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Refolding of Thioredoxin Reductase Assisted by groEL and PDI

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Thioredoxin reductase was unfolded in 2 M guanidine hydrochloride as revealed by fluorescence and CD spectroscopy. Spontaneous refolding of denatured species resulted in low recovery of 10% catalytic activity after 4 h incubation at 25°C. Addition of groEL or protein disulfide isomerase to the renaturation buffer accelerated the rate of recovery of catalytic activity to a level of 35 and 15%, respectively. Fluorescence spectroscopy has been used to investigate the interaction of groEL and protein disulfide isomerase with denatured thioredoxin reductase tagged with a fluorescent probe. The fluorescence emitted by the denatured protein was quenched upon binding to either groEL or protein disulfide isomerase. It is suggested that encapsulation of the protein substrate by the chaperone plays an important role in the process of folding by facilitating the formation of correctly folded species.

Protein disulfide isomerase (PDI) is a multifunctional enzyme that both catalyses the formation of disulfide bonds (1–4) and acts as a subunit of prolyl-4-hydroxylase (5, 6). PDI has been proposed to function as a molecular chaperone by binding to unfolded protein species, thereby preventing aggregation and misfolding (7, 8). More recently, it has been shown that PDI does not only participate in disulfide bond formation but also acts as a molecular chaperone interacting specifically with monomeric procollagen chains (9). Despite these interesting studies, the situation is complicated, since PDI, unlike other chaperones, also catalyses disulfide bond formation. Therefore, it still remains obscure whether its chaperone-like properties are manifestations of its ability to assist protein folding associated with catalysis of disulfide bond formation and isomerization. In the present work we examine the efficiency of groEL and PDI as chaperones in assisting the refolding of the same protein substrate, thioredoxin reductase (TR).

TR, unfolded by guanidine hydrochloride (Gnd/HCl), was selected as the studying model for the following reasons. First, the native enzyme recognizes PDI as a substrate and it catalyzes NADPH-dependent protein disulfide reduction (10). In addition, the denaturation of TR by Gnd/HCl does not result in the formation of insoluble aggregates which greatly facilitates subsequent monitoring study. There is also a great possibility that PDI, acting as a chaperone, would bind denatured TR and assists its refolding. Moreover, a comparison of the efficiency of refolding of two chaperones; i.e., groEL and PDI, acting on the same protein substrate might provide some clues to the structural basis for the chaperone activity of PDI.

MATERIALS AND METHODS

Reagents. DEAE-Sepharose Fast Flow, 2′,5′-ADP-Sepharose, Mono Q HR 5/5, Superdex 200 Prep Grade, CM-Sepharose C-50 were from Amersham Pharmacia Biotech (Sweden). Bovine insulin, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), NADPH, PMSF, and ATP were purchased from Sigma Chemical Co. 5-(iodoacetamido)-fluorescein, guanidine hydrochloride were purchased from Merck. Other reagents used were of analytical grade.

Purification of thioredoxin reductase (TR). Enzyme TR was purified by three successive chromatographic steps developed in our laboratory. Fresh porcine brains (2.5 kg) obtained from local abattoir were homogenized in 0.15 M potassium phosphate, 0.03 mM PMSF, pH 7.4 buffer with a Waring blender. The homogenate was centrifuged at 14,000*g* for 30 min at 4°C. The supernatant was subjected to ammonium sulfate precipitation at 65% saturation. Precipitates so obtained was re-dissolved in 20 mM imidazole/HCl buffer, pH 6.5 (Buffer A) and dialyzed exhaustively in Buffer A. Sample was applied onto DEAE-Sepharose Fast Flow column equilibrated with Buffer A and then eluted with a liner gradient of 0–0.4 M NaCl. Fractions with TR activity were pooled, dialyzed against 20 mM Tris/HCl buffer, pH 7.4 (Buffer B). The crude TR was further applied onto a 2′,5′-ADP-Sepharose column and eluted with a linear gradi-

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Abbreviations used: PDI, protein disulfide isomerase; TR, thioredoxin reductase; IAF-TR, 5-(iodoacetamido)-fluorescein labeled thioredoxin reductase; Gdn/HCl, guanidine hydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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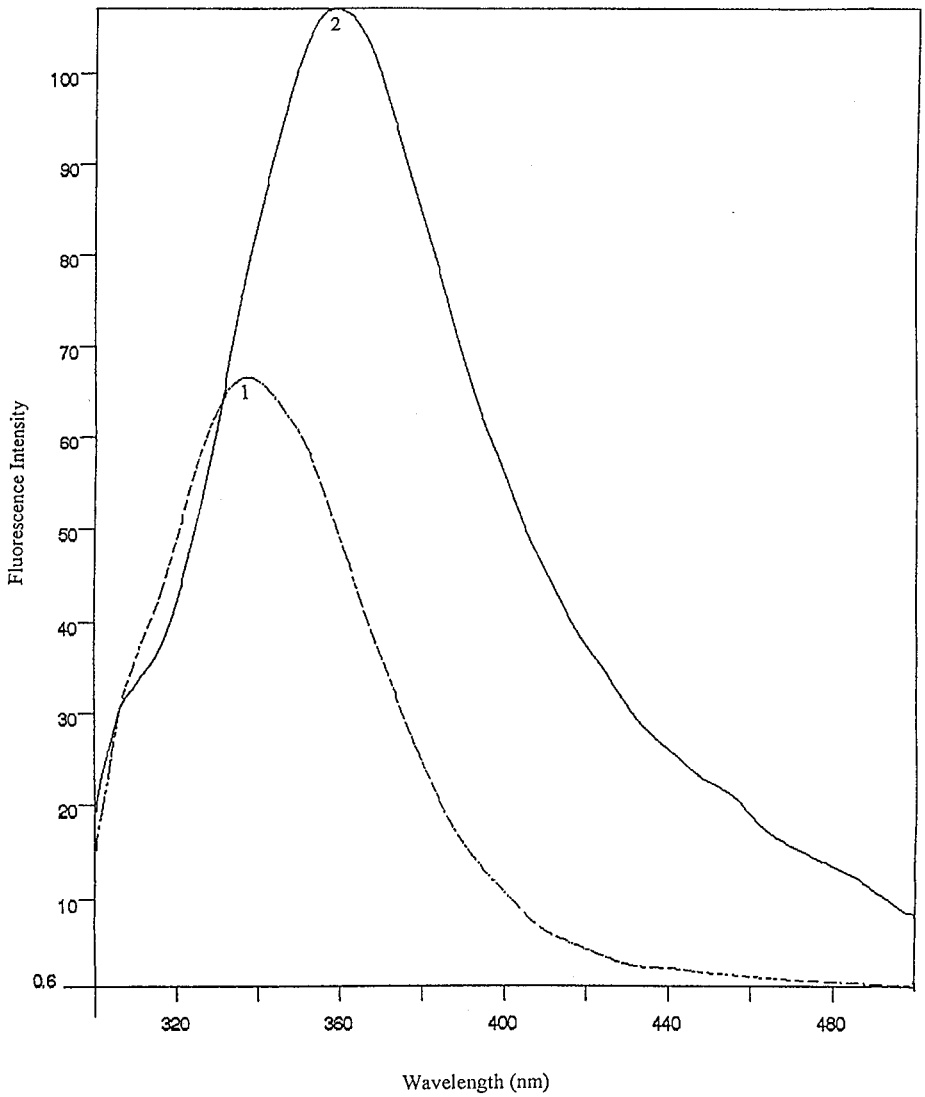

FIG. 1. Emission spectra of thioredoxin reductase (TR). TR in the absence (1) and presence (2) of 2 M Gnd/HCl in 0.1 M phosphate buffer (pH 7.4). Excitation wavelength is 295 nm, excitation and emission slits are 2.5 and 5 nm respectively. Both samples have the same absorbance at the exciting wavelength.

ent of 0–0.7 M NaCl in Buffer B. Fractions displaying TR activity were pooled and dialyzed against buffer B. Final purification was achieved with the use of the Pharmacia ÄKTA Explorer System. Sample was applied onto Mono Q HR 5/5 column equilibrated with Buffer B. Elution was carried out using segmented NaCl gradients (0–0.2, 0–0.21, and 0–0.5 M). An apparent 1000-fold purification was achieved with 12% yield. Protein amount was assayed by Bradford method (11). The TR enzyme activity was monitored by DTNB reduction assay as described by Luthman and Holmgren (12).

Purification of protein disulfide isomerase (PDI). Enzyme PDI was purified according to the method described by Hillson *et al.* (13) with minor modifications. Fresh porcine livers (500 g) obtained from local abattoir were homogenized in 0.1 M phosphate buffer, pH 7.5, containing 1% Triton X-100 and 5 mM EDTA. Homogenate was centrifuged to obtain the supernatant which was then heat treated at 54°C for 15 min. Denatured proteins were removed and the cytosolic content subjected to ammonium sulfate fractionation. Precipitates obtained between 55 and 85% saturation were re-dissolved and dialyzed in 25 mM sodium citrate buffer, pH 5.3 (Buffer C). Sample was applied onto a CM-Sepharose C-50 column and eluted with buffer C. Fractions displaying PDI activity were pooled and dialyzed in 20 mM sodium phosphate, pH 6.3 (Buffer D). It was then further purified by anion exchange chromatography, DEAE-Sepharose Fast Flow column eluted with a linear gradient of 0–0.7 M NaCl in Buffer D. The PDI protein amount was determined using the relationship that 1.0 O.D. at 280 nm is equivalent to 1 mg PDI protein/mL (14). The activity of PDI was determined using the insulin reduction assay as described by Gillbert (14).

Purification of groEL. *E. coli GroESL* gene (15) inserted in plasmid was expressed in *E. coli* strain BL21(DE3) cells. GroEL protein was purified by a published procedure (16) with some modifications. After ammonium sulfate fractionation, the protein was purified by three chromatographic steps; DEAE-Sephacel anion-exchange column, gel filtration through Sepharose CL-6B and affinity column through Red agarose (17). The last step removed contaminating proteins trapped by groEL. Fractions containing groEL were identified by their ATPase activity assay by a coupled enzyme method (18). The protein amount was estimated by UV spectroscopy using the

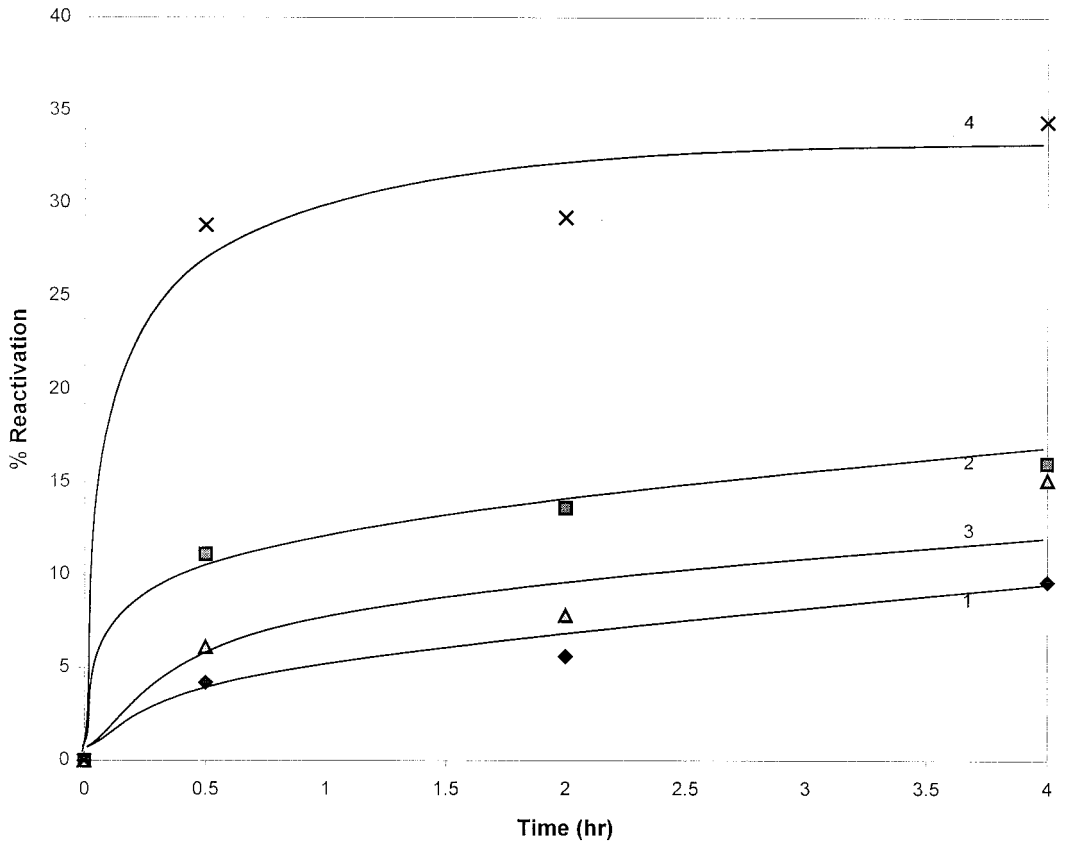

FIG. 2. Refolding of denatured TR. Samples of denatured TR, exposed for 30 min to 2 M Gnd/HCl, were diluted with the renaturation buffer (0.1 M potassium phosphate, pH 7.4) and assayed for enzymatic activity as a function of incubation time (h). The concentration of denatured enzyme in the renaturation buffer was 0.6 μM. Results obtained when the denatured enzyme was diluted with renaturation buffer (1, ◆), with renaturation buffer containing 6 μM PDI (2, ■), 0.6 μM (3, Δ) and 6 μM groEL (4, x) in the presence of Mg^{2+} and ATP (1 mM). Experiments conducted at 25°C.

relationship that 1.0 O.D. at 280 nm is equivalent to 0.25 mg protein/mL.

Unofolding and refolding of TR. Samples of enzyme (0.35 μg/ml) were incubated with various concentrations of Gnd/HCl, ranging from 0.1 to 3 M, for 30 min at 25°C in 0.1 M potassium phosphate buffer, pH 7.4. For refolding experiments, samples of the enzyme treated with 2 M Gnd/HCl were 10-fold diluted with the renaturation buffer (0.1 M phosphate, pH 7.4) in the absence and presence of chaperone proteins. Aliquots withdrawn at several time intervals were used for enzymatic assays using DTNB as a substrate. A Perkin–Elmer fluorometer, Model LS 50B was used for luminescence measurement.

Labeling of thioredoxin reductase. TR (1 mg/mL) was reacted with 5-(iodoacetamido)-fluorescein (0.1 mM) in 0.1 M potassium phosphate buffer, pH 7.4 at 4°C for 12 h. It was then dialyzed extensively in several changes of potassium phosphate buffer, pH 7.4, to remove unreacted dye. The labeled protein displayed catalytic activity, suggesting that nonessential SH groups have been blocked by the reagent. The degree of labeling (0.8–1 mol of dye/mol of monomer) was determined by using an extinction coefficient of $4.9 \times 10^4\ M^{-1}\ cm^{-1}$ at 490 nm. The molecular weight of the monomer of TR is 58,000.

RESULTS

Reactivation of denatured TR. TR contains tryptophanyl residues; upon excitation at 295 nm, the protein exhibits a structureless emission band centered at around 338 nm. Addition of increasing concentrations of Gnd/HCl, followed by incubation for 30 min at 25°C, results in an increase in the fluorescence yield together with a red shift in the band position of the emission spectrum. Maximum perturbation of the emission spectrum was shown to occur at Gnd/HCl concentrations of 2M (Fig. 1). Under similar experimental conditions, the denatured protein exhibited a decrease in ellipticity at 222 nm of approximately 80% indicating that a good deal of the helix content was lost as a result of the exposure to 2 M Gnd/HCl (results not shown). Based on this information it was concluded that 2 M Gnd/HCl is sufficient to induce unfolding of the protein. For the refolding experiments, designed to test the recovery of catalytic activity, samples of TR (6 μM), exposed to 2 M Gnd/HCl, were diluted with the renaturation buffer to a final concentration of 0.6 μM and assayed for catalytic activity. As shown in Fig. 2, a gradual increase in catalytic activity was observed as a function of time; but the level of reactivation did not exceed 10% when compared to the denatured enzyme. Decreasing the temperature to 4°C or increasing the

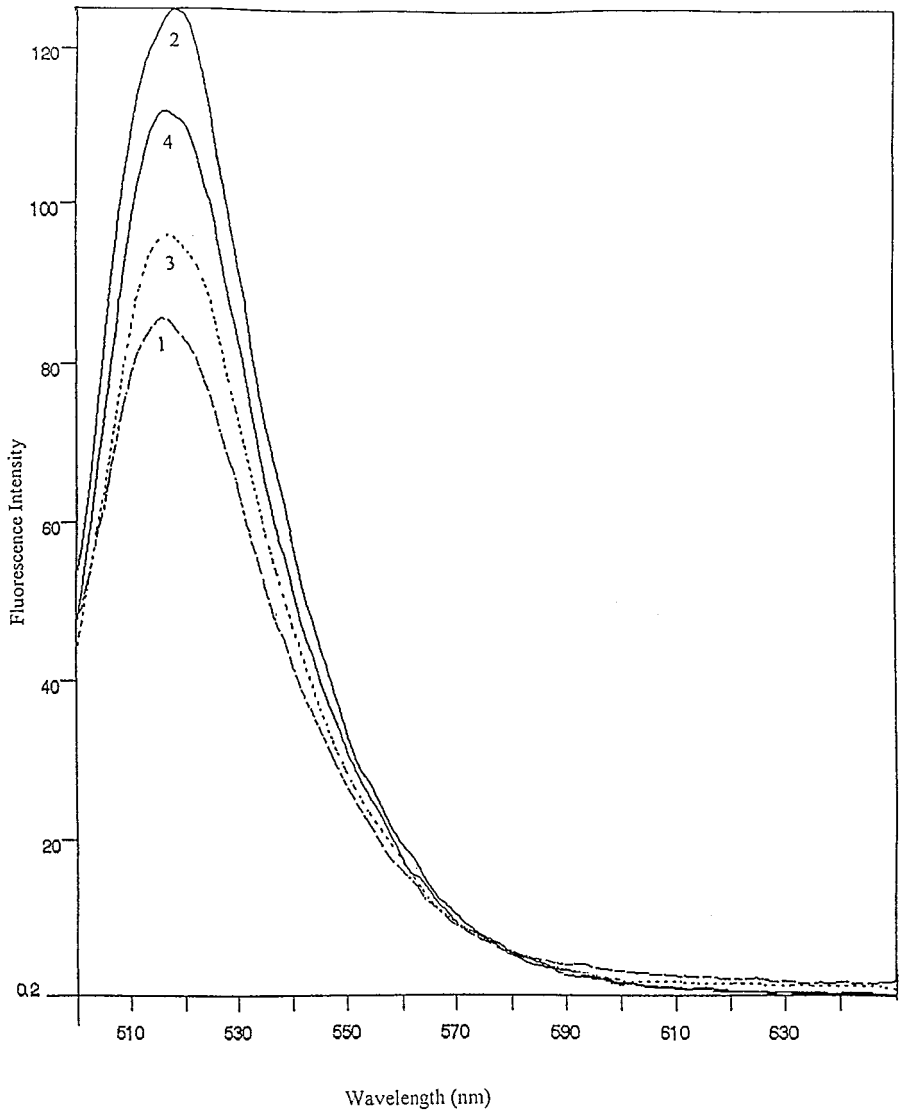

FIG. 3. Emission spectra of 5-(iodoacetamido)-fluorescein-TR (IAF-TR). IAF-TR before (1), and after denaturation (2) with 2 M Gnd/HCl in 0.1 M potassium phosphate (pH 7.4). The concentration of denatured protein was 0.7 μM. After diluting both samples with buffer, emission spectra of denatured IAF-TR were monitored in the presence of 1 μM groEL (3) and in the presence of 3.6 μM PDI (4).

concentration of denatured protein did not improve the level of recovery of catalytic activity. When denatured TR was diluted with the renaturation buffer containing PDI (6 μM), the refolding process was accelerated to the extent that the reactivation reached a level of 15%. The rate of reactivation was not improved by increasing the concentration of PDI to 12 μM. A decrease in the concentration of PDI to 1 μM showed no changes in the level of reactivation either. Refolding of denatured TR was also assisted by groEL (6 μM) in the presence of Mg^{2+} and ATP (1 mM). Under this set of experimental conditions, the refolding process was significantly accelerated leading to 35% recovery of catalytic activity (Fig. 2).

Binding of TR to PDI. To assess the binding of denatured TR to PDI, the enzyme was labeled with the extrinsic probe 5-(iodoacetamido)-fluorescein (IAF), denatured by addition of 2 M Gnd/HCl and allowed to refold in the absence and presence of chaperone proteins. Derivatized TR shows a maximum of fluorescence at 520 nm when excited at 480 nm. The large fluorescence yield of the probe, excited in the spectral region located far away from the absorption of aromatic residues of proteins, can be used to detect the presence of protein-protein complexes. Denaturation of IAF-TR, followed by dilution with buffer, induced an increase in the fluorescence yield of the probe as it became exposed to the solvent (Fig. 3). Addition of groEL to equimolar concentrations of denatured TR was sufficient to bring about a significant decrease in fluorescence yield. Derivatized TR also bound to PDI, but the decrease in fluorescence yield was less pronounced than that ob-

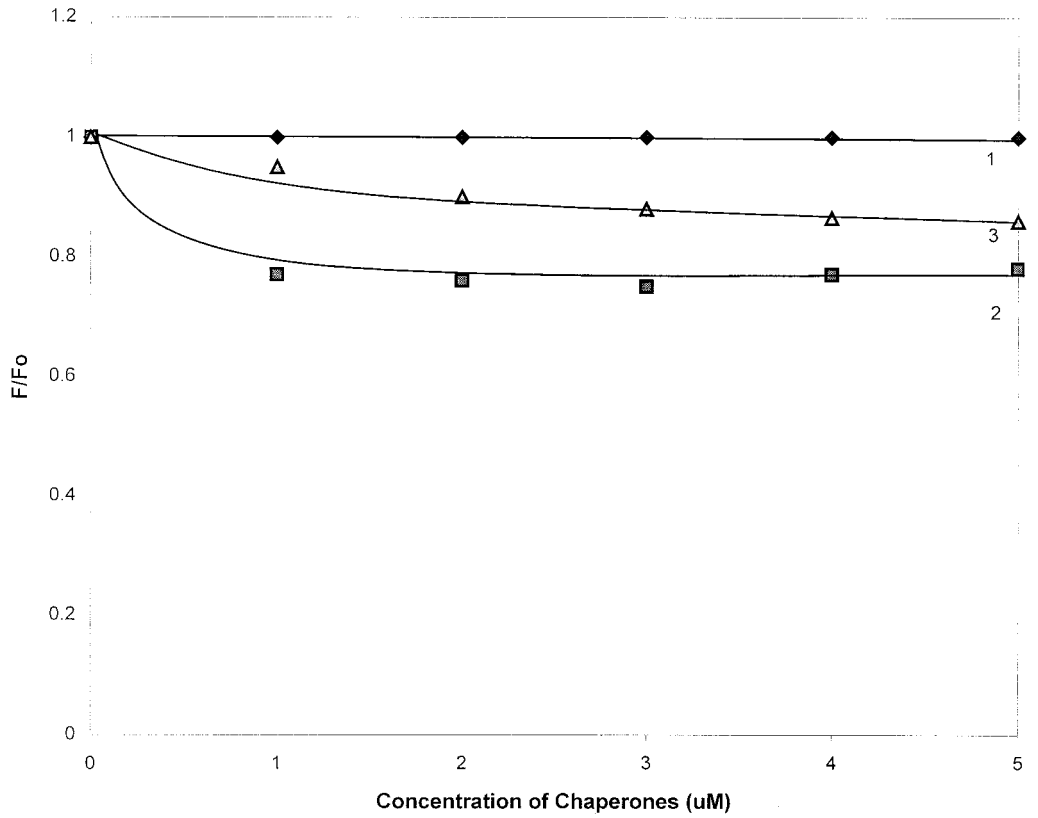

FIG. 4. Effect of increasing concentrations of chaperones on the fluorescence emitted by denatured IAF-TR. Fluorescence emitted by a fixed concentration of IAF-TR, (0.7 μM) was monitored at 520 nm, excitation wavelength 480 nm. The emission were recorded 5 minutes after addition of the chaperone proteins. The fluorescence ratio of IAF-TR with chaperones to IAF-TR is denoted as F/F_o. Native IAF-TR emission in the presence of groEL is denoted as (1, ◆). Denatured IAF-TR in the presence of groEL and PDI are denoted as (2, ■) and (3, Δ) respectively.

served when the protein was trapped inside the cavity of groEL (Fig. 3). The effect of increasing concentrations of chaperone proteins on the fluorescence emitted by a fixed concentration of denatured IAF-TR was also examined by fluorescence spectroscopy. The results included in Fig. 4 indicate that an equimolar ratio of PDI and substrate was sufficient to bring about a decrease in fluorescence but fivefold molar excess of PDI would lead to a maximum decrease in IAF fluorescence. The concentration of PDI required to form a complex was similar to the concentration needed to assist the refolding of the denatured enzyme. It should be emphasized that the fluorescence of IAF-TR that was not denatured by Gnd/HCl remained practically invariant upon addition of chaperone proteins.

DISCUSSION

TR was shown to undergo a structural transition in solutions containing Gnd/HCl. The enzyme was unfolded by 2 M Gnd/HCl resulting in a displacement of the emission maximum together with a decrease in ellipticity at 222 nm. Under optimal experimental conditions, spontaneous refolding of the reductase resulted in 10% recovery of catalytic activity. These results are interpreted to mean that during spontaneous refolding a large percentage of the protein population remains in incorrectly folded state. The rate of recovery of catalytic activity is influenced by the presence of PDI in the renaturation buffer. Under our optimized experimental conditions of temperature and PDI concentration, the recovery of reductase activity amounts to 15%. According to the spectroscopic studies conducted on IAF-TR, fivefold excess of PDI over denatured protein would be sufficient to ensure the formation of a complex. These results are consistent with reports of other laboratories (19) which indicated binding of PDI to several denatured proteins when tested by affinity chromatography.

The refolding studies presented in this work have shown that groEL is more efficient than PDI in assisting the refolding of thioredoxin reductase. There are some aspects of the interaction of groEL with unfolding protein substrates that should be considered in the analysis of the behavior of PDI. GroEL is a protein made up of 14 identical subunits (800 kDa) that binds tightly the unfolded substrate. The binding of ATP drives the chaperone complex through a functional

state in which refolding of the protein substrate occurs inside the cavity of groEL (20, 21). PDI, on the other hand, binds to unfolded TR, but due to its small size (107 kDa) is unable to encapsulate the protein substrate. Judging from the spectroscopic studies reported in the present work, it appears that IAF-TR is not completely shielded from the solvent despite the formation of a complex with PDI. It is our contention that encapsulation of the protein substrate by the chaperone plays an important role in the folding process because it restricts the out-diffusion of unfolded species and their accumulation in solution. The possibility of misfolding increases when the unfolded species are free in solution or partially exposed to the solvent.

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