Study on the interactions between protein disulfide isomerase and target proteins, using immobilization on solid support

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Abstract Interaction between protein disulfide isomerase, possessing not only isomerase but also chaperone-like activity, and olygomeric enzyme, GAPDH, has been studied using technique of immobilization on insoluble support. PDI dimers bound to CNBr-activated Sepharose were shown to posses high TPOR activity as well as the ability to reactivate lysozyme. Immobilized PDI was not found to interact neither with soluble tetrameric GAPDH, nor with soluble denatured GAPDH. However, soluble PDI binds effectively to immobilized GAPDH monomers; K_d was found to be 3.7×10^{-6} M, stoichiometry 0.824 mole PDI monomers per mole GAPDH monomers. Immobilized GAPDH tetramers do not interact with PDI. These observations are also confirmed by the data on electrophoresis of proteins bound to immobilized GAPDH monomers and tetramers. The ability of PDI to interact with denatured protein form, but not with the native one, is considered to be evidence of chaperone-like activity of the enzyme.

Key words: Protein disulfide isomerase; Glyceraldehyde-3-phosphate dehydrogenase; Chaperone; Protein immobilization

1. Introduction

It is now generally accepted that the recognition and binding of folding intermediates of either denatured or nascent polypeptide chains by chaperones is the first step of chaperone-assisted protein folding [1]. Complexes formed by different chaperones and their target proteins have been characterized by a variety of methods. A stable complex of GroEL with the refolding intermediate of dihydrofolate reductase was identified using a HPLC sizing column [2]. A binary complex between GroEL and partially folded rhodanese was characterized by SDS-PAGE [3]. A number of complexes formed by small heat shock proteins [4], including α-crystalline [5], SecB [6], TCP1 complex [7], with their target proteins have also been characterized with many other techniques. The crystal structure of the complex formed by PapD, periplasmic chaperone, with a pilus subunit has been analyzed to reveal the recognition mechanism [8]. Recently, the crystal structure of GroEL fragment (191–376) with a 17-residue N-terminal tag has been solved at 1.7 Å [9]. However, it is not always possible to capture chaperone-target protein complexes by using standard methods, such as sedimentation analysis, gel-filtration, cross-linking etc., because complexes are often unstable.

Protein disulfide isomerase (PDI) is one of the two foldases thus far characterized, assisting protein folding by catalyzing the joining of thiol groups distantly situated in the peptide sequence to form correct disulfide bonds in nascent peptides [10]. It has been suggested to be not only an isomerase but also a molecular chaperone [11]. The in vivo and the in vitro chaperone-like activity of PDI has obtained more and more experimental support [12]. In order to distinguish explicitly the possible chaperone activity of PDI from its isomerase activity in assisting protein folding, protein disulfide D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [13] or rhodanese [14], were employed as target proteins. The presence of PDI in the refolding system at near stoichiometric instead of catalytic amounts indeed increases greatly reactivation yield of both guanidine hydrochloride denatured enzymes upon dilution, and suppresses their aggregation during refolding without being a part of the final functional structure. However, no stable complex between PDI and its target protein has so far been observed. It is quite possible that the interaction of PDI with folding intermediates is not so strong as that of GroEL or other aforementioned chaperones with targets, which lead to formation of stable binary complexes, but is like the interactions formed by other ATP-independent chaperones, such as Hsp-90, that might be transient and not requiring ATP for dissociation.

In this study, we have used the technique of immobilization to investigate the interaction of PDI with its target proteins and to get the direct evidence of binding of PDI with the proteins without disulfide bonds. The immobilized PDI was shown to be able to catalyze oxidative reactivation of disulfide-containing proteins, i.e. to posses the isomerase activity. Not any interaction was discovered between PDI and immobilized tetramers of GAPDH, the enzyme containing no disulfide bonds, but PDI appeared to bind tightly to the denatured immobilized monomeric form of GAPDH. These facts are the additional evidence of the chaperone-like activity of PDI.

2. Materials and methods

Hen egg white lysozyme was purchased from Serva. Sepharose 4B was purchased from Pharmacia. Glutathione (GSH), glutathione disulfide (GSSG), NAD^+ (98%) and NADPH (type III) were from Boehringer Mannheim. HEPES, glycine, EDTA, DTT, glutathione reductase (yeast, type III), glyceraldehyde-3-phosphate (GAP), bovine serum albumin (BSA, 98–99% albumin, fraction V), guanidine hydrochloride (GdnHCl), micrococcus lysodeikticus dried cells and insulin were Sigma products. Cyanogen bromide (CNBr) was freshly synthesized from potassium cyanogen and bromide. All other chemicals were the local products of analytical grade.

PDI was prepared from bovine liver essentially according to Lam-

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Abbreviations: PDI, protein disulfide isomerase; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; TPOR, thiol-protein oxidoreductase

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bert and Freedman [15] and showed one band on SDS-PAGE with a specific activity of more than 800 units/g. GAPDH prepared from rabbit muscle [16] was a generous gift from Ms. X.L. Li of the National Lab of Biomacromolecules, Beijing.

The concentrations of PDI, GAPDH, lysozyme were determined spectrophotometrically at 280 nm with the following absorption coefficients ($A^{0.1\%}_{1\,cm}$): 0.94 for PDI, 0.98 for GAPDH, 2.63 for native lysozyme and 2.37 for denatured lysozyme [17] or according to Bradford [18].

Immobilization of GAPDH, PDI or BSA on CNBr-activated Sepharose was carried out as previously described [19]; 5 or 26 mg CNBr per ml of the packed Sepharose gel were used to activate the matrix. A modified Bradford procedure [20] was employed to determine the concentrations of immobilized protein preparations. Immobilized monomeric and tetrameric GAPDH were prepared according to [21].

Denaturation and activity assay of lysozyme were performed according to Dai and Wang [22].

Renaturation of lysozyme was done as follows.

Batch mode. Refolding of denatured lysozyme was performed by diluting a denatured lysozyme solution to a final concentration of 10 μM with 100 mM sodium phosphate buffer, pH 7.5, containing 1 mM GSSG and 2 mM GSH in the presence or absence of immobilized PDI. The activity recovery was completed and determined 1 h after dilution.

Column mode. The reduced and denatured lysozyme was diluted to a final concentration of 50 μM with acetic acid, pH 4, and 100 μl were immediately loaded onto an immobilized PDI column (0.8×5 cm) or an immobilized BSA column. The column was eluted with 0.1 mM phosphate buffer, pH 7.5, at 0.1 ml/min. The fractions at 0.5 ml were collected and aliquots were taken for protein concentration and activity assays. The time delay of the appearance of the protein peak was taken as an indicator of the interaction between the soluble and immobilized proteins.

Denaturation and refolding of GAPDH were carried out according to Cai et al. [13].

The thiol-protein oxidoreductase (TPOR) activity of PDI was assayed according to Lambert and Freedmen [15] except that continuous stirring was necessary during the activity assay of immobilized PDI.

3. Results and discussion

3.1. Characterization of PDI immobilized on Sepharose 4B

Two PDI preparations immobilized on Sepharose 4B activated using different concentrations of CNBr have been prepared, and the amount of immobilized PDI was found to be 0.304 mg per ml settled gel (in the case of 5 mg CNBr used to activate 1 g wet gel) and 0.430 mg per ml (26 mg CNBr per 1 g wet gel), respectively. Treatment of the PDI immobilized on highly activated Sepharose, with 8 M urea did not alter the amount of protein bound to the matrix, indicating that both subunits of the PDI molecule were immobilized covalently. In contrast, 40–45% of PDI immobilized on the less activated gel was removed by 8 M urea treatment, indicating that only one subunit of dimeric PDI was covalently bound. In the present work the latter preparation has been used and showed TPOR activity of 0.074 unit/mg, which is 42% of soluble PDI (0.176 unit/mg).

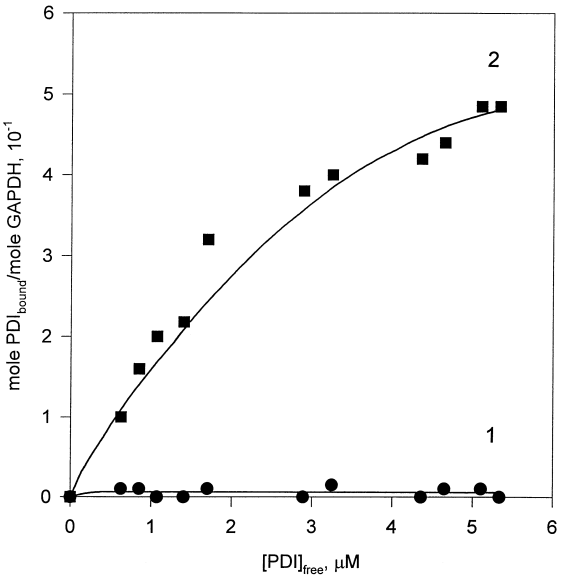

Fig. 1. Binding of soluble PDI with immobilized on Sepharose tetramers (7.8 μM) (1, ●) and denatured monomers of GAPDH (2.0 μM) (2, ■) in 50 mM Tris-HCl buffer, containing 2 mM EDTA, 1 mm DTT, pH 7.5, at 4°C. The experimental results are shown as moles of bound PDI (calculated per monomer with molecular mass 57 kDa) per mole of immobilized GAPDH (calculated per monomer with molecular mass 36 kDa) as a function of free PDI concentration. The solid lines are calculated for a simple one-site binding model with dissociation constant of 3.7×10^{-6} M and stoichiometry of 0.825.

3.2. Lysozyme reactivation assisted by immobilized PDI

As shown in Table 1, the presence of stoichiometric amounts of soluble PDI in the refolding solution increased the extent of reactivation of the full denatured lysozyme from 9% to 35%. In the batch mode the presence of immobilized PDI at 1.75 μM concentration increased the extent of reactivation of lysozyme (10 μM) from 16% (spontaneous reactivation in the presence of a control amount of Sepharose 4B gel) to 25.6% and to 33% if 2.28 μM immobilized PDI was added. Obviously, the immobilized PDI showed some, but not very significant effect on the reactivation of lysozyme probably because of its low concentration. It was difficult to further increase PDI concentration using the present method (batch mode).

In column mode, 55–60% of total amount of the applied protein (preliminary full denatured lysozyme) was recovered by elution and showed 80–90% of that of the native lysozyme. Elution of the lysozyme from the column with immobilized bovine serum albumin (control) resulted in 30–35% recovery of total protein applied.

Noteworthy, the investigation of the immobilized PDI influence on lysozyme was somewhat complicated by the fact

Table 1
Lysozyme reactivation in the presence of soluble and immobilized PDI

	PDI concentration (μM)	Lysozyme reactivation (%)[a]
Control	0	9
Soluble PDI	10	35
Control Sepharose	0	16
Immobilized PDI	1.75	25.6
Immobilized PDI	2.28	33

[a]Lysozyme concentration 10 μM.

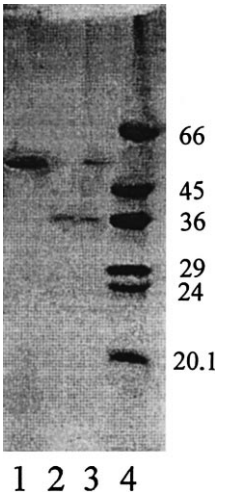

Fig. 2. SDS-PAGE (11%) by Laemmli method modification ('Sigma', Technical Bulletin No. MWS-877L) of PDI bounded with immobilized denaturated monomeric forms of rabbit muscle GAPDH. Protein bands were stained with Coomassie brilliant blue R. Lane 1: Soluble PDI 57 kDa (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT): 8 μg of the pure protein. Lanes 2, 3: PDI was incubated with tetramers (lane 2) or monomers (lane 3) of GAPDH immobilized on Sepharose 4B 60 min at 4°C. Samples obtained after washing of the gel with 50 mM Tris-HCl buffer, containing 2 mM EDTA, 1 mM DTT, pH 7.5, at 4°C, its subsequent treatment with denaturation mixture during 2 h at 60°C, and electrophoresis. Lane 4: SDS molecular weight markers: 66 kDa albumin, bovine; 45 kDa albumin, egg; 36 kDa GAPDH, rabbit muscle; 29 kDa carbonic anhydrase, bovine erythrocytes; 24 kDa trypsinogen, bovine pancreas, PMSF treated; 20.1 kDa trypsin inhibitor, soybean.

that the active lysozyme formed was capable of hydrolyzing the polysaccharide support, Sepharose. This might lead to erroneous results in the absence of the necessary controls. For example, in the only previous work on immobilized PDI [23] the authors could not reveal any reactivating activity of the preparations in the batch system. It should be noted that in this work sufficiently strict immobilization was performed with the use of commercial Sepharose preparations (activation extent was about 70 mg of CNBr per ml), this could lead to decrease of the activity (first of all, the chaperone-like one), whereas in our preparations the enzyme was bound to Sepharose through a single subunit and maintained sufficiently high activity.

Thus, PDI immobilized via one of two subunits was shown to possess rather high isomerase activity and the ability to reactivate standard protein substrates.

The next part of this work was connected with a study on the chaperone-like activity of PDI by means of investigation of its interaction with GAPDH, the protein containing no disulfide bonds. Impossibility of providing sufficient excess of immobilized PDI in the system for GAPDH reactivation together with certain chaperone-like action of the control Sepharose don't permit to make a final conclusion about the presence of the chaperone-like activity in the preparations of the immobilized PDI as it was shown for the soluble enzyme. We can not exclude that immobilization of PDI prevents the enzyme from association-dissociation processes what may influence the process of GAPDH reactivation.

3.3. Interaction of immobilized PDI with GAPDH

Immobilized PDI in both column and batch mode did not appear to interact neither with native GAPDH, nor with a GdnHCl denatured GAPDH. No interaction was observed between these two proteins by comparing the elution rates of the native and denatured GAPDH from the immobilized PDI column and from a Sepharose 4B column.

Possibly, renaturation of the part of GAPDH polypeptide chains proceeds during the experiment, and binding to PDI (if it takes place) can not be revealed because of reversibility of the process. These observations show that it is very difficult to study interaction of chaperones and chaperone-like proteins with various forms of target proteins because of the ability of these forms to interconvert, as well as due to reversibility of the protein-protein interactions of the formed complexes.

3.4. Interaction of GAPDH immobilized as a monomer or a tetramer with PDI

The experiments on the binding of soluble PDI to immobilized GAPDH monomers and tetramers were then performed. Two kinds of immobilized form of GAPDH covalently bound to CNBr-activated Sepharose 4B were titrated with soluble PDI. The procedure was as follows. To a series of samples containing equal amounts of immobilized GAPDH, aliquots of PDI solution containing increasing concentrations of the enzyme were added. After 20 min incubation at 4°C with periodical stirring, the gel was settled by centrifugation, and the protein concentration in the supernatant was determined. Sepharose preparations without immobilized protein served as a control. No interaction was observed in case of the control Sepharose, or immobilized tetramers (Fig. 1, curve 1). The preparations of immobilized monomers were shown to bind PDI sufficiently tightly (Fig. 1, curve 2): the dissociation constant was found to be 3.7×10^{-6} M and stoichiometry of the binding was 0.825 mole PDI monomers (57 kDa) per mole GAPDH monomers (36 kDa).

It was also possible to demonstrate the binding of PDI to immobilized GAPDH monomers in another way. Preparations of immobilized GAPDH monomers and tetramers, as well as samples of control Sepharose were incubated with PDI and then washed under identical conditions until no protein was detected in the eluate. Just after that the samples were put to electrophoretic separation. As seen in Fig. 2, lane 2, there is no PDI in preparations of the immobilized GAPDH tetramers, but considerable amount of this protein with a molecular mass of 57 kDa (monomer of PDI) can be seen in the preparations of GAPDH monomers (Fig. 2, lane 3). Bands corresponding to molecular mass of 36 kDa (monomer of GAPDH) were discovered in the preparations of tetramers and, in small extent, in the preparations of monomers before the incubation with PDI as well as after the incubation. The presence of a small amount of GAPDH in the preparations of GAPDH can be explained by partial destroying covalent bonds between protein and Sepharose under strict conditions and partial releasing monomers to the solution. Such phenomenon was observed with other proteins and enzymes immobilized on CNBr-activated Sepharose [24,25].

We suggest that these were reasons for using the immobilization for fixing of certain conformational state of target protein. GAPDH tetramers immobilized to Sepharose through a single subunit were obtained with the properties of native enzyme. Then dissociation of the tetramers to monomers was performed in quite strict conditions, and binding of PDI to the tetramers as well as to denatured monomeric

forms was investigated. Noteworthy, active GAPDH monomers can be obtained in certain conditions, when all non-covalently bound subunits denature and turn into solution gradually. Stabilizing action of matrix increases the stability of a covalently bound subunit, making possible to get an active subunit [21]. However, further incubation of the subunit under denaturing conditions leads to its unfolding followed by inactivation. Noteworthy, reactivation is impossible after unfolding. Possibly, formation of a covalent bond between a denatured subunit and matrix alters thermodynamics of the refolding process, and denaturation becomes irreversible. These observations shows that immobilization not only provides a possibility to get monomeric forms of oligomeric enzymes in denatured state, but also to prevent their refolding as well as reassociation.

Thus, PDI was shown to interact with only denatured monomeric form of GAPDH, the protein containing no disulfide bonds, that is to possess one of the principal properties of a chaperone – the ability to recognize non-native conformations of proteins.

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