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ANALYSIS OF THE COMPONENTS OF NRF2-KEAP1 SIGNALLING AS POTENTIAL TARGETS OF HEME

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Various regulatory effects of heme (Fe-protoporphyrin IX) revealed in mammals under stress and trauma conditions could be mediated by direct heme binding to signalling cascade members. Transcription factor Nrf2 regulating antioxidant response elements (AREs) is controlled primarily by protein Keap1 and further proteolysis through interaction with Cul3 and Rbx1. Nothing is known today about heme effects on these signaling components so their ability to bind heme as potential regulatory agent was analyzed *in silico*. The most number of heme-binding amino acid residues were predicted in the redox-active protein Keap1 and included Cys77, His96, His225 and Met156 in BTB-dimerization domain; Cys319 and Cys489 in Kelch repeats domain involved in Nrf2 binding and Cys226 in intervening linker region. Docking to Nrf2 regions revealed heme in proximity to Cys514 and ETGE motif (79-82) necessary for Nrf2 ubiquitination. The highest docking scores were found for metalloenzyme Rbx1. So signalling cascade members Keap1 and Rbx1 could bind heme more tightly through aromatic and sulfur-containing amino acid side chains that could prevent Nrf2 degradation and therefore activate ARE-regulated transcription.

Keywords: signalling, Nrf2, Keap1, heme, antioxidant response elements

Heme (Fe-protoporphyrin IX) is the universal molecule working as a prosthetic group in the enzymatic redox reactions, electron transport chains and oxygen carriers. Under normal conditions heme is bound to proteins while free heme is lipophilic pro-oxidant [5]. The significant rise of free heme level usually is the result of hemoproteins degradation by stress factors [5]. In mammals it is caused mostly by hemolytic states with consequent hemoglobin breakdown and the incoming of heme from blood into different tissues. Experimental data indicate that heme molecule has diverse regulatory effects and can mediate oxidative stress response through heme binding to signalling cascade members [5]. Transcription factor Nrf2 is specific to antioxidant response elements (AREs) thus launching dozens of target genes [9]. The regulation of its activity is provided by two mechanisms: the phosphorylation and ubiquitin-dependent proteolysis [2]. Cytosolic protein Keap1 acts as Nrf2 inhibitor: being modified through conservative cysteines it targets Nrf2 to proteosome making the complex with another cytosolic proteins Cul3 and Rbx1 [6]. Nothing is known about affinity of these signalling components to heme so in this study they were analyzed *in silico* for the ability to bind heme as potential regulatory agent under oxidative stress in humans.

Materials and methods

The protein annotations (Table 1) were loaded from UniProt knowledgebase (http://www.uniprot.org/). Known protein structures were loaded from Protein Data Bank knowledgebase (http://www.rcsb.org/pdb/home/home.do).

The prediction of protein Keap1 structure with template (4CXI or 4IFJ) was performed using I-TASSER server [10] (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). Structural alignment was carried out by TM-align server (http://zhanglab.ccmb.med.umich.edu/TM-align).

Table 1

UniProt data for the selected human Nrf2/Keap1 signalling components used in the study (AA – amino acids)

Protein name (abbreviation)	Gene Symbol	UniProt ID	Sequence data	Number of PDB structures
Nuclear factor erythroid 2-related factor 2 (Nrf2)	NFE2L2	Q16236	605 AA (Isoform 1)	4
Kelch-like ECH-associated protein 1 (Keap1)	KEAP1	Q14145	624 AA	20
Cullin-3 (Cul3)	CUL3	Q13618	768 AA (Isoform 1)	10
E3 ubiquitin-protein ligase RBX1 (Rbx1)	RBX1	P62877	108 AA	6

Docking analysis performed by the help of PatchDock, Beta 1.3 Version (http://bioinfo3d.cs.tau.ac.il/PatchDock/) [7]. Structure file for heme was loaded from PubeChem (http://www.ebi.ac.uk/pdbe-srv/pdbechem/chemicalCompound/show/HEM). Visualization and analysis of structures were carried out by the help of The PyMOL Molecular Graphics System (Version 1.3, Schrödinger, LLC) and SwissProt viewer 4.1.0 (http://spdbv.vital-it.ch).

Results and discussion

Sequence motifs known to bind heme specifically consist of histidine (His), cysteine (Cys), tyrosine (Tyr), phenylalanine (Phe), tryptophan (Trp) and methionine (Met) [4]. Heme regulatory motifs containing cysteine and proline are usually surrounded by hydrophobic residues [3]. So the main attention in the analysis of the docking variants was paid to the character of amino acids in the close proximity to heme iron. The most number of potential heme-binding amino acid residues were revealed in Keap1 (Table 2): Cys77, His96 and Phe111 (in 4CXI; Table 2) located in BTB-dimerization domain necessary for Cul3 binding [1]; Cys489, Tyr490 and Tyr491 (in 2FLU:X and 4IFJ) in Kelch repeats domain involved in Nrf2 binding [1]. Cys77 and Cys489 were shown to be modified by several electrophilic agents that caused activation of Nrf2-dependent transcription [2]. Docking of heme to the Keap1 model structures predicted by I-Tasser (with 4CXI or 4IFJ as templates) revealed additional potential heme binding sites: Phe139 or Phe221, His225, Met156 (in BTB-dimerization domain), Cys226 (in Intervening linker region) and Cys319 (in Kelch repeats region, data not shown in the table 2). It's remarkable that docking scores for these solutions are much higher (6076-6376) than those for known structures of Keap1 (Table 2).

Table 2

The selected results of heme binding sites prediction in PDB structures (by PatchDock)

PDB ID	Chain: protein (sequence range)	Contact area	Total Score	Amino acids number predicted to be within 6,5 Å to heme iron / Amino acids with potential heme-binding activity	
4CXI	A: KEAP1 (48-180)	742.6	5558	8	C77; H96; V98; F111
4IFJ	A:KEAP1 (321-609)	703.8	5310	8	V561; H562; V608
		662.4	5266	8	L471; Y473; C489; Y490; Y491
1U6D	X:KEAP1 (321-609)	806.8	5416	11	Y525; Y572; F577; V604
4IFL	P: NFE2L2 (69-84) X: KEAP1 (321-609)	724.0	5332	Chain P: 6 Chain X: 1	Chain P (NFE2L2): L76; L84
2FLU	P: NFE2L2 (69-84) X: KEAP1 (321-609)	701.2	5380	Chain P: 0 Chain X: 9	Chain X (KEAP1): C489; Y490; Y491; W497; M499; I500
2LZ1	A: NFE2L2 (445-523)	761.1	5870	10	C514; L519
		719.2	5628	7	H443; M444; H446
4AP2	B:CUL3 (1-388) (A: KLHL11)	804.9	5930	Chain B: 11 Chain A: 0	Chain B (CUL3): L97; F101; L102; H160
4APF	B:CUL3 (23-388) (A:KLHL11)	766.3	5786	Chain B: 1 Chain A: 11	Chain B (CUL3): Q133 Chain A: H209; H213; L217; L220; L222
2LGV	A:RBX1 (12-108)	717.1	6146	9	C53; C56 (near-Zn10); C68; F81; H82; C83
3DPL	R:RBX1 (5-108) (C:CUL5)	848.7	6218	Chain R: 7 Chain C: 5	R: L32; W33; W35; V39; W72 C: H572

Docking to C-end region of Nrf2 (2LZ1) revealed heme in proximity to either Cys514 or His443; Met444 and His446 (Table 2). In 4IFL:P heme was posed close to ETGE motif (79-82) known as one of two necessary regions interacting with Keap1 for consequent Nrf2 ubiquitination [2]. Complex of Cul3 with KLHL11 of Kelch-like family docked heme either to N-end part of Cul3 through Phe101 and His160 (in 4AP2:B) or to the contacting site of KLHL11 with Cul3 through His209 and His213 in BACK-domain (positions 163-265) of KLHL11 (4APF:A; Table 2). Highly conservative BACK domain is suggested to take part in the substrate orientation in Cul3-based E3 ligase complexes [8]. Heme docking to metalloenzyme Rbx1 (2LGV) revealed most probable heme binding in 53-86 region through Cys53 and Cys56 (Zn ligands). In Rbx1 complex with Cul5 of cullin family (3DPL) these residues become involved in cullin binding so heme contacted with Trp33, Trp35 and Trp72 of Rbx1 and His572 of Cul5. In the complexes of another composition (data not shown) heme preferred KLHL3 in Cul3-KLHL3 dimer (4HX1) or Cul1 in Rbx1-Cul1 dimer (1LDJ). The most high scores (>6000) of heme docking were found for Rbx1 complexes.

So Keap1 and Rbx1 could bind heme more tightly providing aromatic and sulfur-containing amino acid side chains. Heme binding to these Nrf2-Keap1 signalling cascade members could prevent ubiquitin-dependent proteolysis of Nrf2 and therefore activate ARE-regulated transcription.

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АНАЛІЗ КОМПОНЕНТІВ NRF2-KEAP1 СИГНАЛІНГУ ЯК ПОТЕНЦІЙНИХ МІШЕНЕЙ ГЕМУ

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Різноманітні регуляторні ефекти гему (Fe-протопорфірин IX) у ссавців в умовах стресу і травми можуть бути опосередковані прямою взаємодією гему з компонентами сигнальних каскадів. Транскрипційний фактор Nrf2, який регулює антиоксидантні відповідні елементи (AREs), контролюється насамперед білком Keap1 і подальшим протеолізом через взаємодію з Cul3 і Rbx1. Оскільки на цей момент ефекти гему на дані сигнальні компоненти невідомі, їхня здатність зв'язувати гем як потенційний регуляторний агент була проаналізована *in silico*. Найбільша кількість амінокислотних залишків, які зв'язують гем, була передбачена у редокс-активному білку Keap1, в тому числі Cys77, His96, His225 і Met156 у BTB-димеризаційному домені; Cys319 й Cys489 у домені Kelch повторів, якій бере участь у зв'язуванні Nrf2 і Cys226 у проміжній лінкерній ділянці. Докінг до ділянок Nrf2 виявив гем поблизу Cys514 і мотиву ETGE (79-82), необхідного для Nrf2 убіквітинування. Найвищі очки докінгу були виявлені для металоферменту Rbx1. Таким чином, члени сигнального каскаду Keap1 і Rbx1 можуть зв'язувати гем більш міцно через ароматичні та сірковмісні бокові ланцюги амінокислот, що може запобігти деградації Nrf2 й таким чином активувати ARE-регульовану транскрипцію.

Ключові слова: сигналінг, Nrf2, Keap1, гем, антиоксидантні відповідні елементи.

АНАЛИЗ КОМПОНЕНТОВ NRF2-KEAP1 СИГНАЛИНГА КАК ПОТЕНЦИАЛЬНЫХ МИШЕНЕЙ ГЕМА

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Различные регуляторные эффекты гема (Fe-протопорфирин IX) у млекопитающих в условиях стресса и травмы могут быть опосредованы прямым взаимодействием гема с компонентами сигнальных каскадов. Транскрипционный фактор Nrf2 регулирует антиоксидантные ответные элементы (AREs) и контролируется прежде всего белком Keap1 и последующим протеолизом через взаимодействие с Cul3 и Rbx1. Поскольку на данный момент эффекты гема на данные сигнальные компоненты неизвестны, их способность связывать гем как потенциальный регуляторный агент была проанализирована *in silico*. Наибольшее число гем-связывающих аминокислотных остатков были предсказаны в Keap1: включая Cys77, His96, His225 и Met156 в BTB-димеризационном домене; Cys319 и Cys489 в домене Kelch повторов, участвующем в связывании Nrf2, и Cys226 в промежуточном линкерном участке. Докинг к участкам Nrf2 выявил гем вблизи Cys514 и мотива ETGE (79-82), необходимого для Nrf2 убиквитинирования. Наивысшие очки докинга были выявлены для металлофермента Rbx1. Таким образом, Keap1 и Rbx1 могут связывать гем более прочно, предоставляя ароматические и серосодержащие боковые цепи аминокислот, что может предотвратить деградацию Nrf2 и привести к активации ARE-регулируемой транскрипции.

Ключевые слова: сигналинг, Nrf2, Keap1, гем, антиоксидантные ответные элементы.