PhD. THESIS

Examination of transglutaminase activity of protein disulphide isomerase in *C. elegans*

Bernadett Blaskó

UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
DEPARTMENT OF BIOCHEMISRTY AND MOLECULAR BIOLOGY
DEBRECEN, 2003

1. INTRODUCTION

1.1. Protein disulphide isomerase

Reducing environment can be found within the cell. However, there are some proteins which should be transported to the outer surface of the membrane, or in the intercellular space. Both of these cases originally reduced protein will be oxidized at its final destination. If the disulphide bonds form too quickly, the protein could be stabilized due to not proper disulphide bond formation. Disulphide bond formation in linked to the endoplasmatic reticulum (ER), and the key enzyme is: the protein disulphide isomerase. The protein disulphide isomerase is associated with two kinds of activities: on one hand, they catalyze the disulphide bond formation, izomerization, and reduction, and on the other hand, as the molecular chaperone, help to instable protein with disulphide bond, to gain its correct structure. The proteins of the PDI-family are characterized by a multidomain structure. Each domain has the typical thioredoxin fold, that is: β-α-β-α-β-α-β-β-α. The **a** and **a'** are redox-active thioredoxin fold domains, **b** and **b'** are redox-inactive thioredoxin fold domains, and **c** is a putative calcium-binding domain. The **a** and **a'** domains have high sequence similarity to the thioredoxin, each of which contains one copy of the C-X-X-C active site sequence. The **b** and **b'** domains may have lost the C-X-X-C active site sequence via point mutations during evolution. The redox/isomerase activities of PDI, as in thioredoxin, are due to the reactivity of the N-terminal Cys residue in two thioredoxin-like boxes, within the **a** and **a`** domains of the protein. The **b** domain of PDI have been shown to be responsible for the interaction with other unfolded proteins.

Additional multifunctionality of PDI has been shown that PDI is also able to bind small ligands, like estradiol, 3,3`,5-triiodo-$_L$-thyronine and ATP. It has been also identified as structural subunit of other enzymes, e.g.: prolyl-hydroxyalse and microsomal triglyceride transfer protein complex. PDI has amino acid sequence acting as an ER retention signal , which is KDEL. It was found that ERp57 is present in significant amounts not only in the ER but also in the cytosol. Nevertheless, PDI and ERp57 were detected in the nuclear compartment, and it was also demonstrated that these proteins could influence the DNA binding of transcription factors. PDIs are also present in mitochondria and outside of the cell, either as a secreted proteins or as proteins located on the cell surface.

1.2. Transglutaminases of the lower organism

The Ca^{2+}-dependent transglutaminase enzymes (TGase) crosslink proteins in an acyl transfer reaction. The enzyme catalyzes the crosslinking of proteins through Glu and Lys residue, forms insoluble protein-links in apoptotic cells. The enzyme has a Cys active site, which forms the catalytic triad with His and Asp, similarly to the catalytic triad of papain (cysteine) protease-family. The enzyme reaction is also similar to catalyzed reaction of cysteine proteases, just in a reversed form. In recent years, transglutaminase activity had been detected in several lower organisms (e.g.: bacteria, nematodes, plants). Some of the genes encoding transglutaminases have been successfully cloned from these organisms. Although some TGases from these organism have exhibited no structural and functional similarities to the known mammalian TGases. For example, TGases that have been cloned from two bacteria (*Bacillus subtilis* and *Streptovertcillium mobaraense*) exhibit no homology to any known family of TGase. Similarly, the Cytotoxic necrotizing factor 1 from Escherichia coli and the dermonecrotic toxin from Bordetella pertussis, both of which act by deamidation of a specific glutamine residue in Rho GTPases, contain the catalytic cysteine and histidine residues and catalyze the transglutamination reaction, but have no similarity to mammalian TGases. The N-terminal amino acid sequence of the first TGase homologue that was purified from the *Brugia malayi* nematode showed no homology with any known TGases or other protein sequences in the Genebank database. An antibody raised against a synthetic N-terminal peptide of *Brugia spp*. TGase recognized a 56-kDa protein in immunoblots of crude extract from *Brugia spp*. and other nematode parasites. This antibody was then used to purify TGase from *Dirofilaria immitis*, and then a cDNA clone encoding full-length TGase protein was obtained. The nucleotide sequence or the deduced amino acid sequence of this cDNA clone showed no homology to any of the known TGases. The nematode TGase showed significant homology to a protein disulfide isomerase (PDI)-related endoplasmic reticulum protein, ERp60. Another interesting aspect of nematode TGase was that it contained two distinct regions that are identical to the active-site sequences of the PDI/thioredoxin family of proteins. The protein expressed in *E. coli*, and the recombinant protein had both protein disulfide isomerase and Ca^{2+}-dependent transglutaminase activity. In addition, its activity could be inhibited by TGase-specific inhibitors. Similarly, three isoforms of PDI that were recently isolated from another eukaryote, *Giardia lamblia*, exhibited Ca^{2+}-dependent transglutaminase activity. Biochemical and molecular biological examination of transglutaminases of parasite nematodes have been revealed, that their characteristic features

are very similar to each other, however they are definitely separated from the "classical" transglutaminases, they form a novel family of the enzyme. The identification of these novel TGase proteins with two PDI-like active sites suggests an interesting aspect of protein post-translational modification in primitive forms of life.

Biochemical examination of TGase of *C. elegans* showed that the enzyme was different from mammalian tissue TGase, but it was similar to other enzyme of nematodes. We have little information about evolution of tissue transglutaminase. Upon investigation of through enzyme from some invertebrate species, we could observe, that TGase could exist in homologue (related function and structure) and in analogue form (related function, but different structure) in the living world.

2. AIMS

During our experiments we asked the following questions:

1. *Does the protein disulfide isomerase of C. elegans –similarly to other nematodes-have transglutaminase activity?*

   We tired to carry out our experiment by molecular genetics assets; we would like to produce the recombinant protein in fusion form by means of bacterial expression. We adjusted a new system for isomerase measurement of recombinant PDI. We wanted to test the transglutaminase activity of recombinant protein by known ELISA method. We tried to characterize the recombinant protein from the point of the transglutaminase activity.

2. *If the PDI has the transglutaminase activity, which domain of the enzyme is responsible for the transglutaminase activity?*

   We planed molecular genetic examination of PDI active site. We tried to localize the region which is responsible for transglutaminase activity by different deletion forms of PDI.

3. *We would like to find a molecular biological explanation, how can enzyme catalyze two, different kind of reaction.* We tried to model different protein structure method and to explain the two different kinds of mechanism catalyzation.

METHODS

2.1. Plasmid constructs, expression and purification of recombinant PDI.

PDI-3 was amplified with Pfu polymerase using the yk83g12 EST-clone as a template and subcloned into pGEX-4T3 applying SalI and NotI linkers. This construct was transformed to The GST-PDI-3 fusion protein (rPDI) was over expressed in and purified from cell free extract of *E. coli* JM83 by affinity chromatography using glutathione agarose. Site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit) was utilized for the generation of single and double mutant enzymes.

2.2. PDI enzyme assays.

PDI activity was determined by the measurement of the reactivation of reduced, denaturated RNase. PDI catalyses inter-and intramolecular disulfides in scrambled RNase, results in regain of native disulfide pairing, native conformation. The formation of active RNase was measured spectrophotometrically by monitoring hydrolysis of the RNase substrate, cCMP, at 296 nm as described.

2.3. DsbA complementation.

E. coli dsbA^{-} mutants have defects in the formation of functional alkaline phosphatase that requires disulfide bond formation for proper folding in the periplasm. *C. elegans* PDI-3 was subcloned into pMALp2 for targeting the periplasmatic space of *E. coli.* The construct was transformed into *E. coli* dsbA^{-} strain, and alkaline phosphatase activity of bacterial extract was measured to detect complementation.

2.4. Transglutaminase assays.

Transglutaminase activity of rPDI was detected by a microtiter plate assay measuring the incorporation of biotinylated-pentylamine (1 mM) into dimethylated casein in the presence of 5mM Ca^{2+}, 10 mM DTT, at pH 8.5. The reaction mixture was incubated at 55 °C for 1h, and then the covalently incorporated amine substrate was detected by an indicator reaction using streptavidine/alkaline phosphatase measuring the absorbance at 405 nm following the addition of p-nitrophenyl phosphate. The above assay was also carried out in test tubes using casein at 20mg/ml concentration and aliquots from the reaction mixture were dropped onto an Immobilon^{TM}-P Transfer membrane (Millipore); following the addition of avidin and

horseradish peroxidase-conjugated anti-mouse IgG (Vectastain ABC Kit, Vector Laboratories) signals were detected by ECL (Amersham Pharmacia Biotech). Transglutaminase activity was also measured by the colometric hydroxamate procedure using Z-glutaminylglycine (10 mM) and hydroxylamine (50 mM) as substrates in presence of 5mM Ca^{2+} at pH 6.0.

2.5. Chemical modification of protein-bound histidine.

Modification of exposed histidine residue was carried out using diethyl-pyrocarbonate in 50 mM sodium phosphate buffer, pH 6.8, at 25 °C.

2.6. Sequence alignment and homologue modeling.

Representative set of sequences from different species were used to generate a multiple alignment with CLUSTAL program. Using the structure of the **a** domain of human PDI as a template and the Swiss-PdbViewer program, we generated a homology model for the *C. elegans* PDI-3 proteins.

2.7. Spectroscopic technique.

Circular dichroism (CD) spectra were recorded on a Jasco-810 spectropolarimeter equipped with thermostatic cell holder (RTE-111, NesLab). Samples were at a concentration of 5 μM (PDI) and 25 μM (Trx) in 0.1 M Tris-HCl, pH 8.5.

4. RESULTS AND DISCUSSION

4.1. Biochemical and molecular genetic examination of recombinant PDI-3

There are three different PDIs in the *C. elegans* genome. We wanted to select the most homologue gene to ERp60, so we made multiple sequence alignments. PDI-3 was more similar to ERp60 than to *C. elegans* PDI-1, PDI-2. We identified the cDNA clone of PDI-3 in *C. elegans* Genome Sequencing Center (yk83g12). We cloned PDI-3 into an IPTG-inducible GST-fusion expression vector, and the recombinant protein was expressed and purified.

The isomerase activity of the recombinant PDI-3 was measured using an RNase refolding assay. PDI-3 was able to refold denaturated RNase, and therefore has disulfide isomerase activity.

It was also verified that *C. elegans* PDI-3 could complement the $dsbA^-$ defective *E. coli* mutant JCB571. DsbA is the *E. coli* periplasmatic PDI homologue, and the $dsbA^-$ mutants have defects in the formation of functional alkaline phosphatase, that require disulfide bond formation in the periplasm. PDI-3 was cloned into a pMALp2 vector for targeting to the periplasmic space. After induction, PDI-3 could complement $dsbA^-$ strain, the alkaline phosphatase activity of bacterial extract was measured to detect complementation.

PDI-3, similarly to previously studied *C. elegans* PDIs, have shown transglutaminase activity in an indirect ELISA assay incorporating biotinylated pentylamine into surface-bound casein in the presence of Ca^{2+} with a temperature optimum of 55°C. We repeated the measurement at 37°C and 25°C, but in both cases the transglutaminase activity was below the detection level. This activity could be inhibited by iodoacetamide, $MgCl_2$ and NH_4Cl as it had been observed in case of TGase activity of *C. elegans* tissue homogenate.

Incorporation of primary amines into proteins may occur spontaneously breaking internal thioester bonds, as it occurs in human α_2-macroglobulin. However, PDI-3 did not incorporate the biotinylated amine into itself, but catalyzed transamidation of casein as shown in dot blot demonstration. To exclude the possibility that other than glutamine residues of casein are modified by the enzyme, transglutaminase activity was also demonstrated by a colorimetric assay using CBZ-L-glutaminylglycine as the amine acceptor and hydoxylamine as the amine donor. In this assay the specific activity of PDI-3 was 12.3 μmol/min/μg enzyme protein comparable to that of guinea pig liver transglutaminase (34.7 μmol/min/μg). K_M values for the two enzymes were found to be 0.33 mM and 0.62 mM, respectively.

4.2. TGase activity of recombinant PDI-3 is linked to its thioredoxin domains

To determine which domain of PDI-3 was responsible for transglutaminase activity several deletion mutants were produced. Analysis of these deletion derivatives clearly demonstrated that only mutants containing either the **a** or **a`** or both **a** and **a`** domains showed transglutaminase activity. This result was in agreement with the finding that one isoform of the three PDIs from *G. lamblia* (gPDI-3), where the small 13 kDa protein consists of only a thioredoxin domain, had TGase activity.

C. elegans PDI-3 has two active site CysGlyHisCys sequences present in both the **a** and **a`** domains. The first Cys residues in each of these thioredoxin motifs are responsible for initiating disulphide isomerase activity of PDIs. Using site-directed mutagenesis these Cys residues were replaced with alanine in both the **a** and **a'** domains and the activity of various forms ($rPDI_{\mathbf{A}GHC\text{-}C'GHC}$, $rPDI_{C'GHC\text{-}\mathbf{A}GHC}$ and $rPDI_{\mathbf{A}GHC\text{-}\mathbf{A}GHC}$) were compared to that of the the wild type ($rPDI_{C'GHC\text{-}C'GHC}$). Isomerase activity of the single-mutant enzyme was half of the wild type and the double-mutant enzyme was inactive. However, there were no significant differences between the measured TGase activities of the wild type and mutant forms. Therefore, the active site of the transglutaminase reaction in PDI-3 is different from the active site essential for initiating disulphide isomerase activity.

4.3. Determination of essential catalytic residues of TGase activity within thioredoxin domain

To clarify whether the active site for the detected transglutaminase activity is the second cysteine of the thioredoxin motif several mutants (**a** $_{AGHC,}$ **a** $_{CGH\mathbf{A}}$ and $\mathbf{a}_{\mathbf{A}GH\mathbf{A}}$ **)** of the rPDI-3 **a** domain were prepared and compared to $\mathbf{a}_{CGHC}$. Elimination of the second cysteine in the CysGlyHisCys motif resulted in loss of TGase activity as determined by both the indirect ELISA and the hydroxamate assays suggesting that this is the cysteine which serves as the active site for the transamidation reaction.

Transglutaminases usually require three properly positioned specific residues for their catalytic activity, such as the Cys-277, His-335, Asp-358 catalytic triad in the recently revealed structure of human TGase 2. In each of the thioredoxin domain of *C. elegans* PDI-3 there is only one histidine residue located within the thioredoxin motif. When this histidine

was modified with the group-specific reagent diethyl-pyrocarbonate almost complete loss of transglutaminase activity was observed.

The thioredoxin domains of PDIs are closely related to the thioredoxin proteins which are protein disulphide reductases utilizing CXXC thioredoxin motifs in their active site. Assaying recombinant *E. coli* and human thioredoxin (each contains a single His residue in addition to the thioredoxin motif) it was found that they also displayed Ca^{2+}-dependent TGase activity.

4.4. PDI and Trx aminoacid sequence alignment

Recently, several publications demonstrated that PDIs of both higher and lower organisms can act as TGases. Presumably, PDIs may form a transglutaminase-like catalytic triad within their structure. As it is shown on the sequence multiple alignment of PDIs from representative species the Cys residues and His of the CGHC motif are absolutely conserved. We have also found two Asp-rich conserved regions in all **a** domains of the PDIs; the conservation was particularly strong in the one located at C-terminal direction from the CGHC box.

The possibility that Trxs can also act as TGases has emerged after TGase activity of PDIs was linked to their thioredoxin domains. Sequence analysis of Trxs reveals that their thioredoxin box contains the distal Cys needed for TGase activity. In the N-terminal direction from this box conserved His residues can be found which are very likely the ones which participate in transglutamylation. The third member of the catalytic triad is also available since there are conserved Asp residues located in both the N- and C-terminal directions from the thioredoxin box.

4.5. Potential transglutaminase active site regions in protein disulphide isomerases and thioredoxins

Although experimental and sequence data have provided some details on the catalytic mechanism of TGase activities displayed by PDIs and Trx, final evidence of their resemblance to the that of the traditional TGases can be obtained only by studying the 3D structure of these proteins. Using the known 3 D structure of the human PDI **a** domain is known the homologue model of the *C. elegans* PDI-3 could be obtained. Both structures

reveal that the catalytic residues for TGase activity are accessible for substrate proteins and can be found in the same region.

The human and bacterial Trx structures are also known. The catalytic triad for the TGase reaction could be found around the thioredoxin box in these proteins, too.

The distances between these catalytic residues in the human and nematode PDI **a** domains are 7.0 and 7.5 Å for Cys-His. The distances between the participating residues are also larger than those in the traditional TGases 8.2 and 9.8 Å for Cys-His in the human and bacterial Trx, respectively. The corresponding data for human TG2 the distance is 3.9 Å. Therefore, in both case of PDIs and thioredoxins the induction of a conformational change is necessary for the movement of these residues to a close enough proximity for transglutamylation.

4.6. Conformational change of PDI and Trx

The above detailed 3 dimensional analysis clearly show that both PDI **a** domains and Trxs need conformational changes before being capable of efficient catalyses of TGase reactions. These changes may be induced by the binding of Ca^{2+} to these enzymes and/or by the higher temperature required for optimal TGase activity of both PDIs and Trxs. In case of PDIs the **b** and **c** domains may contribute to the induction and maintaining of an optimal structure for transglutamylation (particularly the presumed Ca^{2+}-binding site of **c** domains) - though the **a** domain by itself is sufficient for the activity. The suggested conformational changes occurring in either PDI or Trx has been supported by our circular dichroism measurements. Curves of the CD spectrum showing characteristic features of α-helices, which are one of the dominant structural elements in the thioredoxin fold, have significantly changed upon heating the enzyme solutions to 55^{o}C, that is the temperature optimal for the TGase reaction.

4.7. PDI and Trx, as multifunctional enzymes

It is quite a general phenomenon that proteins have multiple functional activities including the presence of related or completely unrelated enzymatic activities. The logical question arises whether the TGase activities of PDIs and Trxs are linked or not to the so far recognized physiological functions of these proteins. First of all, it should be realized that members of the three superfamilies have more than one overlapping functional activities. In

addition to transglutamylation they are all capable to act as redox catalysts/proteins; TGases have unusually large number of free cysteine-SH residues which are very reactive and indeed, the natural redox function of PDIs and Trx has been also displayed by a transglutaminase. Furthermore, the non-catalytic interaction of TGases with proteins both inside and outside of cells has raised the strong possibility of their chaperoning function which has been already described for PDIs and Trxs. While no direct evidence has been provided one may presume that in case of unfolded, damaged, and oxidized proteins a rescue process is attempted by the isomerase and/or chaperon activities of PDIs or by the disulphide reductase function of Trxs. These rescue attempts may include stabilization of the target protein by transglutamylation (deamidation, crosslinking, incorporation of primary amines) as well provided by either the TGase activity of PDIs and Trxs. TGases themselves may act on proteins as redox catalysts, chaperons and crosslinking enzymes. It may be also presumed that when the protective attempts made by these multifunctional proteins fail, they crosslink the damaged proteins – perhaps in parallel to ubiquitination sending them to their final destination in the proteasomal system.

Several additional shared characteristics of the PDI, Trx and TGase family members should be also noticed. They are present in all types of living organisms, and in all compartments of eukaryotic cells - including their release into the extracellular space following secretion in spite of the lack of signal sequence. They are also inducible enzymes responding to – among other signals – stress conditions, oxygen radicals, cellular and tissue damages. Based on all these data it is tempting to speculate that these three big protein/enzyme families are part of a general cellular defense and protective system into which convergent evolution has brought together gene products to perform a broad variety of shared enzyme activities for the purpose of protecting and stabilizing proteins, cells and tissues.

PDI and Trx proteins are in large quantities inside and outside of the cell and on the cell surface. These proteins have two different activities in bacteria (*E. coli* Trx), in nematode (*C. elegans*) and in vertebrate (human Trx, PDI) shows conserved function in the protein motif. Several reaction of PDI is already known: reduction, isomerization, chaperone function, consequently a kind of post-translational modification. The role of thioredoxin is also wide: redox signaling, photosynthesis, regulation of apoptosis, development and immune modulation. More and more transglutaminase function become well-known: cross-linking protein, signal protein, (as a G-protein) role in apoptosis, and in phagocytosis. The role of

proteins have not yet been cleared up, to reveal its regulation, interaction – that decide which function of protein “ switch on”- may be a big step within in vivo research.

Papers, serving the basis of the thesis

Blasko B, Madi A, Fesus L. (2003) Thioredoxin motif of *Caenorhabditis elegans* PDI-3 provides Cys and His catalytic residues for transglutaminase activity. *Biochem. Biophys. Res. Commun.* 303, 1142-1147. IF: 3.26

Blasko B, Madi A, Fesus L. (2003) Structural elements responsible for transglutaminase activity of protein disulphide isomerases and thioredoxins. *J. Biol. Reg. Homeo. Ag.* (accepted) IF: 0.71

Other papers

Szegezdi E, Kiss I, Simon A, **Blasko B**, Reichert U, Michel S, Sandor M, Fesus L, Szondy Z. (2003) Ligation of retinoic acid receptor alpha regulates negative selection of thymocytes by inhibiting both DNA binding of nur77 and synthesis of bim. *J. Immunol.* 170, 3577-84. IF: 7.06

Posters and lectures on national and international conferences

Blaskó B., Kovács A., Mádi A. és Fésüs L.: Characterization of protein disulphide isomerase in *Caenorhabditis elegans*, Sárospatak, 2001. (poster)

Blaskó B., Kovács A., Mádi A. és Fésüs L.: Characterization of protein disulphide isomerase in *Caenorhabditis elegans*, Siófok, 2002. (poster)

Blaskó B.: Does protein disulfide isomerase of *Caenorhabditis elegans* function as a transglutaminase? TDK/PhD conference, Debrecen, 2002. (lecture)

B. Blaskó, A. Mádi and L. Fésüs: The *C. elegans* transglutaminase; is it the protein disulfide isomerase? COST 884 conference, Nottingham, 2002. (lecture)

B. Blaskó, A. Mádi and L. Fésüs: Does protein disulfide isomerase of *Caenorhabditis elegans* function as a transglutaminase? 7th International Conference on Transglutaminases and Protein Crosslinking Reactions, Ferrara, 2002. (lecture)

Blaskó B.: Transglutaminase activity of protein disulfide isomerase is linked to the thioredoixn motif, TDK/PhD conference, Debrecen, 2003. (lecture)

Blaskó B., Mádi A. és Fésüs L.: Determination of critical aminoacids for transglutaminase activity of protein disulfide isomerase. Tihany, 2003. (poster)

B. Blaskó, A. Mádi and L. Fésüs: Structural basis of the transglutaminase activity exhibited by protein disulphide isomerases and thioredoxins. 2nd Japanese-Hungarian Transglutaminase Conference, Hévíz, 2003. (lecture)