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Investigation of the interaction of fusion Trx-Aβ40 proteins with heme

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Aβ amyloids (Aβ) are known for their key role in Alzheimer's disease. There are two main species of Aβ – Aβ40 and Aβ42 – consisting of 40 and 42 amino acids, respectively. It has been previously shown that Aβ42 binds heme, and the resulting Aβ42-heme complex acts as a peroxidase. In this work we have tested whether the thioredoxin-Aβ40 (Trx-Aβ40) fusion protein binds heme and how they interact. His residues are known to be the best candidates to coordinate the iron of heme. We changed His codons of Aβ40 to other amino acid codons (Ala, Cys, Ser) to see whether a particular His residue is participating in a binding process. The heme binding experiments showed that mutants bind heme, but less efficiently than does Trx-Aβ40. Thus, it may be concluded as His residues participate in heme binding, but its full mechanism is still unclear. Also, we demonstrate that the Trx-Aβ40-heme complex functions as a peroxidase.

Key words: Aβ amyloids, heme, thioredoxin, mutants, peroxidase

INTRODUCTION

The amyloid β peptide (Aβ) is normally soluble 4.3-kDa 40- and 42-residue peptides found in cerebrospinal fluid and plasma. These peptides, denoted as Aβ40 and Aβ42, are generated by the proteolytic processing of the amyloid precursor protein (APP). The production of the Aβ peptide is a normal occurrence, although its function remains unknown. Aβ accumulates as the major constituent of the extracellular deposits that are the pathologic hallmarks of Alzheimer's disease (AD) [1]. Aβ peptides are neurotoxic [2], but the mechanism of toxicity and the responsible Aβ species have not been clearly defined. Aβ *in vitro* binds metal ions, including Zn, Cu, and Fe. The discovery that metal binding to Aβ may be responsible for some of the pathological effects of AD makes the characterization of the metal-binding site of interest as a potential therapeutic target [3].

Amyloid-β specifically binds catalase with high affinity and inhibit catalase breakdown of H_2O_2 [4]. It is possible that Aβ binds to the heme group of the catalase active center. Howlett and co-workers have established that heme interferes effectively with the aggregation of β-amyloid peptides and inhibits the aggregation process [5]. Heme metabolism was altered in the brains of AD patients.

Heme is an iron protoporphyrin IX complex which is essential to the function of a number of proteins. For instance, hemoglobin contains four non-covalently bound heme molecules. Cytochrome, peroxidase, catalase and nitric oxide synthase also contain a heme moiety. The physiological processes involving these proteins and mediated by heme include electron transport and other redox mechanisms [6].

Previous reports have suggested that the pathology of Alzheimer's disease may be linked to oxidative stress. Iron is known to be a facilitator of oxidative stress due to the production of hydroxyl radicals from hydrogen peroxide via the Fenton reaction [6]. Iron in the brain is mainly associated with ferritin, and its relatively very low levels are free and in reactive form. Nevertheless, the ferritin and iron ratio decreases in Alzheimer's disease, correlating with the severity of the neurological damage. Aβ42 binds to heme to form an Aβ-heme complex [7]. Small Aβ–heme concentrations (in a normal brain) play a physiological role. The Aβ–heme complex is a peroxidase. The Aβ–heme peroxidase was efficient in oxidizing serotonin and DOPA. Heme binding with Aβ42 is an endogenous mechanism that prevents Aβ42 aggregation [8]. However, excessive production of Aβ, as in the case of AD, may have detrimental consequences if the Aβ–heme peroxidase is produced.

So far, amino acids participating in the Aβ–heme interaction have been unknown. The three histidine residues in Aβ can potentially bind heme. In this study, we investigated the interaction of heme with thioredoxin-Aβ40 (Trx-Aβ40) fused protein. We have concluded that heme interacts with such fused protein, and this protein complex is a peroxidase as in the case of the Aβ42–heme complex. Histidine mutation in the Aβ40 peptide influences the interaction between fused protein and heme, demonstrating that histidine residues of Aβ40 are participating in heme binding.

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MATERIALS AND METHODS

Bacterial plasmids pET3a-abeta40 were a donation of Dr. A. Olofsson. Hemin, imidazole were from Fluka, Germany. NaOH was from Merck, Germany. β-mercaptoethanol was purchased from Ferak, Germany. Tris-HCl, TMB were from Sigma, USA.

NA (Nutrient Agar) was purchased from Oxoid, England. Primers were from Metabion, Germany. Restriction endonucleases and ligation enzymes, isopropyl-β-D-thiogalactopyranoside (IPTG), *Pfu* polymerase, PCR mix were from Fermentas, Lithuania.

Escherichia coli DH5α strain [ϕ80dlacZΔM15 Δ(lacZYargF)U169 *deoR recA1 endA1 hsdR17*($r_K^- m_K^+$) *sup E44 thi-1 gyrA96 relA1*] was from Pharmacia, *E. coli* BL21(DE3) was from Avidis, USA.

Construction of mutants. Mutants were obtained by site-directed mutagenesis (Table 1). Site-directed mutagenesis of Trx-Aβ40 was performed by PCR amplification of pET3a-TrxAβ40 containing a fused gene of thioredoxin and Aβ40 using *Pfu* polymerase. Pairs of primers were used (Table 1) to amplify the fused gene of thioredoxin and Aβ40 with introduced mutations. In the first step, two separate PCRs were used to generate the primary PCR products having overlapping ends (the mutating primer F and the flanking primer R in one tube and the mutating primer R and the flanking primer F in the second tube). PCR products were then used as templates to generate the full-length sequence. Flanking primers were designed with the recognition sites of restriction endonucleases XbaI and BamHI.

The amplified and XbaI-BamHI digested fragments were ligated into vector pET3a and subsequently used to transform competent DH5α cells. Positive clones were screened, and plasmids containing a fused Trx-Aβ40 gene with certain mutations were confirmed by DNA sequencing (the Sequencing Centre, Institute of Biotechnology, Lithuania).

Expression and purification of fused Trx and Aβ40 protein and mutants. BL21 (DE3) *E. coli* cells were transformed with pET3a-TrxAβ40 or mutant constructs. From freshly transformed cells, some colonies were used to inoculate 5 ml of NB medium containing 50 μg/ml of carbenicillin. The culture was shaken overnight at 30 °C, 180 rpm, and then was used to inoculate 1 l of NB medium in flasks with 50 μg/ml of carbenicillin. The cells were grown at 30 °C until OD_{600} reached 0.8, then the flasks were transferred to a temperature of 24 °C, 0.4 mM of IPTG was added to induce the expression, and the culture was incubated overnight. Cells were harvested by centrifugation at 3000 g. The pellets were washed and resuspended in 50 mM Tris-HCl buffer, pH 8.5. The cells were sonicated on ice and cell debris was removed by centrifugation. The clear supernatant was applied on a HiTrap chelating column (Amersham Biosciences) prepared according to the manufacturer's instruction. If inclusion bodies were formed during the induction, the pellets were washed twice – first with a buffer containing 0.5 M urea and then dissolved in 6 M urea. The column was preequilibrated with a buffer containing 50 mM Tris-HCl, pH 8.5, and 100 mM NaCl. The fusion protein Trx-Aβ40 and mutants were eluted with the same buffer with 500 mM imidazole. The fractions were collected and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions with the purified fusion proteins were desalted by dialysis against 50 mM Tris-HCl, pH 8.5.

Preparation of heme. A stock solution of heme was prepared with freshly solved hemin in 0.1 M NaOH. The heme solution was diluted with PBS buffer, pH 9.0, to obtain a heme concentration of 6 mM. The solutions were kept in the dark.

Interaction of heme and fusion proteins. Protein concentrations were calculated according to absorption at 280 nm (mo-

Table 1. **Primers used for construction of mutants. The annealing temperature was 50 °C. Mutated codons are marked in bold-italic**

Mutation	Used primers 5′→3′ direction
6His→6Cys	6CYSF CTGAATTCCGT***TGC***GACTCC 6CYSR GGAGTC***GCA***ACGGAATTCAG
13His→13Cys	13CYSF CGAAGTT***TGC***CACCAGAAACTG 13CYSR CAGTTTCTGGTG***GCA***AACTTCG
14His→14Cys	14CYSF CGAAGTTCAC***TGC***CAGAAACTG 14CYSR CAGTTTCTG***GCA***GTGAACTTCG
14-13 His→14-13Cys	14-13CYSF CGAAGTT***TGCTGC***CAGAAACTG 14-13CYSR CAGTTTCTG***GCAGCA***AACTTCG
6His→6Ala	6ALAF CTGAATTCCGT***GCG***GACTCC 6ALAR GGAGTC***CGC***ACGGAATTCAG
6His→6Ser	6SERF CTGAATTCCGT***AGC***GACTCC 6SERR GGAGTC***GCT***ACGGAATTCAG
14His→14Ala	14ALAF CGAAGTT***GCG***CACCAGAAACTG 14ALAR CAGTTTCTGGTG***CGC***AACTTCG
15His→15Ala	15ALAF CGAAGTTCAC***GCG***CAGAAACTG 15ALAR CAGTTTCTG***CGC***GTGAACTTCG
14-15His→14-15Ala	14-15ALAF CGAAGTT***GCGGCG***CAGAAACTG 14-15ALAR CAGTTTCTG***CGCCGC***AACTTCG
Flanking	4612R TCCTTTCGGGCTTTGTTAGC 4036F CACAACGGTTTCCCTCTAGA

lar extinction coefficient 15460). Formation of the Aβ–heme complex was determined by heme binding to Aβ fusion proteins at a ratio 1 : 1 (the final concentration in PBS solution, pH 7.5, was 6 μM). The spectrum was recorded between 350 and 750 nm. The peak at 380 nm was observed when heme bound to Aβ fusion proteins [8].

Peroxidase activity of the Aβ fusion protein–heme complex. Peroxidase activity of the Aβ fusion protein–heme complex was measured via the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by H_2O_2 following the increase in absorbance at 652 nm every 5 and 10 minutes. The peroxidase activity of Aβ fusion protein–heme complex was tested at a concentration of 1.2 μM in PBS solution, pH 7.5. The reaction mixture (1 ml) contained 100 μl of TMB, 200 μl of mixture (6 μM of protein, 6 μM heme in PBS, pH 7.5) and 700 μl of PBS, pH 7.5).

RESULTS AND DISCUSSION

It was supposed that three histidine residues in Aβ (Fig. 1) can potentially bind heme. Heme was shown to inhibit the *in vitro* aggregation of Aβ42 and protected neuronal cells against Aβs toxicity [5]. To see if these three histidine residues are crucial for binding heme, we substituted histidine codons in the plasmid encoding Trx-Aβ40 protein with other codons for alanine, serine or cysteine in all different positions. Ten mutant plasmids were constructed carrying out site-directed mutagenesis as described in Materials and methods (Table 2).

The constructed plasmids were used to transform BL21 (DE3) *E. coli* cells for the expression of mutants and Trx-Aβ40.

Table 2. **The list of constructed Trx-Aβ40 mutants**

	His codon	Altered codon	Mutant name
Mutants			
Single:	6His	6Ala	H6A
		6Ser	H6S
		6Cys	H6C
	13His	13Ala	H13A
		13Cys	H13C
	14His	14Ala	H14A
Double:	13-14His	13-14Ala	H(13-14)A
	6-13His	6-13Ala	H(6-13)A
	6-14His	6-14Ala	H(6-14)A
Triple:	6-13-14His	6-13-14Ala	H(6-13-14)A

The proteins were purified with a HiTrap chelating column. All proteins were of approximately 18 kDa according to SDS-PAGE, but the level of expression of Trx-Aβ40 and its mutants was different. The highest expression was observed in the case of Trx-Aβ40 protein, and the productivity was lower in the case of all mutants. The triple mutant was produced at the lowest level.

The proteins were desalted in dialysis buffer (50 mM Tris-HCl, pH 8.5, containing 100 mM NaCl). It has been known that Aβ42 has a propensity to form aggregates more than Aβ40. This can be explained by conformational differences between those two peptides, although the amino acid sequences differ only in two amino acids in C terminal. Atamna and Boyle have shown that Aβ42 forms a complex with heme, and this interaction dismantles Aβ aggregates [8].

Measuring the interaction between TrxAβ40 and heme, the ratio of concentrations was 1 : 1 (the final concentration 6 μM: 6 μM). The spectra of heme and TrxAβ40 with heme were immediately recorded between 350 nm and 750 nm at room temperature (Fig. 2).

Heme rapidly bound with TrxAβ40 and its mutants, although the binding intensity differed, suggesting that mutated His is important in binding the iron atom of heme. Nevertheless, His is known to bind with heme in several heme proteins; additionally, the amino acid sequence of Aβ contains hydrophobic amino acids (two Leu and three Ile residues) and amino acids Asn and Gln which are usually found in heme-binding pockets of heme proteins [8]. Moreover, there are more histidines in the sequence of TrxAβ40 fusion protein: thioredoxin has two histidines, and there is a 6 His tag between thioredoxin and Aβ40 (Fig. 1).

Trx with 6 His alone tag also interact with heme, but this interaction is weak (data not shown). So, in this case they also can participate in heme binding, although from the differential spectra one can see that His alterations with other amino acids (6His→6Ala, 6Ser, 6Cys; 13His→13Ala, 13Cys, 14His→14Ala) in the Aβ40 sequence influences heme binding. Furthermore, an increase in absorbance was observed at 530–550 nm only in the case of TrxAβ40. This can be explained by some redox reactions between the iron ion of heme and Aβ [10], but more experiments are necessary to elucidate the origin of increase at 530–550 nm. Atamna and Boyle [8] also observed such an increase in this range with Aβ42.

Previously, it has been determined that the Aβ42 and heme complex acts as a peroxidase [8]. We wanted to elucidate whether TrxAβ40 could also bind heme and function as a peroxidase.

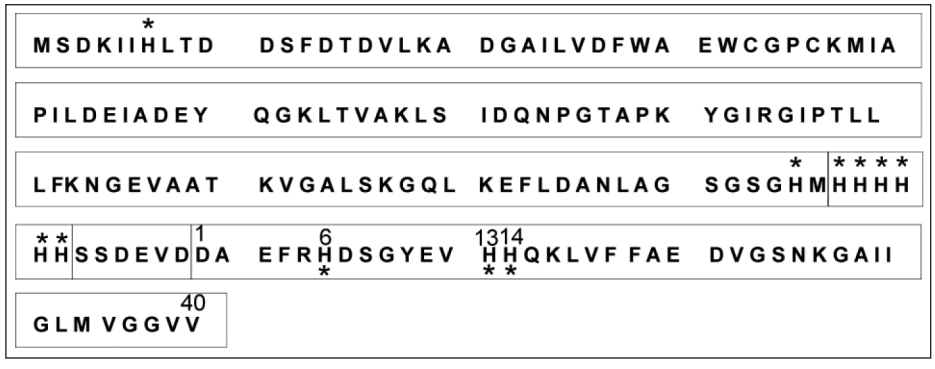

Fig. 1. Sequence of fusion protein Aβ40-Trx. Histidines of Trx and six His tag and Aβ40 are marked with asterisks. Numbers indicate the first and the last amino acid residues and His positions in the Aβ40 peptide

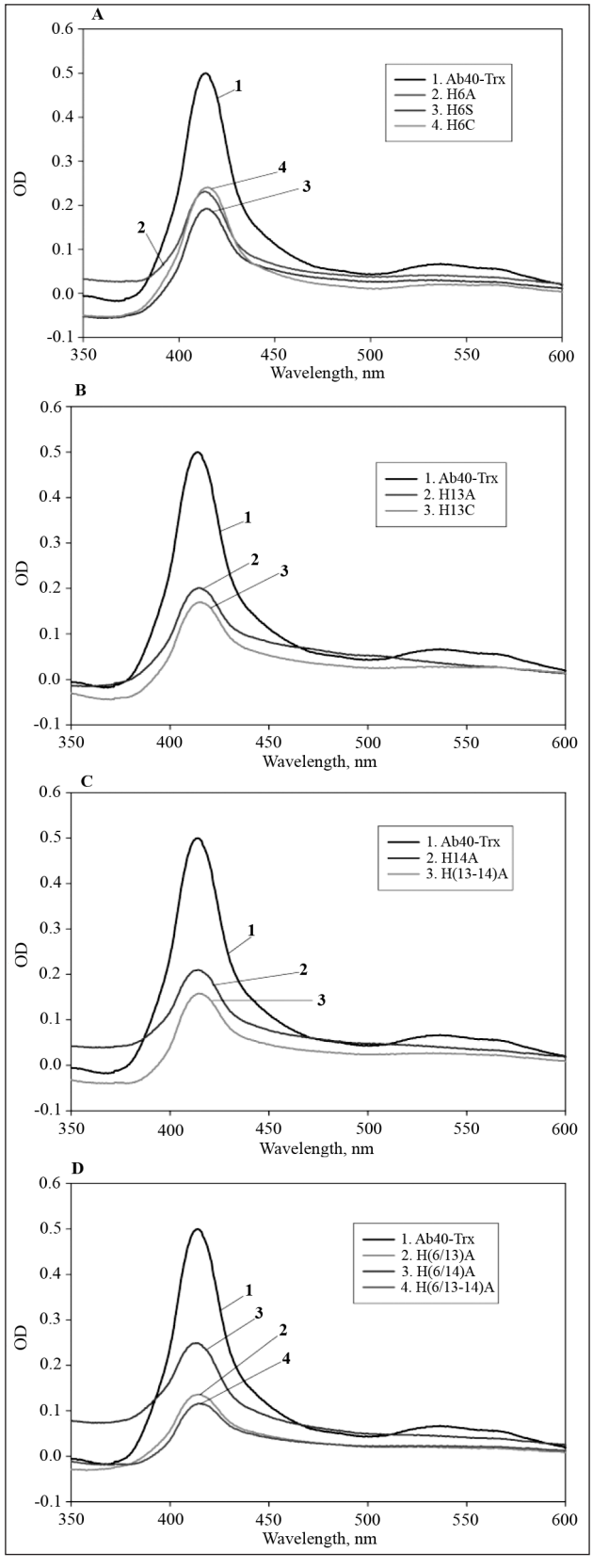

Fig. 2. Differential spectra of fused protein and heme. The spectra were measured between 350 and 750 nm. A – differential spectra of single 6His mutants, B – differential spectra of single 13His mutants, C – differential spectra of single 14His and double 13-14His mutants, D – differential spectra of double 6-13His and 6-14His and triple 6-13-14His mutants

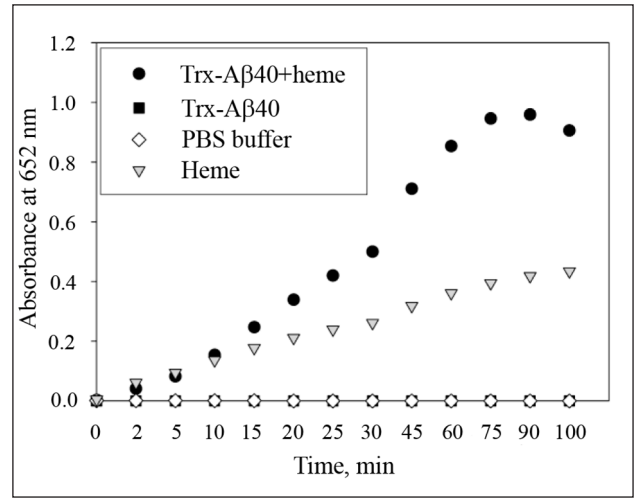

Fig. 3. Peroxidase activity of (Aβ40-Trx) + Heme complex. TMB oxidation by (Aβ40-Trx) + Heme peroxidase was observed as an increase in the absorbance of 652 nm. All tubes contained H_2O_2

Peroxidase activity of the TrxAβ40–heme complex was measured via TMB oxidation (a standard substrate for peroxidases) by H_2O_2 and the following increase in absorbance at 652 nm (Fig. 3).

The amino acid sequence of Aβ contains Arg, His, and Phe, which participate in the H_2O_2 binding and catalyze the heterolytic split of the O–O bond of peroxidase H_2O_2 [9]. Heme deficiency leads to an increased production of H_2O_2 which could serve as a substrate for the peroxidase Aβ-heme [8].

In this work, we have demonstrated that histidine residues in the Aβ40 protein sequence participate in heme binding. The binding efficiency decreased when histidine codons were substituted with other amino acid codons, while the interaction between TrxAβ40 and heme was very strong, showing that Aβ40 is responsible for the interaction of TrxAβ40 with heme. Moreover, the complex of fusion protein TrxAβ40–heme showed peroxidase activity, but more experiments are necessary to fully explain the mechanism of heme binding.

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HIBRIDINIŲ Aβ40-TRX BALTYMŲ SĄVEIKOS SU HEMU TYRIMAS

Santrauka

Aβ40 ir Aβ42 amiloidai yra 40-ies ir 42-iejų amino rūgščių peptidai – pagrindiniai veiksniai, sukeliantys Alzheimerio ligą. Iš literatūros žinoma, kad Aβ42 peptidai gali jungtis su hemu, o susidaręs kompleksas pasižymi peroksidaziniu aktyvumu. Iki šiol nėra aiškus šių peptidų sąveikos su hemu mechanizmas. Hemą surišančiuose baltymuose būtent histidinas dalyvauja prisijungiant hemo sudėtyje esantį geležies atomą. Aβ peptidų sekoje yra trys histidino amino rūgšties liekanos. Šiame darbe, tiriant hibridinių Aβ40-Trx baltymų sąveiką su hemu, buvo konstruojami hibridinio baltymo Aβ40-Trx mutantai, kuriuose histidino amino rūgštis buvo pakeista kitomis amino rūgštimis (alaninu, serinu, cisteinu). Mutantinių hibridinių baltymų analizė rodo, kad Aβ40 peptido sekoje esantys histidinai dalyvauja prisijungiant hemą, tačiau sąveikos mechanizmas dar neaiškus. Susidaręs Aβ40-Trx ir hemo kompleksas taip pat pasižymėjo peroksidaziniu aktyvumu.