino acids while stable attachment of heme is provided by its covalent binding predominantly to histidine or cysteine residues (Smith et al., 2010). Under significant free heme accumulation the glutathione redox cycle enzymes could become the targets for heme-mediated modification but their affinity to heme molecule as well as susceptibility of their cysteine residues to oxidation have not been investigated yet.

Taking into account direct and indirect prooxidant effects of heme on protein conformation and activity, *in silico* study of putative heme-binding sites and oxidizable cysteine residues in human proteins

involved in glutathione redox cycling was performed as a part of analysis of the mechanisms of heme action on the redox homeostasis under hemolysis.

Materials and methods

The amino acid (AA) sequences and annotations of the proteins selected for study (Table 1) were downloaded from UniProt knowledgebase (http://www.uniprot.org/).

Table 1.

Selected UniProt annotations data on the proteins analyzed in the study

Official protein name (gene symbol; UniProt ID)	Length, isoform	Oligomerization	Tissue/organelle specificity
Glutathione reductase, mitochondrial (GSR; P00390)	479 AA, Isoform cytoplasmic	Homodimer disulfide linked (C134)	Cytosol
Glutathione peroxidase 1 (GPX1; P07203)	203 AA, Isoform 1	Homotetramer	Erythrocytes
Glutathione peroxidase 3 (GPX3; P22352)	226 AA	Homotetramer	Secreted in plasma
Glutaredoxin-1 (GLRX1; P35754)	106 AA, Isoform 1	Monomer	Cytosol
Glucose-6-phosphate 1-dehydro-genase (G6PD; P11413)	515 AA, Isoform short	Homotetramer (dimer of dimers)	Cytosol
6-phosphogluconate dehydrogenase, decarboxylating (PGD; P52209)	483 AA, Isoform 1	Homodimer	Cytoplasm

Free online tool HemeBIND (http://mleg.cse.sc.edu/hemeBIND/) was used to predict amino acids heme-binding propensity by analysis of protein sequences in *.fasta format. HemeBind performs predictions through comparison with sequences of the known heme-binding complexes (Liu, Hu, 2011).

The experimental data on protein structures (Table 2) was downloaded from Protein Data Bank (PDB) knowledgebase (http://www.rcsb.org/pdb/home/home.do).

Table 2.

PDB data on the protein structures analyzed in the study (method for all: X-ray diffraction)

PDB ID	Reso-lution	Chains (Protein)	Sequence region	Ligands and Mutations
3DK9	0.95 Å	A (GSR)	44–522	Ligands: FAD, sulphate
3DJJ	1.10 Å	A (GSR)	45–522	Ligands: phosphate, sulphate; FAD, glycerol, NADP
1DNC	1.70 Å	A (GSR)	45–522	Ligands: FAD, reduced GSH, phosphate
2GRT	2.70 Å	A (GSR)	62–522	Ligands: FAD, oxidized glutathione disulfide. Mutations: E17A, W20R
2GH5	1.70 Å	A/B (GSR)	62–522	Ligands (A,B): FAD, 6-(3-methyl-1,4-dioxo-1,4-dihydro-naphthalen-2-yl)hexanoic acid; phosphate, glycerol
3SQP	2.21 Å	A/B (GSR)	45–522	Ligands (A,B): FAD, sulphate, pyocyanin, glycerol
2F8A	1.50 Å	A/B (GPX1)	14–198	Ligands: Malonic acid. Mutation: G47C
2R37	1.85 Å	A/B (GPX3)	25–223	Ligands: Na, Cl. Mutation: G50C
4RQR	1.08 Å	A (GLRX1)	2–106	Ligand: 1-thioethanesulfonic acid
2BH9	2.50 A	A (G6PD)	27–515	Ligands: NADP, glycerol. Mutation: V1H
5UKW	2.65 A	A (G6PD)	29–511	Ligands: β-D-glucose-6-phosphate; glycerol Mutation: C254A
2BHL	2.90 A	A/B (G6PD)	28–515	Ligands (A,B): β-D-glucose-6-phosphate, glycerol
4GWG	1.39 Å	A (PGD)	2–483	Ligand: 2-(N-morpholino)-ethanesulfonic acid
4GWK	1.53 Å	A (PGD)	2–483	Ligands: 3-phosphoglyceric acid; 2-(N-morpholino)-ethanesulfonic acid

Among structures known for this moment for human GSR six ones with highest resolution having coenzymes and substrate (GSSG) or product (GSH) as ligands were selected for analysis. PDB-files with dimeric structures of GPX1 and GPX3 were edited in text redactor for analysis of single protein chains. Structure of tetrameric forms of glutathione peroxidase hasn't been yet experimentally investigated.

Free online tool TM-Align (http://zhanglab.ccmb.med.umich.edu/TM-align/) was used for structural alignment which was estimated in Å as RMSD (root mean squire deviation) of the distances between α-carbons of the aligned protein chains (Zang, Scholnik, 2005).

Docking of heme as a ligand to PDB-structures was carried out by free on-line tool PatchDock, Beta 1.3 Version (http://bioinfo3d.cs.tau.ac.il/PatchDock/) with clustering RMSD 1.5 Å as it was recommended for protein-ligand docking. First 20 docking solutions with the highest scores for each target PDB-structure were analyzed. Scoring was based on both geometric fit and atomic desolvation energy calculation mostly oriented on molecular shape complementarity (Schneidman-Duhovny et al., 2005). PDB-file for heme molecule was downloaded from PubeChem (http://www.ebi.ac.uk/pdbe-srv/pdbechem/chemicalCompound/show/HEM). Visualization of PDB-structures in cartoon view with amino acid side chains was performed by the help of PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC, downloaded from https://sourceforge.net/projects/pymol/); analysis of amino acid residues arranged in close proximity to heme iron as well as distances to heme iron was carried out by SwissProtViewer 4.1.0 (SPDBV; http://spdbv.vital-it.ch).

Cysteines oxidation prediction was performed by free online tool Cy-Preds (Soylu, Marino, 2016) that provided energy and similarity evaluations and functional characterization of the cysteine reactivity based on the experimental data collected in RedoxDB. Results of both HAL_C and COPA schemes of Cy-preds algorithm were used for calculations according to the tool documentation.

Results and discussion

Protein sequence analysis by HemeBind predicted predominantly hydrophobic amino acid residues as potential heme-binding sites: 10 hydrophobic amino acids (AA) of totally 14 AA predicted for GSR sequence; 18 AA of 21 AA for GPX1; 21 of 26 AA for GPX3; all 6 AA for GLRX1; 13 AA of 24 AA for G6PD and 44 AA of 57 AA predicted for PGD. Cys235 of GSR and Cys289 of PGD predicted to bind heme are not located in CP/PC or CXXC motives while Cys170 belongs to PC motif and is neighbor to Cys171 (169PCC) in PGD. Moreover only in this sequence histidines (H453, H466) were scored as putative targets for heme binding. Abovementioned histidines and Cys289 are more accessible than Cys170 or Cys171 according to PDB-files. Proline but not cysteine of PC motif (CP77) was predicted to bind heme in GPX1. Percentage of predicted heme-binding sites (as a ratio to the total number of amino acids in the chain) was the biggest in PGD – 12% against 3% in GSR, 5% in G6PD; 6% in GLRX1 and 10% in GPX1. Summarizing the results of HemeBind analysis it can be concluded that specific heme-binding sites able to covalent heme attachment were predicted only for GSR and PGD sequences.

Structural alignment of PDB structures known for one protein revealed very close conformations. Thus for GSR variants used in the study (including 2GRT with mutation) RMSD range varied from 0.20 Å (3DK9 aligned to 3DJJ) to 0.45 Å (3SQP aligned to 2GH5); for glucose-6-P dehydrogenase RMSD was close to 1.00 Å; for glutathione peroxidase isoforms RMSD=1.10 Å (alignment of 2F8A and 2R37). Two structures known for human 6-P-gluconate dehydrogenase were found almost identical (RMSD=0.1 Å).

Molecular docking of heme molecule to monomeric GSR (1DNC, 3DJJ, 3DK9 and 2GRT) revealed putative heme-binding sites located predominantly in four areas (Fig. 1A): the loop region 80–96 with Cys90 and His80 neighbor to α-helix (*a*), the cavity in the centre of the molecule near FAD and glutathione disulfide binding sites close to Cys58 and Cys63 (*b*), near the hydrophobic regions 45–50 (Arg-Ala-Ala-Val-Val) and 123–127 (His-Ile-Glu-Ile-Ile) organized into two adjacent beta-strands (*c*) and the region with the loop 354–360 and part of α-helix with His351 (*d*).

Chains in the dimers (3SQP and 2GH5, analyzed in SPDBV) revealed rather big contacting areas formed by more than 140 amino acids with about 70 ones from each chain. Some of them were predicted (Table 3) as heme-binding sites in monomeric models, including redox-active Cys58 and Cys63 as well as regions near His80, Thr339, Phe372 and Glu442. Thus heme might interfere dimerization and thereby disturb the formation of the active form of glutathione reductase. In the enzyme dimers (3SQP and 2GH5) the cavity formed by two chains was predicted to become more preferable site for heme binding than other areas (Fig. 1, boxes *a*–*c*). Significant portion of these amino acids was also involved in binding of

coenzymes (FAD and NADP) and substrate (GSSG). Among amino acids found in these sites only His351 arranged near the surface could provide long-term specific heme binding.

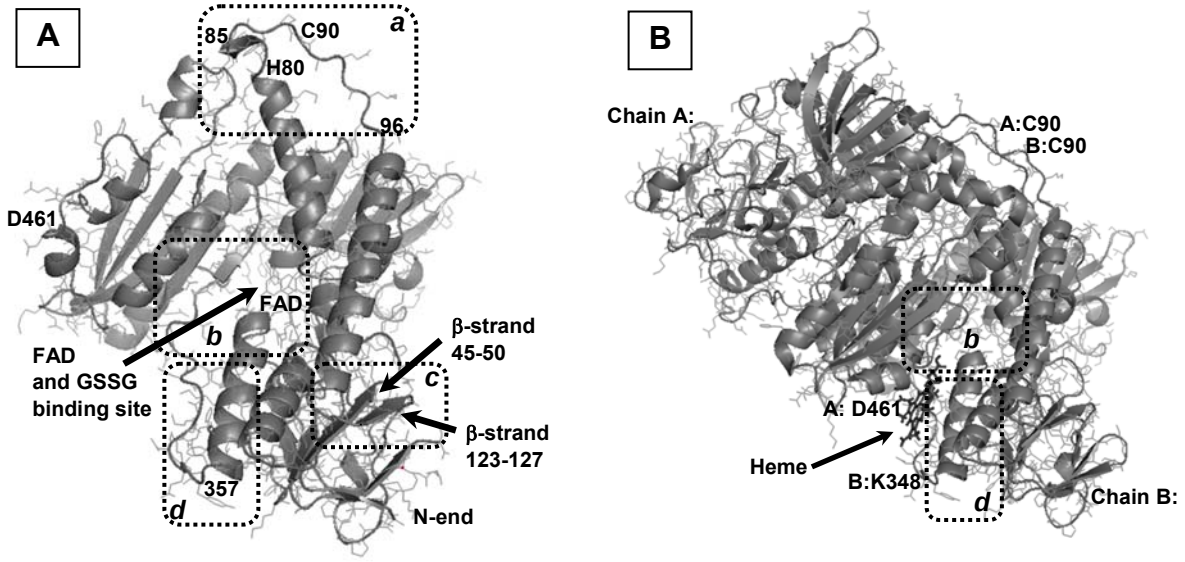

Fig. 1. Human glutathione reductase (cartoon view in PyMol) with predicted heme-binding areas shown as the boxes with dotted borders. A: Monomeric GSR (1DNC is taken as an example), box *a* – the loop region 80–96; box *b* – the cavity in the centre of the molecule; box *c* – two beta-strands 45–50 and 123–127; box *d* – area near the loop 354–360 and α-helix with His351. B: Dimeric GSR docked with heme (3SQP, solution 3), Cys90 form interchain disulfide bridge. Here and hereafter numbers that indicate amino acid residues are taken from pdb-files, A and B parts have different scale

Table 3.

Representative results of heme docking to glutathione reductase (GSR). Here and hereafter the amino acids (AA) arranged mostly close to heme iron are marked by bold font, distances to Fe are given in brackets (as calculated in SPDBV), amino acids numbers are taken from pdb-files

PDB ID (docking solution)	Total Score	Contact area	Amino acids of GSR predicted to be at 6 Å or less from the heme ring	Location of binding region
3DK9 (solution 2)	5910	696.0	A: Ser76, Glu77, Phe78, Met79, **His80** (1,35 Å), Asp81, Ala208, Arg413	The loop region 80–96
3DJJ (solution 10)	5566	654.6	A: Ser30, **Cys58** (4,83 Å), Val59, Cys63, Val64, Tyr106, Leu110, Tyr114, Thr339, Ile343	NADP and GSSG binding sites
2GRT (solution 4)	5816	781.8	A: Lys67, Val370, **Phe372** (3.91 Å), Asp441, Glu442	GSSG and FAD binding sites
1DNC (solution 13)	5744	671.8	A: Tyr21, Arg45, Val48, Ile123, Glu124, **Ile125** (4,40 Å), Ile126, Arg127	β-strands 45–50 and 123–127
3SQP (dimer, solution 3)	6500	857.5	A: Ala458, **Asp461**(4.69 Å), Asn462, Thr464 B: Ala344, Arg347, **Lys348** (3,00 Å), His351, Ser360, Lys361, Leu362	Chain A: N-end, Chain B: loop near K348
2GH5 (dimer, solution 8)	6342	845.5	A: Arg461, Thr463, Ala465, Glu472, Glu473, Val475, **Thr476** (3,24 Å), Leu477, Arg478 B: Ile343, Ala344, **Arg347**(3.47 Å)	Chain A: N-end, Chain B: loop near K348

Molecular docking of heme to glutathione peroxidase isozymes revealed different heme-binding areas for dimeric and monomeric forms (Fig. 2, Table 4). In both dimers heme was predicted to bind predominantly in the interchain region (boxes *a* and *c*) thereby interacting with amino acids of both chains. Heme ring was attached to the GPX1 dimer surface just in the region of active centre near Gly47.

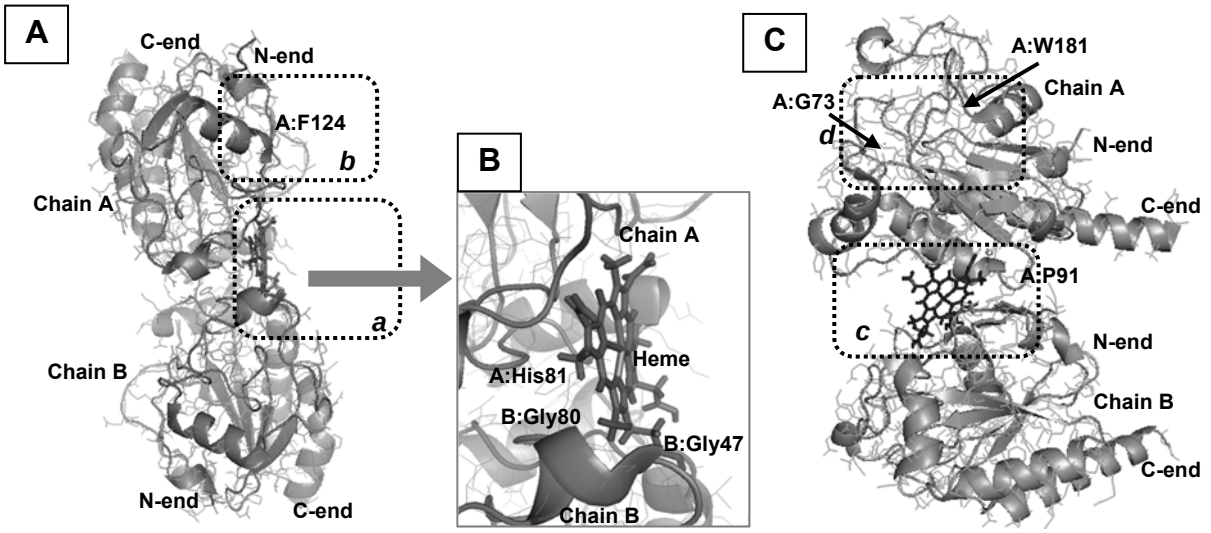

Fig. 2. Human glutathione peroxidase isoforms GPX1 and GPX3 (cartoon view in PyMol) with predicted heme-binding areas shown as the boxes with dotted borders. A: GPX1 dimer (2F8A), box *a* – heme-binding region predicted for dimeric form, box *b* – heme-binding region predicted for 2F8A monomer. B: enlarged view of docking site *a* in 2F8A (solution 1) with Gly47 inserted by mutation instead of selenocysteine shown in the active site of GPX1. C: GPX3 dimer (2R37) with predicted heme-binding area in the cavity between chains (box *c*, solution 1) and in monomeric model (box *d*). Gly73 inserted by mutation instead of selenocysteine shown in the active site of GPX3.

Table 4.

Representative results of heme docking to human glutathione peroxidase isozymes (GPX1, GPX3) and glutaredoxin 1

PDB ID (protein, docking solution)	Total Score	Contact area	Amino acids predicted to be at 6 Å or less from the heme ring	Location of binding region
2F8A (GPX1 dimer; solution 1)	6078	751.1	A: **Asn77** (3,38 Å), Gln78, **His81** (4,20 Å), Glu83, Asn84, Lys112, Glu144 B: Gly80, Gln82	Dimer contact area, active site
2F8A, chain A (GPX1 monomer; solution 1)	5454	709.6	A: Val115, Gly117, Ala118, Gly119, Ala120, His121, Pro122, Leu123, Phe124, **Ala125** (2,51 Å), Phe126, Arg128, Glu129	Central part of the molecule
2R37 (GPX3 dimer; solution 2)	5992	707.5	A: Gln86, Glu87, Pro130, **Asn131** (3,39 Å), Phe132 B: Ala45, **Leu46** (3,81 Å), Tyr53, Glu136	In the cleft between two chains
2R37, chain A (GPX3 monomer; solution 1)	5660	765.40	A: Leu162, Leu163, Gly164, Thr165, Arg168, Arg180, **Trp181** (5,44 Å), His199, His200	Active site of enzyme
4RQR (GLRX1; solution 1)	5376	660.2	A: Glu3, **Phe4** (4,42 Å), **Val5** (4,71 Å), Asn6, Cys7, Lys8, Thr50	In the N-end region
4RQR (GLRX1; solution 7)	5316	725.7	A: Pro23, **Tyr24** (1,62 Å), Cys25, Val59, Thr68, Pro70, Gly81, **Cys82** (4.46 Å), Ser83	Near redox-active disulfide

GPX1 monomer (2F8A:A) was predicted to bind heme predominantly near Phe124 (box *b*) that didn't participate in dimerization. Thus erythrocyte-specific GPX1 in dimer form could be directly inhibited by heme. Lack of information about GPX tetramer structure didn't allow to predict heme action on the assembly of glutathione peroxidase isomers.

GPX3 dimer (2R37) is not symmetrical thereby protein chains interact by different residues: Lys62 and region 87–93 from the side of chain A and Ile36 together with regions 41–44 and 53–57 from chain B.

Heme could be fitted into the cleft between chains in dimer while GPX3 monomer (2R37:A) more probably could bind heme in the region of active site (Fig. 2, box *d*).

Glutaredoxin 1 is the smallest protein among ones investigated in this study. Heme was predicted to bind almost exclusively (19 of 20 best solutions) to short α-helix at the N-end (Table 4). Only one solution predicted heme interaction with Tyr24 just near redox-active disulfide Cys22-Cys25 that is close to the surface. No cavities were found in GLRX1 PDB-structure thereby all cysteines are rather accessible to heme binding including CPYC motif in active site.

Molecular docking of heme molecule to glucose-6-phosphate dehydrogenase revealed two main heme-binding areas (Fig. 3A) for monomer (2BH9): NADP+ binding region in the active site (box *a*) and α-helix 437–447 (box *b*) found in the dimer 2BHL at the interchain surface. G6PD chains in the dimer are bound symmetrically by the interaction of more than 130 amino acids (equally about 65 from each chain) mostly (more than 50%) hydrophobic. Thus heme binding could more probably directly inhibit activity of G6PD at the active centre than disturb tetramer assembly.

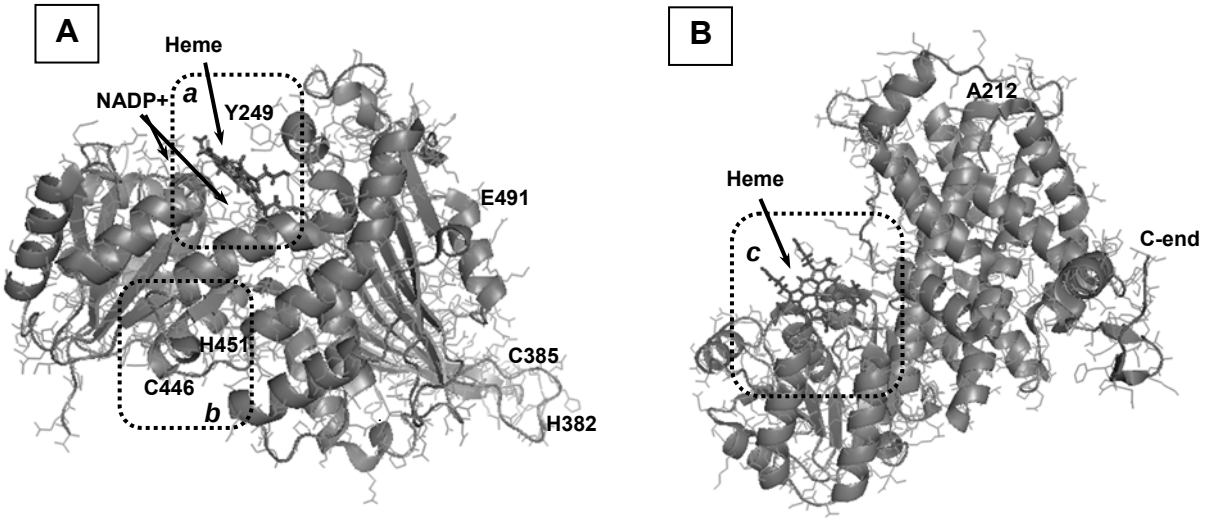

Fig. 3. Human glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (cartoon view in PyMol) with predicted heme-binding areas shown as the boxes with dotted borders. A: G6PD monomer (2BH9) docked with heme (solution 4), box *a* – the cavity with NADP+ binding site at Lys171; box *b* – region 440–446 in α-helix near the loop with His451. B: PGD monomer (4GWK) docked with heme (solution 1), box *c* – substrate binding region containing Gly131 and His 187 with docked heme

Heme binding to 6-phosphogluconate dehydrogenase monomer (4GWK) was predicted at only one area (Fig. 3B, box *c*) known as substrate binding region. This region contains two groups of amino acid residues: S129-G130-G131 and His187-Asn188 (Table 5). No data is available about residues involved in dimerization so only direct inhibition of PGD activity might be suggested as the mechanism of heme action. Almost total identity of two PDB structures available for human PDG (RMSD=0.1 Å) explains coinciding docking results for 4GWK and 4GWG.

Summarizing the results of docking studies performed for human enzymes of glutathione redox cycle it should be concluded that direct heme binding in the active sites areas is highly probable. Regions involved in substrates or/and coenzymes attachment were revealed in the majority of heme docking variants. Heme binding probability was affected by dimerization. Analysis of the first 20 solutions for all variants of PDB structures used in the study revealed approximately 10% higher scores ($p<0,001$) in the case of dimers compared to monomer forms. GSR dimers 2GH5 and 3SQP had the highest scores (6381±229 and 6330±137) and the largest contact areas (840+33 and 847+26, correspondently) as well as G6PD dimer 2BHL (6226±171 score with contact area 819+38). The lowest scores with small contact areas were found for monomeric variants of GPX1 and GPX3 (5281±89 and 5338±108, areas 686+33 and 686+53) as well as for GLRX1 (5276±124, areas 712+34).

Table 5.

Representative results of heme docking to human glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

PDB ID (protein, docking solution)	Total Score	Contact area	Amino acids predicted to be at 6 Å or less from the heme ring	Binding area
2BH9 (G6PD monomer; solution 4)	5918	680.2	A: Leu142, Pro143, Pro144, Lys171, Pro172, Arg246, **Tyr249** (5,33 Å), Phe253	NADP+ binding site
2BHL (G6PD dimer; solution 3)	6490	736.2	A: Lys47, Lys171, Arg246, **Asp258** (5,82 Å), Glu239, **Lys360** (5,65 Å)	NADP+ binding site
4GWG (PGD monomer; solution 1)	6464	794.0	A: Val13, Met14, Asn103, Gly130, **Gly131** (4,49 Å), Glu132, Glu133, Ala135	Active site area

It should be mentioned that PatchDock algorithm didn't take into account the types of interactions but scored mostly geometrical fitting of ligand therefore only the presence of certain amino acid in docking area could be used for type of bond predictions.

Cysteines oxidation potential was estimated by the Cy-Preds online tool (Table 6).

Table 6.

Potentially oxidized cysteines in human proteins of the glutathione redox cycle predicted by Cy-Preds. Predictions with total score ≥ 50 (max 100) are presented for corresponding PDB structure. Numbers for amino acids are given according to sequence data

Protein isoform	Number of Cys in mature sequence, CP/PC motifs, disulfide bonds	PDB (chains)	Potentially oxidized Cys
GSR, Isoform cytoplasmic	Totally 10 cysteines (C3, C59, C64, C91, C235, C285, C334, C418, C424, C441); no CP/PC; intrachain disulfide C102–C107, C134 in the interchain disulfide	3DJJ (A)	C64(75), C91(50)
		1DNC (A)	C59(75), C91(50)
		2GRT (A)	C91(50)
		2GH5 (A, B)	C59(50)
		3SQP (A, B)	C91(50)
GPX1, Isoform 1	Totally 5 cysteines (C2, C78, C115, C156, C202) and U49 selenocysteine; C78 in PC motif; no information on disulfides	2F8A (A, B)	C78(50), C156(50)
GPX3	Totally 3 cysteines (C32, C101, C156) and U73 – selenocysteine; C156 in CP motif; no information on disulfides	2R37 (A,B)	C101(50), C156(75)
GLRX1, Isoform 1	Totally 5 cysteines (C8, C23, C26, C79, C83); C23 inside of CPYC motif; disulfides C23-C26 (redox-active) and C79-C83.	4RQR (A)	C23(100) in S-S bond, C83(50)
G6PD, Isoform short	Totally 8 cysteines (C13, C158, C232, C269, C294, C358, C385, C446), no CP/PC, no data on disulfides.	2BH9 (A)	C294(50), C446(50)
		5UKW (A)	C277(50), C294(50)
		2BHL (A, B)	C294(50), C385(50)
PGD, Isoform 1	Totally 9 cysteines (C30, C113, C170, C171, C199, C289, C366, C402, C422), 2 PC (C170, C422); no data on disulfides	4GWG (A)	C288(50)
		4GWK (A)	C288(50)

The covalent heme binding to proteins is known to be realized predominantly through cysteine, histidine or tyrosine (Smith et al., 2010). These three types of amino acids were predicted at the distances close enough for iron coordination in several solutions for GSR (His81, Cys59, Table 3), G6PD (Tyr249, Table 5), GPX1 (His80) and GLRX1 (Cys83, Table 4). Moreover, Cy-Preds found at least one cysteine susceptible to oxidation in each protein studied (Table 6). Three oxidizable cysteines were predicted in GSR (Cys59, Cys64, Cys91).

So analysis carried out in this study testifies that glutathione reduction is more likely to be inhibited under hemolysis by heme binding and oxidation of cysteines in glutathione reductase and glucose-6-phosphate dehydrogenase. These results are in agreement with the experimental data on the increase of GSSG/GSH ratio in the cells and blood plasma under action of agents causing oxidative stress in mammals (Pandey, Rizvi, 2011). Inhibition of glutathione peroxidases by heme might temporally redirect GSH to non-enzymatic reactions including Fe^{3+} reduction that might have pro-oxidant effect through acceleration of Fenton reaction. On the other hand, inhibition of glucose-6-phosphate dehydrogenase in

erythrocytes could result in glucose utilization predominantly in glycolysis for energy production that is critical for red blood cell survival under stress (Handala et al., 2017).

Taking into account the hemolytic action of various xenobiotics, the heme-mediated oxidation of glutathione redox cycling enzymes in erythrocytes and blood plasma could be considered to be important mechanism of hemolysis progression under stress and pathologies.

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