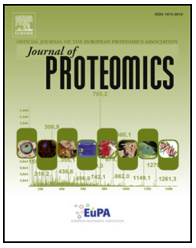
JOURNAL OF PROTEOMICS XX (2014) XXX–XXX

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot



Probing the structure of human protein disulfide isomerase by chemical cross-linking combined with mass spectrometry

Li Peng[a], Morten Ib Rasmussen[a], Anna Chailyan[a], Gunnar Houen[b], Peter Højrup[a,*]

[a]Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark
[b]Department of Clinical Biochemistry, Immunology and Genetics, Statens Serum Institut, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 10 February 2014
Accepted 24 April 2014

Keywords:
Cross-linking
BS3
Protein structure
Calnexin
ERp72
Placenta

ABSTRACT

Protein disulfide-isomerase (PDI) is a four-domain flexible protein that catalyzes the formation of disulfide bonds in the endoplasmic reticulum. Here we have analyzed native PDI purified from human placenta by chemical cross-linking followed by mass spectrometry (CXMS). In addition to PDI the sample contained soluble calnexin and ERp72. Extensive cross-linking was observed within the PDI molecule, both intra- and inter-domain, as well as between the different components in the mixture. The high sensitivity of the analysis in the current experiments, combined with a likely promiscuous interaction pattern of the involved proteins, revealed relatively densely populated cross-link heat maps. The established X-ray structure of the monomeric PDI could be confirmed; however, the dimer as presented in the existing models does not seem to be prevalent in solution as modeling on the observed cross-links revealed new models of dimeric PDI. The observed inter-protein cross-links confirmed the existence of a peptide binding area on calnexin that binds strongly both PDI and ERp72. On the other hand, interaction sites on PDI and ERp72 could not be uniquely identified, indicating a more non-specific interaction pattern.

Biological significance
The present work demonstrates the use of chemical cross-linking and mass spectrometry (CXMS) for the determination of a solution structure of natural human PDI and its interaction with the chaperones ERp72 and calnexin. The data shows that the dimeric structure of PDI may be more diverse than indicated by present models. We further observe that the temperature influences the cross-linking pattern of PDI, but this does not influence the overall folding pattern of the molecule.

1. Introduction

Elucidating the structure of proteins is an essential part of molecular structure-function studies. However, despite the fact that the amino acid sequence is known for most proteins of many species, the structure of many proteins remains unknown or only partly characterized. This is due mainly to limitations in the availability of the proteins and in the methods used for protein structure elucidation.

X-ray crystallography has for many years been the major method for detailed structure elucidation of proteins [1–6]. This method requires relatively large amounts of pure protein

* *Corresponding author*. Fax: +45 65 50 24 67.
E-mail address: php@bmb.sdu.dk (P. Højrup).

http://dx.doi.org/10.1016/j.jprot.2014.04.037
1874-3919/

and is limited to proteins, which can be made to form suitable crystals. Large amounts of protein can usually be obtained by recombinant technology; however, this often does not lead to suitable crystals. Obstacles to this include inherent mobility or flexibility in multi-domain proteins, heterogeneity, or a lack of a well-defined structure. In such cases, structural information may be obtained for crystallizable domains or for defined parts of the protein. Even so, X- ray crystallography can only give information about solid state structures of proteins and for many proteins, particularly enzymes, molecular dynamics is vital to the function, e.g. for PDI, which must be able to break improper disulfide bonds and to join the proper ones.

Protein disulfide isomerase (PDI), which is a 57 kDa protein composed of four thioredoxin domains, two oxido-reductase domains (**a** and **a′**) with CGHC active sites, two non-catalytic domains (**b** and **b′**), a linker region between domains **b′** and **a′**, and a C-terminal acidic tail [7–9]. The main responsibility of disulfide bonds is to stabilize protein structures, while in some proteins the cysteines perform redox regulation of enzymatic activity. Approximately one third of human proteins contain disulfide bonds [10], and the formation of disulfide bond limits the rate of protein synthesis. Protein disulfide isomerase (PDI), was published in 1963 as the first folding catalyst [11]. It catalyzes disulfide bond formation, breakage and rearrangement, and thus assists proper protein folding [12,13]. PDI is mainly expressed in the endoplasmic reticulum (ER), but also on the cell surfaces of lymphocytes, hepatocytes, and platelets [14,15]. In the ER It also takes part in peptide loading onto major histocompatibility complex class I [16], as well as regulating NAD(P)H oxidase [17].

Although PDI was discovered more than 50 years ago, a complete X-ray structure was not solved until 7 years ago for yeast PDI (yPDI) [18] and has only recently been obtained for human (hPDI) PDI [19]. On the way to solve the full structure, X-ray crystallography models for individual domains were solved and yielded important information about the active sites and substrate binding sites [18,20–22].

Nuclear magnetic resonance (NMR) spectroscopy can yield information about structure and dynamics for proteins in solution and can be used for proteins up to 100 kDa but also requires large amounts of pure protein, which must not aggregate in solution [23–32]. For PDI, NMR spectroscopy has yielded important information about the structure and dynamics of several domains [33–38] but not yet the whole enzyme.

Small angle X-ray scattering (SAXS) can yield low resolution structural information for proteins in solution, but has the same requirements for sample amount and purity as NMR spectroscopy [31,39–43]. For PDI, SAXS has shown an annular arrangement of the four domains [44].

Thus, the above methods each have their advantages and disadvantages, but all are challenged by the requirement for sample purity. Here, we show, using PDI and other endoplasmic reticulum (ER) proteins, that chemical cross-linking in combination with mass spectrometry (CXMS) can yield relevant information about the structure, internal dynamics and intermolecular interactions for low concentrations of mixtures of native proteins in solution. CXMS is particularly useful in combination with known structural models obtained by the methods described above and when handling proteins from natural sources, that have not been obtained in a very high purity.

2. Materials and methods

2.1. Materials

NaH_2PO_4, Na_2HPO_4, NH_4HCO_3, CH_3CN and TFA were from Sigma Aldrich (St. Louis, MO). Bis[sulfosuccinimidyl] suberate (BS^3) was obtained from Thermo Scientific Pierce (Rockford, IL). Porcine trypsin was a gift from Novo Nordisk (Copenhagen, Denmark). Spin filters were from Sertorius Stedim Biotech (Bohemia, NY). Poros Oligo R2 and R3 50 were from Applied Biosystems (Foster City, CA). ReproSil Pur C18 AQ 3 μm was from Dr Maisch GMBH (Ammerbuch-Entringen, Germany). Pure water was obtained from an Elga PureLab Flex (Marlow, UK).

2.2. Purification of PDI

PDI was obtained during purification of human placenta chaperones as described elsewhere [45]. In short, a placenta was homogenized with Triton X-114, the supernatant was separated by temperature-dependent phase separation, and the supernatant from this was precipitated by ammonium sulfate. The new supernatant was ultradiafiltrated, and the retentate was applied to a Q Sepharose Fast Flow column equilibrated with 20 mM Tris, 1 mM $CaCl_2$, and pH 7.5. Proteins were eluted with a gradient of NaCl (0–0.5 M). Eluted proteins were monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked immunosorbent assay (ELISA) using antibodies directed against calreticulin, calnexin, ERp57, ERp72 and PDI. The fraction used for the current analysis corresponds to fractions 132–136 in Fig. 3 of [37]. Two different preparations were used, one for the room temperature experiment, and one for the low/high temperature experiments.

2.3. Cross-linking

50 μg sample (fraction 132–136) was transferred to phosphate buffer (25 mM, pH 7.5) to a concentration of 2 μg/μL using 10 kDa spin filter. Before adding cross-linkers, samples were equilibrated for 30 min under different temperatures, 0 °C on ice water, room temperature and 37 °C separately in a thermostat. Following equilibration, an equal volume of phosphate buffer containing 2500 μg BS^3 was added to the samples to a final ratio of 1:50 w/w protein: cross-linker (approximate molar ratio of 1:376), and the cross-linking reactions were carried out under the same temperatures as the equilibration step. After 1 h, the reaction was terminated by the addition of 1 M NH_4HCO_3 to a final concentration of 20 mM NH_4HCO_3. After 20 min, the samples were reduced, alkylated and digested by 2% trypsin w/w at 37 °C overnight. Two different batches containing the same proteins in different amounts were used, one for the room temperature assay and another for the 0 °C and 37 °C experiments.

Please cite this article as: Peng L, et al, Probing the structure of human protein disulfide isomerase by chemical cross-linking combined with mass spectrometry, J Prot (2014), http://dx.doi.org/10.1016/j.jprot.2014.04.037