Chaperone properties of *Escherichia coli* thioredoxin and thioredoxin reductase

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Thioredoxin, thioredoxin reductase and NADPH form the thioredoxin system and are the major cellular protein disulphide reductase. We report here that *Escherichia coli* thioredoxin and thioredoxin reductase interact with unfolded and denatured proteins, in a manner similar to that of molecular chaperones that are involved in protein folding and protein renaturation after stress. Thioredoxin and/or thioredoxin reductase promote the functional folding of citrate synthase and α-glucosidase after urea denaturation. They also promote the functional folding of the bacterial galactose receptor, a protein without any cysteines. Furthermore, redox cycling of thioredoxin/thioredoxin reductase in the presence of NADPH and cystine stimulates the renaturation of the galactose receptor, suggesting that the thioredoxin system functions like a redox-powered chaperone machine. Thioredoxin reductase prevents the aggregation of citrate synthase under heat-shock conditions. It forms complexes that are more stable than those formed by thioredoxin with several unfolded proteins such as reduced carboxymethyl α-lactalbumin and unfolded bovine pancreatic trypsin inhibitor. These results suggest that the thioredoxin system, in addition to its protein disulphide isomerase activity possesses chaperone-like properties, and that its thioredoxin reductase component plays a major role in this function.

Key words: oxidoreduction, protein folding, protein renaturation.

INTRODUCTION

The thioredoxin system, composed of thioredoxin, thioredoxin reductase and NADPH, is ubiquitous from bacteria to humans [1]. In this system, thioredoxin reductase uses the reducing potential of NADPH to maintain thioredoxin in a reduced state, so that thioredoxin in turn can reduce substrate proteins. The thioredoxin system is a hydrogen donor for several reductive enzymes such as ribonucleotide reductase, methionine sulphoxide reductase and sulphate reductase [2]. It also plays a key role in maintaining the reduced state of cytoplasmic proteins [3,4] {in contrast with cytoplasmic proteins, which are reduced, extracytoplasmic proteins are oxidized by protein disulphide isomerase (PDI) in eukaryotes and Dsb ('disulphide bond') enzymes in bacteria [5]}. Thioredoxins are also involved in the redox regulation of photosynthesis by light [6] and that of transcription factor nuclear factor-κB [7]. Thioredoxins can also form complexes with several enzymes, such as T7 DNA polymerase, to which it confers processivity [8], or apoptosis signalling kinase, which is inhibited by reduced thioredoxin [7]. The other major factor generally responsible for the low redox potential inside cells is glutathione, which is maintained in its reduced state by NADPH and glutathione reductase [2]. Glutathione-dependent disulphide reductions are catalysed by glutaredoxins whose functions partially overlap those of thioredoxins [2,9].

All thioredoxins show strong sequence homology to *Escherichia coli* thioredoxin 1, and have the same overall three-dimensional structure, the thioredoxin fold, which consists of a central core of five β-strands enclosed by four α-helices [10]. The Cys-Gly-Pro-Cys active site is located on the surface of the protein, at the end of a β-strand and at the beginning of a long α-helix. One part of the area around the active site is hydrophobic and has been suggested to be a main interaction site for other proteins [10,11]. Thioredoxin reductase is a member of the family of dimeric flavoenzymes, which includes lipoamide dehydrogenase and glutathione reductase, which catalyse the transfer of electrons between NADPH and disulphide/dithiols compounds and promote catalysis via FAD and a redox-active disulphide [1]. The thioredoxin reductases in lower and higher organisms are different. In higher organisms, the enzyme is larger (subunit molecular mass of 55 000 Da instead of 35 000 Da), and has a broader substrate specificity (including low-molecular-mass substrates such as ascorbate) and an additional redox motif which, in mammals, contains selenocysteine [1]. Thioredoxin reductases reduce not only thioredoxins, but also the glutaredoxin-like protein NrdH, in the case of the *E. coli* thioredoxin reductase [12], and non-disulphide substrates such as selenite, lipid hydroperoxides and H_2O_2, in the case of mammalian thioredoxin reductases [1].

Molecular chaperones form a class of polypeptide-binding proteins that are implicated in protein folding, protein targeting to membranes, protein renaturation or degradation after stress and the control of protein–protein interactions (reviewed in [13]). Several protein disulphide isomerases, such as PDI, DsbC and DsbG, have been shown to display a chaperone-like activity [14–16]. PDI, DsbC and DsbG can assist the refolding of denatured proteins, and the crystal structure of DsbC reveals a large hydrophobic cleft which might be involved in peptide binding and foldase activities [16]. Although no chaperone-like properties have been reported for thioredoxin, it is able to interact with a great number of proteins [1], and enhances the solubility of fusion proteins [17], suggesting that it interacts favourably with proteins. In the present study, we show that thioredoxin and thioredoxin reductase increase severalfold the folding of urea-denatured

Abbreviations used: PDI, protein disulphide isomerase; Hsp, heat-shock protein; BPTI, bovine pancreatic trypsin inhibitor; R-CMLA, reduced carboxymethyl α-lactalbumin.

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proteins, and that thioredoxin reductase efficiently protects citrate synthase against thermodenaturation and discriminates between unfolded and native proteins.

EXPERIMENTAL

Materials

Citrate synthase (from porcine heart), α-glucosidase (from yeast), bovine pancreatic trypsin inhibitor (BPTI), reduced carboxymethyl α-lactalbumin (R-CMLA), BSA, ovalbumin, lysozyme and all other chemicals were from Sigma and were of reagent grade. D-[^{3}H]Galactose was obtained from Amersham Biosciences, and was used at 1 Ci/mmol.

Thioredoxin, thioredoxin reductase, MglB and DnaK/DnaJ/GrpE

E. coli thioredoxin and thioredoxin reductase were obtained from Sigma and from IMCO (Stockholm, Sweden; catalogue no. S-10031) respectively. Reduced and oxidized thioredoxin were prepared as described in [18]. DnaK was prepared as described previously [19–21], from an overproducing strain of *E. coli* bearing plasmid pLNA2 derived from plasmid pDM38 [22] (a gift from Dr O. Fayet, Microbiologie et Génétique Microbienne CNRS, Toulouse, France). DnaJ and GrpE were purchased from Stressgene. The galactose receptor MglB was purified as described previously [23].

Refolding of citrate synthase and α-glucosidase

Denaturation and renaturation reactions were carried out at 20 °C. For both proteins, renaturation was initiated by pouring the renaturation solvent on to the unfolded protein, under vortex agitation, in Eppendorf polyethylene tubes. Citrate synthase was denatured at a concentration of 10 μM in 8 M urea, 50 mM Tris/HCl, 2 mM EDTA and 20 mM dithiothreitol (pH 8.0) for 50 min. Renaturation was initiated by a 100-fold dilution in 40 mM Hepes, 50 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM potassium acetate (pH 8.0). The enzymic activity of citrate synthase was measured as described previously [24]. α-Glucosidase was denatured at a concentration of 1.5 μM in 8 M urea, 0.1 M potassium phosphate, 2 mM EDTA and 20 mM dithiothreitol (pH 7.0) for 15 min. Renaturation was initiated by a 30-fold dilution in 40 mM Hepes/KOH (pH 7.8) at 20 °C. The enzymic activity of α-glucosidase was measured as described previously [24].

Refolding of the galactose receptor

MglB was unfolded in 1 M guanidine hydrochloride at 20 °C, and allowed to refold at a concentration of 1 μM by a 60-fold dilution of the denaturation solution in 10 mM Hepes/KOH (pH 7.0)/0.5 mM EGTA, at 16 °C [25]. Thioredoxin and thioredoxin reductase (2 μM each) were added to the renaturation buffer as indicated. To measure the extent of MglB renaturation, its galactose-binding activity was determined within 5 s. Galactose was added for 3 s, followed by saturated $(NH_4)_2SO_4$, and the mixture was filtered on nitrocellulose filters as described previously [23]. $(NH_4)_2SO_4$ leads, in less than 1 s, to a conformation of the MglB–galactose complex that prevents further galactose binding or dissociation, and gives galactose-binding results identical to those obtained by equilibrium dialysis [23].

Thermal aggregation of citrate synthase

The native enzyme (80 μM) was diluted 100-fold in 40 mM Hepes, 50 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM potassium acetate (pH 8.0) at 43 °C in the absence of added proteins, or in the presence of DnaK, thioredoxin or thioredoxin reductase. Citrate synthase aggregation was monitored by measuring the absorbance at 650 nm as described in [24].

Size-exclusion chromatography

For binding assays of radiolabelled R-CMLA and unfolded BPTI to thioredoxin, thioredoxin reductase and DnaK, three gel-permeation columns [TSK G-2000 HPLC, 30 cm × 7.5 mm; Bio-Gel P-200 (Bio-Rad); and Sephadex G-75 (Amersham Biosciences)], each with a 300 μl bed volume, were equilibrated with column buffer containing 50 mM Tris/HCl (pH 8.2 for studies with R-CMLA, pH 7.4 for studies with BPTI), 50 mM KCl, 1 mM dithiothreitol and 100 μg/ml BSA. Reaction mixtures containing thioredoxin, thioredoxin reductase or DnaK, and radiolabelled unfolded BPTI or radiolabelled R-CMLA at indicated concentrations were incubated for 20 min at 23 °C in column buffer without serum albumin, and applied to the column at room temperature. Fractions were collected at a flow rate of 1 ml/min in HPLC experiments, and 1 drop/fraction per 30 s in low-pressure chromatography experiments, and then counted for radioactivity. DnaK was incubated for 3 h at 37 °C before use. Unfolded BPTI was prepared as described previously from native BPTI [26]. Unfolded BPTI, native BPTI and R-CMLA were ^{3}H-labelled by reductive methylation [27].

RESULTS

Thioredoxin increases the amount of correctly folded citrate synthase and α-glucosidase

We first investigated whether thioredoxin acts as a molecular chaperone in the folding of proteins. Citrate synthase and α-glucosidase, whose refolding is facilitated by several chaperones such as GroEL, DnaK, heat-shock protein (Hsp) 90 and small Hsps [24,28,29], were chosen as substrates for this reaction. They were unfolded in the presence of 8 M urea, and allowed to refold upon dilution of the denaturant, in the absence or in the presence of thioredoxin (protein folding in the presence of DnaK was studied in parallel). Under our experimental conditions, the refolding yield of 0.1 μM citrate synthase is increased from 5 % in the absence of added proteins to 20 % in the presence of 2 μM thioredoxin, 27 % in the presence of 2 μM DnaK and 30 % in the presence of 2 μM DnaK and 0.2 mM ATP (Figure 1, top-left panel). In all cases, the maximal renaturation of citrate synthase is attained in about 10 min. The refolding yield of citrate synthase in the presence of thioredoxin is similar to that obtained by others in the presence of Hsp25 [29].

The dependence of citrate synthase reactivation on the concentration of thioredoxin is shown in Figure 1 (top-middle panel). Maximal recovery of citrate synthase activity attains 22 % in the presence of 5 μM thioredoxin. Half-maximal reactivation occurs at 0.4 μM thioredoxin, a concentration similar to that of citrate synthase. This concentration is also similar to that of DnaK (1 μM) required for half-maximal reactivation of citrate synthase in similar conditions (results not shown, and [24]), and is similar to the concentration of Hsp25 (expressed as monomers) used for the renaturation of citrate synthase in similar conditions [29]. The thioredoxin concentration required for half-maximal reactivation of citrate synthase is similar to those of classic chaperones used in the same renaturation reaction, and lower

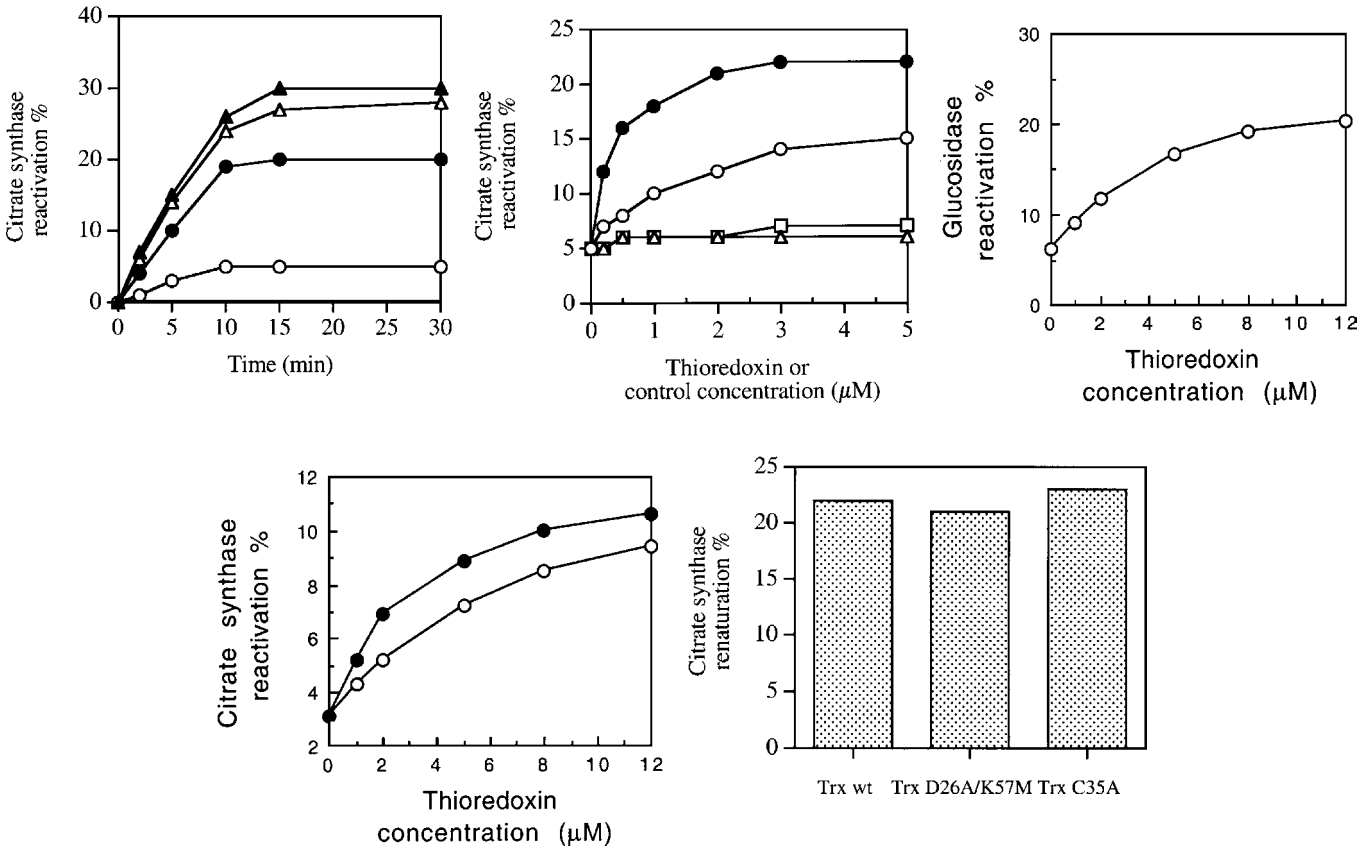

Figure 1 Influence of thioredoxin on the refolding of urea-denatured citrate synthase and α-glucosidase

Top-left panel: kinetics of refolding. Citrate synthase was denatured in urea and then renatured by dilution of the denaturant as described in the Experimental section, at a concentration of 0.1 μM in the absence of additional protein (○) and in the presence of either 3 μM thioredoxin (●), 3 μM DnaK (△) or 3 μM DnaK, 0.2 mM ATP and 1 mM $MgCl_2$ (▲). ATP and $MgCl_2$ alone had no significant effect on citrate synthase renaturation. Top-middle panel: dependence of citrate synthase refolding on thioredoxin concentration. Citrate synthase was denatured in urea and subsequently renatured for 20 min by dilution of the denaturant in the presence of thioredoxin (●) at the indicated concentrations. As a control, we measured citrate synthase renaturation in the presence of lysozyme (△), ovalbumin (□) or serum albumin (○). Top-right panel: dependence of α-glucosidase refolding on thioredoxin concentration. α-Glucosidase was denatured in urea and then renatured for 40 min by dilution of the denaturant as described in the Experimental section, at a concentration of 0.05 μM in the presence of thioredoxin at the indicated concentrations. Bottom-left panel: dependence of citrate synthase refolding on oxidized (○) or reduced (●) thioredoxin. Citrate synthase was denatured in urea and then renatured for 20 min by dilution of the denaturant in the presence of oxidized or reduced thioredoxin at the indicated concentrations. Oxidized and reduced thioredoxin were prepared as described in the Experimental section. Bottom-right panel: citrate synthase renaturation by redox thioredoxin mutants. Citrate synthase was denatured in urea and subsequently renatured for 20 min by dilution of the denaturant in the presence of 1 μM wild-type thioredoxin (Trx wt), 1 μM D26A/K57M thioredoxin or 1 μM C35A thioredoxin. D26A/K57M and C35A mutant thioredoxins were prepared as described in [30,31].

than the concentrations of thioredoxin in the cytoplasm (around 15 μM [2]). Control proteins such as ovalbumin and lysozyme were unable to stimulate citrate synthase renaturation, whereas serum albumin could stimulate it to some extent (Figure 1, top-middle panel).

As shown in Figure 1 (top-right panel), the refolding of α-glucosidase is increased from 6 % in the absence of added protein to 20 % in the presence of thioredoxin and half-maximal reactivation of α-glucosidase occurs at 3 μM thioredoxin. In similar conditions, the refolding of α-glucosidase was 27 % in the presence of 2 μM DnaK (results not shown). These results suggest that, like molecular chaperones (with a similar efficiency, and at similar concentrations), thioredoxin specifically interacts with unfolded proteins, and increases their productive folding.

Citrate synthase renaturation by reduced and oxidized thioredoxin

We investigated the activity of oxidized and reduced thioredoxin in the reactivation of urea-denatured citrate synthase. The structural difference between oxidized and reduced thioredoxin is subtle [11], but some substrates interact preferentially with reduced thioredoxin [1]. For this experiment, citrate synthase was denatured in the absence of dithiothreitol. Whereas the renaturation of citrate synthase is limited to 3 % in the absence of thioredoxin, oxidized thioredoxin reactivation of citrate synthase reaches 9 % (half-maximal reactivation is reached at 4 μM thioredoxin), and reduced thioredoxin reactivation of citrate synthase reaches 10 % (half-maximal reactivation is reached at 2 μM thioredoxin; Figure 1, bottom-left panel). This result suggests that the reduced and oxidized states of thioredoxin interact with similar efficiencies with citrate synthase, and that the redox cysteines of thioredoxin do not play a major role in the reactivation of citrate synthase.

Citrate synthase renaturation by redox thioredoxin mutants

We investigated the chaperone properties of two redox thioredoxin mutants, D26A/K57M and C35A. The double mutant D26A/K57M has a very low activity as a disulphide reductase because both residues influence the redox mechanism. It is, however, similar in its overall structure to the wild-type protein, and the structure of its active-site hydrophobic surface is

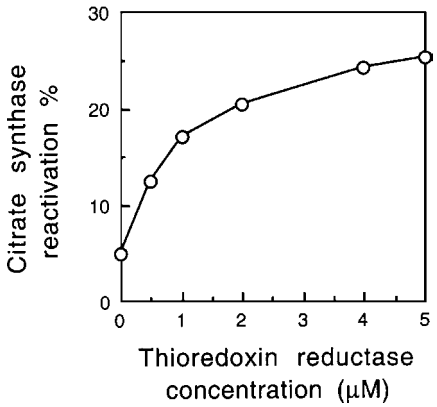

Figure 2 Dependence of citrate synthase refolding on thioredoxin reductase concentration

Citrate synthase was denatured in urea and then renatured for 20 min by dilution of the denaturant in the presence of thioredoxin reductase at the indicated concentrations.

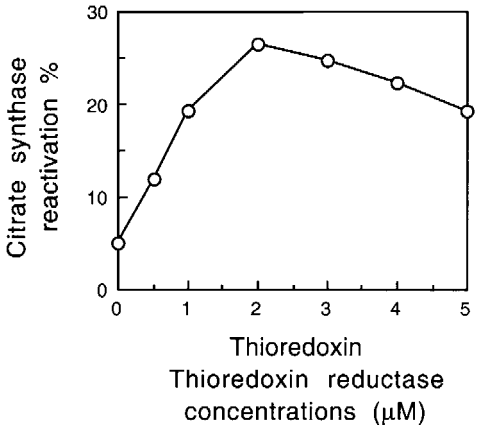

Figure 3 Refolding of citrate synthase by both thioredoxin and thioredoxin reductase

Citrate synthase was denatured in urea and then renatured for 20 min by dilution of the denaturant in the presence of both thioredoxin and thioredoxin reductase at the indicated concentrations (concentrations of thioredoxin monomers and thioredoxin reductase dimers).

unchanged, as deduced from its normal interaction with T7 DNA polymerase [30]. The C35A mutant cannot catalyse the normal redox reactions of thioredoxin but shows a similar structure to wild-type thioredoxin [31]. As shown in Figure 1 (bottom-right panel), each of these mutant thioredoxins (at a concentration of 1 μM) display similar chaperone properties to wild-type thioredoxin for the renaturation of urea-denatured citrate synthase. These results suggest that the redox function of thioredoxin is not involved in its chaperone function, as it is not involved in the stimulation of T7 polymerase [30] or in the assembly of filamentous phages [32].

Thioredoxin reductase increases the amount of correctly folded citrate synthase

We investigated the renaturation of citrate synthase in the presence of thioredoxin reductase in conditions similar to those described above for thioredoxin. The dependence of citrate synthase reactivation on the concentration of thioredoxin reductase is shown in Figure 2. The maximal recovery of citrate synthase activity is 25 % in the presence of 5 μM thioredoxin reductase, and half-maximal reactivation occurs at $\approx$0.7 μM thioredoxin reductase, a low concentration like that of other chaperones in similar reactions. We investigated the renaturation of α-glucosidase in the presence of thioredoxin reductase in conditions similar to those described for thioredoxin. The refolding of α-glucosidase was inhibited in the presence of 6 μM thioredoxin reductase (results not shown); the inhibition of protein renaturation by chaperones is frequently observed, and reflects a strong interaction between the unfolded protein and the chaperone [28], which can be relieved by co-chaperones or nucleotides (in fact, thioredoxin plus thioredoxin reductase stimulates α-glucosidase renaturation; see below). Thus thioredoxin reductase, with an efficiency similar to that of thioredoxin, increases the productive folding of urea-denatured citrate synthase, and interacts with unfolded α-glucosidase.

Renaturation of citrate synthase and α-glucosidase by both thioredoxin and thioredoxin reductase

The renaturation of citrate synthase in the presence of both thioredoxin and thioredoxin reductase was studied at different concentrations (in equimolar amounts of monomeric thioredoxin and dimeric thioredoxin reductase). The maximal recovery of citrate synthase activity is 25 % in the presence of 5 μM each of thioredoxin and thioredoxin reductase, and half-maximal reactivation occurs at around 1 μM (Figure 3). Thus the interaction between thioredoxin and thioredoxin reductase does not prevent the thioredoxin/thioredoxin reductase system from catalysing the refolding of citrate synthase. The renaturation of α-glucosidase was also stimulated from 6 to 22 % in the presence of both thioredoxin and thioredoxin reductase (5 μM each; results not shown).

Renaturation of the MglB galactose receptor, a protein without cysteine, by thioredoxin and thioredoxin reductase

Since the main function of the thioredoxin system is to catalyse oxidoreduction of protein thiols, we wanted to check whether thioredoxin/thioredoxin reductase could stimulate the renaturation of a protein that did not contain any cysteines. We studied the renaturation of guanidine-denatured MglB. MglB is the periplasmic bacterial galactose receptor involved in galactose transport and chemotaxis; it has been reported to interact with the chaperone SecB, which accelerates its renaturation [25]. MglB was denatured in the presence of 1 M guanidine hydrochloride, as described previously [25], and renatured in the presence of thioredoxin and thioredoxin reductase. As shown in Figure 4, the renaturation of 1 μM MglB is accelerated 6-fold in the presence of thioredoxin and thioredoxin reductase (2 μM each). Since MglB contains no cysteines, its interaction with thioredoxin/thioredoxin reductase should not involve any dithiol–disulphide exchange, and a *bona fide* chaperone-like interaction should occur between MglB and the thioredoxin/thioredoxin reductase system. Thioredoxin or thioredoxin reductase alone accelerate the refolding of MglB 1.7- and 1.9-fold, respectively (results not shown).

Stimulation of MglB renaturation by redox cycling of the thioredoxin system

We tested whether redox cycling of the thioredoxin/thioredoxin reductase system stimulates MglB renaturation. The refolding of MglB was studied in the presence of both thioredoxin and thioredoxin reductase, either unsupplemented (as above) or supplemented with NADPH and cystine. (Under the latter conditions, NADPH and cystine are transformed to NADP

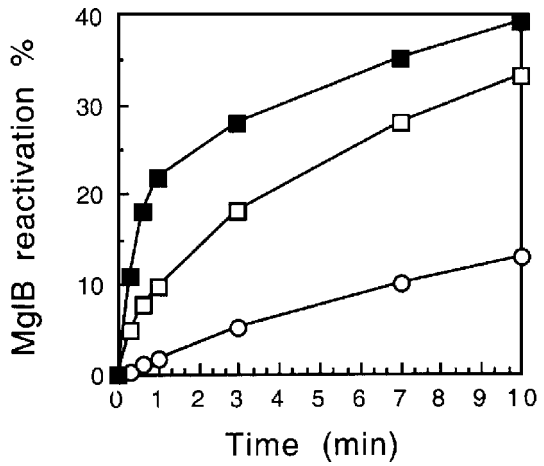

Figure 4 Refolding of the MglB galactose receptor by both thioredoxin and thioredoxin reductase

MglB was denatured in guanidine hydrochloride and then renatured (at a concentration of 1 μM) by dilution of the denaturant, as described in the Experimental section, without any additional protein (○) or in the presence of 2 μM each of thioredoxin and thioredoxin reductase (□), or 2 μM each of thioredoxin and thioredoxin reductase supplemented with 0.4 mM NADPH and 0.4 mM cystine (■).

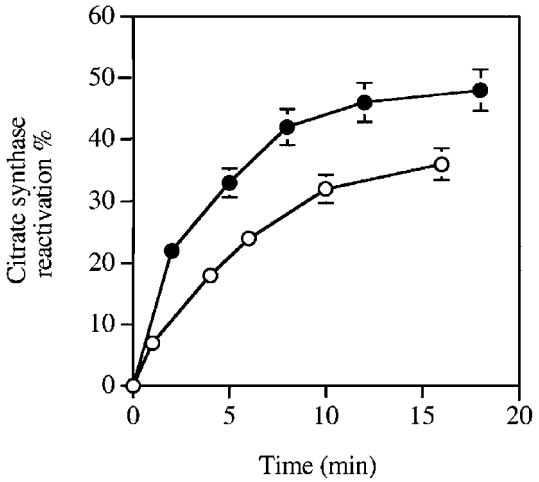

Figure 5 Synergistic refolding of citrate synthase by the DnaK/DnaJ/GrpE and thioredoxin/thioredoxin reductase chaperone machines

Citrate synthase was denatured in urea and then renatured by dilution of the denaturant as described in the Experimental section, at a concentration of 0.1 μM in the absence of additional protein (○) and in the presence of either 1 μM DnaK, 0.4 μM DnaJ, 0.4 μM GrpE, 0.2 mM ATP and 1 mM $MgCl_2$ without (○) or with 1 μM thioredoxin, 1 μM thioredoxin reductase, 0.4 mM NADPH and 0.4 mM cystine (●). The results are the mean values of three experiments.

and cysteine, and the thioredoxin/thioredoxin reductase system undergoes important conformational changes during catalysis [33]). As shown in Figure 4, MglB renaturation is 3-fold accelerated when redox cycling of thioredoxin/thioredoxin reductase occurs in the presence of NADPH and cystine, thus displaying a 15-fold acceleration over spontaneous refolding (no acceleration of MglB renaturation was observed in the presence of thioredoxin/thioredoxin reductase and one of the following: NADPH, NADP, cystine, cysteine, or NADPH and cystine without thioredoxin/thioredoxin reductase; results not shown). Thus the redox activity of the thioredoxin reductase/thioredoxin system appears to stimulate its chaperone activity (even in the absence of dithiol–disulphide reactions between the chaperone system and its substrate protein), suggesting that redox-induced conformational changes of thioredoxin reductase/thioredoxin favours cycles of binding and release of unfolded proteins, in a manner that is reminiscent of the ATP-dependent cycles of conventional chaperones [34,35]. Such redox-dependent cycles of substrate–protein binding and release to PDI have been reported recently [14].

Synergy between the DnaK/DnaJ/GrpE and thioredoxin/thioredoxin reductase chaperone machines

We tested whether the thioredoxin/thioredoxin reductase/NADPH chaperone machine contributes to synergistic refolding when added to DnaK/DnaJ/GrpE/ATP. The renaturation of citrate synthase increases from 35 % in the presence of DnaK/DnaJ/GrpE/ATP to 43 % in the presence of additional thioredoxin/thioredoxin reductase/NADPH/cystine, suggesting that some synergistic refolding of citrate synthase occurs in the presence of both chaperone machines (Figure 5).

Thioredoxin reductase, but not thioredoxin, protects citrate synthase from irreversible aggregation during thermal stress

We investigated the function of thioredoxin and thioredoxin reductase under heat-shock conditions. As reported previously

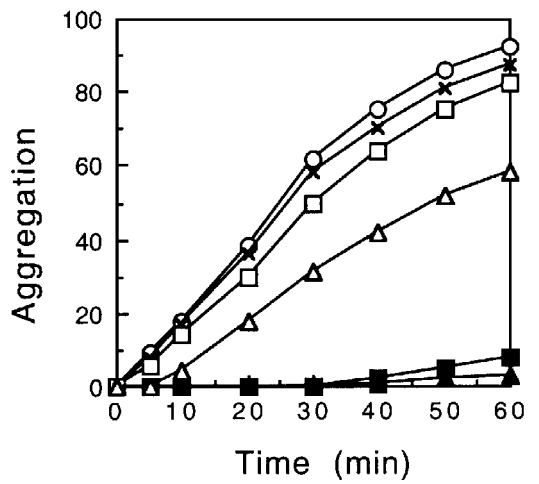

Figure 6 Thermal aggregation of citrate synthase in the presence of thioredoxin and thioredoxin reductase

The kinetics of citrate synthase aggregation were determined by light scattering at 650 nm. Native citrate synthase was diluted to a final concentration of 0.8 μM at 43 °C, as described in the Experimental section, in the absence of additional protein (○), or in the presence of 10 μM thioredoxin (×), 1 μM thioredoxin reductase (□), 7 μM thioredoxin reductase (■), 1 μM DnaK (△) or 7 μM DnaK (▲).

[24,29], citrate synthase loses its native conformation and undergoes aggregation during incubation at 43 °C. The addition of 10 μM thioredoxin (and up to 30 μM; results not shown) does not significantly protect 0.8 μM citrate synthase against thermal aggregation (Figure 6). In contrast, 1 μM thioredoxin reductase reduces citrate synthase aggregation, and 7 μM thioredoxin reductase completely suppresses citrate synthase aggregation (in a similar experiment, 1 μM DnaK partially reduces citrate synthase aggregation, and 7 μM DnaK suppresses citrate synthase aggregation; Figure 6). Thus whereas thioredoxin, like serum

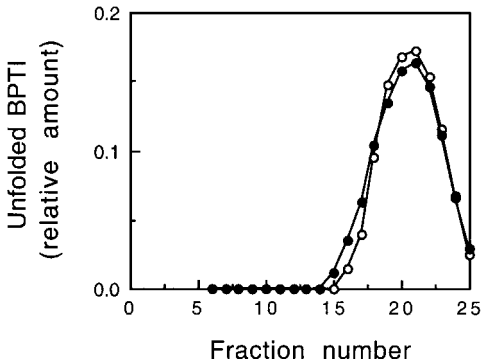

Figure 7 Gel-permeation chromatography and thioredoxin–unfolded BPTI complexes

Unfolded [^{3}H]BPTI (0.5 μM) was incubated either alone (○), or with 4 μM thioredoxin (●), and the mixture was loaded on a TSK G-2000 HPLC gel-permeation column as described in the Experimental section. Fractions were collected and counted for radioactivity.

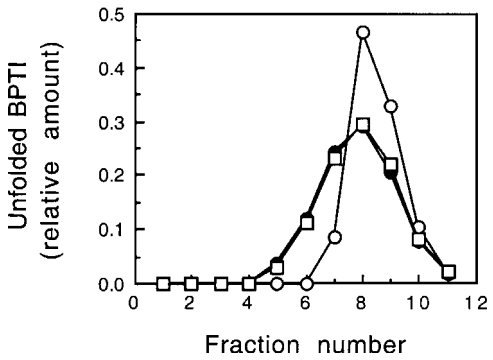

Figure 8 Gel-permeation chromatography and thioredoxin reductase–unfolded BPTI complexes

Unfolded [^{3}H]BPTI (0.5 μM) was incubated alone (○), with 4 μM thioredoxin reductase (●) or with 4 μM thioredoxin reductase and 10 μM thioredoxin (□), and the mixture was loaded on a G-75 gel permeation column as described in the Experimental section. Fractions were collected and counted for radioactivity.

albumin and lysozyme [24], is inefficient in protecting citrate synthase from thermal denaturation, thioredoxin reductase is nearly as efficient as DnaK. This suggests that thioredoxin reductase can interact with partially unfolded proteins more efficiently than thioredoxin.

Interaction between thioredoxin, thioredoxin reductase and unfolded proteins

One characteristic of molecular chaperones is their preferential interaction with unfolded proteins [27,35]. Unfolded BPTI, but not native BPTI, interacts with chaperones, including DnaK [36,37]. The formation of complexes between 0.5 μM unfolded BPTI (6000 Da) and 4 μM thioredoxin (12 000 Da) was studied by gel filtration on a gel-permeation HPLC column TSK G-2000. A small percentage (4 %) of unfolded BPTI reproducibly fractionates as material of a higher molecular mass than unfolded BPTI alone (Figure 7; under similar conditions 4 μM DnaK retained 34 % of unfolded BPTI, results not shown). The amount of unfolded BPTI bound to thioredoxin increases at higher flow rates of the column (results not shown), suggesting that a greater fraction of unfolded BPTI is bound to thioredoxin at equilibrium, and dissociates during the chromatographic procedure. When complex formation between 0.5 μM native BPTI and 4 μM thioredoxin was studied by gel filtration on the same column, 6 % native BPTI fractionated as material of a higher molecular mass than native BPTI alone (results not shown). Thus thioredoxin, in contrast with DnaK and other chaperones, displays only a moderate affinity for unfolded proteins, and does not discriminate efficiently between unfolded and folded BPTI. Although unfolded and folded BPTI bind to thioredoxin with a similar efficiency, the interactions involved in its binding are probably different, especially since unfolded BPTI exposes hydrophobic surfaces, and native BPTI contains numerous cysteines (blocked by iodoacetamide in unfolded BPTI), which could possibly interact with the active cysteines of thioredoxin [38].

We also studied the interaction between unfolded BPTI and thioredoxin reductase. Formation of complexes between 0.5 μM unfolded BPTI (6000 Da) and 4 μM thioredoxin reductase (72 000 Da for the dimer) was studied by gel filtration on a gel permeation G-75 column; a high percentage (28 %) of unfolded BPTI fractionates as material of a higher molecular mass than unfolded BPTI alone (Figure 8). In similar conditions 4 μM DnaK retained 29 % of unfolded BPTI (results not shown), and a binding of unfolded BPTI to thioredoxin could hardly be detected (results not shown). Unfolded BPTI fractionates also as material of a high molecular mass in the presence of 4 μM thioredoxin reductase and up to 10 μM thioredoxin (Figure 8) suggesting that the interaction between thioredoxin and thioredoxin reductase does not compete for the binding of unfolded BPTI to thioredoxin reductase. In contrast with unfolded BPTI, native BPTI did not bind to thioredoxin reductase: when 0.5 μM native BPTI and 4 μM thioredoxin reductase were loaded on a gel-permeation G-75 column, no native BPTI eluted as a high-molecular-mass complex (results not shown).

We also studied by gel filtration the interaction between thioredoxin, thioredoxin reductase and the permanently denatured protein R-CMLA. Thioredoxin reductase displayed a strong interaction with R-CMLA whereas thioredoxin interacted only weakly with the latter protein (results not shown). Thus whereas thioredoxin appears to interact only weakly with both unfolded and folded proteins, thioredoxin reductase displays a strong interaction with unfolded proteins, and thus efficiently discriminates between unfolded and folded proteins.

DISCUSSION

We present biochemical evidence that *E. coli* thioredoxin and thioredoxin reductase have a chaperone-like function. Thioredoxin and thioredoxin reductase both increase by $\approx$3-fold the yield of active citrate synthase and α-glucosidase renaturation, like molecular chaperones. The stimulation factors of protein renaturation (more than 3-fold), and the thioredoxin and thioredoxin reductase concentrations required for half-maximal protein renaturation (below 1 μM), are not significantly different from those of DnaK, Hsp90 and small Hsps (this study and [24,28,29]).

The oxidized and reduced states of thioredoxin stimulate the renaturation of citrate synthase with similar efficiencies, suggesting that they interact with unfolded proteins in a similar manner. In fact, the structures of oxidized and reduced thioredoxin are very similar, their backbones being essentially identical except for slight differences in the active site, which contains Cys^{32} and Cys^{35}. Meanwhile, the affinity of several tight-binding proteins, such as gene 5 protein of T7 DNA polymerase, is dramatically higher for reduced thioredoxin than for its oxidized form [11].

The refolding of citrate synthase by thioredoxin redox mutants, the double mutant D26A/K57M and the C35A mutant, suggests that the redox function of thioredoxin is not involved in its chaperone function, as it is not involved in the stimulation of T7 polymerase [30] or in the assembly of filamentous phages [32].

Thioredoxin and thioredoxin reductase increase the refolding of the MglB galactose receptor, a protein without any cysteines. This suggests (as above) that the chaperone properties of thioredoxin and thioredoxin reductase are at least partially independent from their active-site cysteines. Previous reports concerning the chaperone properties of PDI showed that this latter could stimulate the refolding of glyceraldehyde-3-phosphate isomerase, a protein with no disulphide bonds [13], but this protein contains a reactive cysteine which might possibly interact with the active cysteines of PDI.

The combination of both thioredoxin reductase and thioredoxin is active in protein renaturation, and this activity is increased in the presence of NADPH and cystine, which allow redox cycling of the system. The interaction between thioredoxin reductase and thioredoxin involves the active cysteines of thioredoxin and a hydrophobic pocket of thioredoxin formed by Trp^{31}, Ile^{60}, Gly^{74} and Ile^{75}. Another hydrophobic patch of thioredoxin, however, proposed to be important in the interaction of thioredoxin with other proteins, is exposed to the solvent and does not contact thioredoxin reductase [33]. Furthermore, several domains of thioredoxin reductase could also interact with substrate proteins, and the large conformational changes between the FO (flavin oxidation by enzyme disulphide) and the FR (flavin reduction by NADPH) conformers of thioredoxin reductase [33] might accelerate protein renaturation. The increase of MglB renaturation in the presence of NADPH and cystine extends to the thioredoxin system, the concept of redox-regulated chaperones described for PDI, in which renaturation of substrate proteins is powered by a redox rather than an ATPase cycle [14].

The thioredoxin/thioredoxin reductase/NADPH chaperone machine contributes to synergistic refolding of citrate synthase when added to DnaK/DnaJ/GrpE/ATP, leading to a reproductive 25 % overstimulation of the DnaK/DnaJ/GrpE/ATP-dependent citrate synthase refolding.

Thioredoxin reductase protects citrate synthase from thermal denaturation. This protection occurs at micromolar concentrations of thioredoxin reductase, similar to those of DnaK [24] and of small Hsp (expressed as monomers) required for a similar protection [29]. In contrast, thioredoxin, like BSA, ovalbumin and lysozyme [24], does not protect citrate synthase efficiently against thermodenaturation, suggesting that it interacts less efficiently than thioredoxin reductase with partially unfolded proteins.

Thioredoxin reductase forms more stable complexes with unfolded proteins than thioredoxin. The thioredoxin reductase-unfolded BPTI complexes display a stability similar to that of the DnaK-unfolded BPTI complexes. Like DnaK [24,36], thioredoxin reductase interacts with unfolded BPTI but not with native BPTI, and discriminates efficiently between unfolded and native proteins. Furthermore, the interaction between thioredoxin reductase and unfolded BPTI is not decreased in the presence of thioredoxin, suggesting that its thioredoxin-binding site is not involved. In contrast with thioredoxin reductase, thioredoxin forms only weak complexes with unfolded BPTI, and does not discriminate efficiently between unfolded and native BPTI. The interaction between thioredoxin and substrate proteins has been suggested to involve the Cys-Gly-Pro-Cys active site and several residues, including *cis*-Pro_{76} and Gly_{92}, which form a moderately hydrophobic surface around the active site and facilitate interactions with other enzymes [33]. A conserved ligand-binding motif has been identified in the thioredoxin superfamily of proteins [40], which includes thioredoxins, PDI, glutaredoxins, glutathione S-transferases and glutathione peroxidase [10]. This involves alignment of a peptide as a β-sheet-like arrangement stabilized by conserved backbone–backbone hydrogen bonds [40]. This may well operate also in the chaperone activity of thioredoxin with unfolded proteins. In addition to hydrophobic interactions, hydrogen bonds and salt bridges probably participate in substrate binding [39]. Our results favour the hypothesis of weak hydrophobic interactions between thioredoxin and substrate proteins, which explains its chaperone-like activity in protein folding, but does not allow the isolation of stable complexes between thioredoxin and unfolded proteins during gel-filtration experiments. Conversely, *E. coli* thioredoxin reductase appears to form stable complexes with unfolded proteins, suggesting that it binds substrate–proteins, and possibly unfolds them, thus facilitating the access of their disulphides to the redox site of thioredoxin. This may explain why an enzymic system of reduction of protein disulphides using NADPH, thioredoxin reductase and thioredoxin works efficiently to allow fast selective disulphide reduction [41,42].

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