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# Dimeric Trigger Factor Stably Binds Folding-competent Intermediates and Cooperates with the DnaK-DnaJ-GrpE Chaperone System to Allow Refolding*

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**Trigger factor (TF) is the first chaperone encountered by the nascent chain in bacteria and forms a stoichiometric complex with the ribosome. However, the functional significance of the high cytosolic concentration of uncomplexed TF, the majority of which is dimeric, is unknown. To gain insight into TF function, we investigated the TF concentration dependence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reactivation yield in the presence and absence of the DnaK-DnaJ-GrpE chaperone system *in vitro*. Cross-linking results indicate that the observed decrease in the reactivation yield of GAPDH at high concentrations of TF is due to the formation of a stable complex between TF dimer and GAPDH intermediates. In the absence of TF, or at low TF concentrations, the DnaK-DnaJ-GrpE chaperone system had negligible effect on the GAPDH refolding yield. However, GAPDH intermediates bound and held by dimeric TF could be specifically rescued by the DnaK-DnaJ-GrpE chaperone system in an ATP-dependent manner. This indicates the potential of TF, in its dimeric form, to act as a binding chaperone, maintaining non-native proteins in a refolding competent conformation and cooperating with downstream molecular chaperones to facilitate post-translational or post-stress protein folding.**

Protein folding in the cell occurs with high efficiency and precision because of the activity of a network of molecular chaperones. In the *Escherichia coli* cytosol, the major chaperones are trigger factor (TF),[1] IbpA/B (small Hsps), HtpG (Hsp90), ClpA/B/X (Hsp100), and the DnaK (Hsp70) and GroEL (Hsp60) chaperones with their respective co-chaperones (1–4). TF is an ATP-independent chaperone and exhibits chaperone and peptidyl-prolyl *cis/trans* isomerase activities, both of which contribute to protein folding (5–7). TF binds to ribosomes at the exit site of the peptide tunnel via interaction with the L23 protein of the ribosome (8, 9) and acts as the first chaperone encountered by nascent chains (10, 11). After interaction with TF, most proteins fold rapidly upon completion of synthesis, and only 20–35% of polypeptide chains require the further assistance of other chaperones (12, 13). Deletion of TF results in an increase in protein aggregation in the cytosol (13, 14), whereas overproduction of TF depresses the formation of inclusion bodies and increases the productive yield of aggregation-prone proteins (15, 16). TF has overlapping chaperone function with DnaK, and only if both are deleted is it lethal for bacteria (12, 13). When functioning as a molecular chaperone *in vitro*, TF has been shown to have a preference for random coil or loosely structured substrates (6, 17, 18), and the binding motif of TF has been identified as a short sequence enriched in hydrophobic and basic amino acids (19).

Most ribosomes exist in a 1:1 complex with TF, consistent with the role of TF in co-translational protein folding (8, 9). However, TF is present in a 2–3-fold molar excess over ribosomes in the cell, with the majority of free TF present as a dimer (20). The functional significance of the free TF is still unknown (20, 21), and a role of TF in mediating post-translational folding has not yet been demonstrated (1, 22). Here, based on our previous results (17, 23), the possible role of the TF dimer in post-translational protein folding was investigated, which may provide insight into the function of TF *in vivo*.

## EXPERIMENTAL PROCEDURES

*Materials*—DL-Glyceraldehyde-3-phosphate, ADP, dithiothreitol, disuccinimidyl suberate (DSS), dithiobis(succinimidyl propionate) (DTSP), β-NAD, and bovine serum albumin (fraction V) were purchased from Sigma. ATP was from Amresco. GdnHCl was from ICN. Anti-TF polyclonal antibody was prepared by Shenzhen Bioinforbody Inc. (Shenzhen, China). All other chemicals were local products of analytical grade.

Plasmid pQE60 containing the wild-type *tig* gene that encodes *E. coli* TF was donated by Professor G. Fischer. TF was expressed in *E. coli* JM109 and purified as described (24). Purification of rabbit muscle glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was as described previously (25). DnaK and DnaJ were provided by Prof. Philipp Christen and Prof. Chih-chen Wang, respectively. GrpE was purchased from Stressgen.

*Denaturation and Renaturation of GAPDH*—GAPDH was denatured in 3 M GdnHCl for 12 h at 25 °C. Reactivation was initiated by rapid 50-fold dilution to a final GAPDH concentration of 2.73 μM, with 0.1 M phosphate buffer, pH 7.5, containing 5 mM dithiothreitol and different concentrations of TF or other additives. The reactivation mixture was kept at 4 °C for 30 min and then for a further 3 h at 25 °C to allow reactivation to go to completion before the reactivation yield of GAPDH was determined. The reactivation yield of GAPDH was defined as the percentage of the activity of native GAPDH, which was determined in a Beckman DU7500 spectrophotometer as described previously (25). Aggregation of GAPDH during refolding was monitored at 25 °C by 90° light scattering at 488 nm in a Hitachi F4500 fluorescence spectrophotometer 3 h after being transferred to 25 °C.

*Reactivation of Denatured GAPDH with TF and the KJE System*—3 M GdnHCl-denatured GAPDH was rapidly diluted 50-fold into 0.1 M

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[1] The abbreviations used are: TF, trigger factor; DSS, disuccinimidyl suberate; DTSP, dithiobis(succinimidyl propionate); GdnHCl, guanidine hydrochloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KJE, DnaK-DnaJ-GrpE.

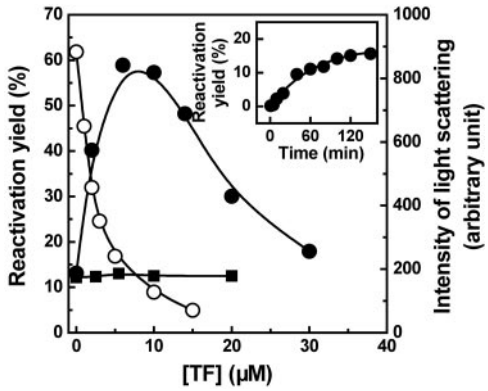



FIG. 1. **Effect of TF concentration on GAPDH reactivation.** Reactivation (*filled circles*) and aggregation (*empty circles*) of 2.73 $\mu$M GAPDH in 100 mM phosphate buffer, pH 7.5. *Squares* represent the effect of bovine serum albumin on the reactivation yield of GAPDH. GAPDH was denatured in 3 M GdnHCl for 12 h at 25 °C. Refolding was initiated by 50-fold dilution into buffer containing TF or bovine serum albumin at 4 °C, followed by preincubation at 4 °C for 30 min before transfer to 25 °C for 3 h, after which the GAPDH reactivation yield was determined. The reactivation yield was defined as a percentage of the activity of native GAPDH. *Inset*, time course of reactivation of GAPDH in the presence of 30 $\mu$M TF at 25 °C, subsequent to preincubation at 4 °C for 30 min.

phosphate buffer, pH 7.5, containing 5 mM dithiothreitol, 5 mM $MgCl_2$ and different amounts of TF to a final GAPDH concentration of 2.73 $\mu$M. The reactivation mixture was kept at 4 °C for 30 min and then transferred to 25 °C. At different reactivation times, the sample was supplemented with refolding buffer containing the KJE system (30 $\mu$M DnaK, 6 $\mu$M DnaJ, 3 $\mu$M GrpE, and 10 mM ATP) and 5 mM $MgCl_2$, at a volume ratio of 1 to 1. To ensure complete reactivation of GAPDH in the presence of the KJE system, the reactivation mixture was kept at 25 °C for 5 h before the reactivation yield of GAPDH was determined.

*Cross-linking and Electrophoresis*—Reactivation of denatured GAPDH was carried out in 15 mM phosphate buffer, pH 7.5, at 4 °C; the final GAPDH and TF concentrations were 3.6 and 36 $\mu$M, respectively. After proteins were incubated for 30 min at 4 °C and 3 h at 25 °C, DSS or DTSP dissolved in $Me_2SO$ was added, and the final concentration of cross-linker was 6 mM. The cross-linking reaction was stopped after 30 or 60 min with 150 mM Tris-HCl, pH 7.0, and samples were desalted using a Sephadex G-25 column (26). Samples were separated by 8–15% gradient SDS-PAGE and detected by silver staining and Western blot using anti-TF polyclonal-antibody.

After non-reducing SDS-PAGE of the TF-GAPDH product cross-linked with DTSP, a strip of gel containing the ~200-kDa cross-linked product was excised and washed with water. The strip was incubated with 15 ml of equilibration buffer (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, and 2% SDS) containing 1% dithiothreitol (freshly prepared) for 15 min and then rinsed with water. The strip was then incubated with 15 ml of equilibration buffer containing 2.5% iodoacetamide (freshly prepared) for 15 min. After rinsing with water, the strip was loaded onto the reducing SDS-PAGE gel and electrophoresed. The electrophoresis result was detected by silver staining.

In the case of cross-linking of GAPDH intermediates with different concentrations of TF, denatured GAPDH was diluted into the buffer containing different concentrations of TF and incubated at 4 °C for 30 min, followed by addition of DSS. The cross-linking reaction was stopped with Tris-HCl after 2 h. Samples were desalted, and 2 M urea was added to prevent aggregation. Samples were then separated by gradient SDS-PAGE and detected by silver staining. All operations were carried out at 4 °C to avoid aggregation.

## RESULTS

*Effect of TF Concentration on the Reactivation and Aggregation of Denatured GAPDH*—The standard refolding assay for GAPDH, as used here and previously (17, 27–29), involves 50-fold dilution of 3 M GdnHCl denatured GAPDH at 4 °C, preincubation for 30 min, and then transfer to 25 °C for 3 h to allow reactivation. As shown in Fig. 1, the spontaneous reactivation yield of GAPDH was very low (~10%). When denatured GAPDH was diluted into buffer containing TF, the reactivation yield of GAPDH increased with increasing TF concentration, reaching a maximum (60%) at 6 $\mu$M TF. However, with a further increase in TF concentration, the reactivation yield decreased to ~20% in the presence of 30 $\mu$M TF, indicating that high concentrations of TF actually suppress reactivation of GAPDH. On the other hand, the extent of GAPDH aggregation decreased steadily with increasing TF concentration over the same concentration range, and aggregation of GAPDH was almost completely suppressed in the presence of 15 $\mu$M TF (Fig. 1). This shows that the reduction in the reactivation yield of GAPDH is not because of aggregation. This, therefore, suggests that at high TF concentrations a stable complex is formed between TF and GAPDH intermediates (17). To further understand this unusual phenomenon, the composition of the TF·GAPDH refolding complex was investigated.

*Characterization of the Complex between GAPDH Intermediates and TF*—Earlier studies detected formation of a TF dimer by treatment with the cross-linker DSS, consistent with a monomer-dimer equilibrium of TF (20, 23).[2] To investigate whether a complex is formed between TF and GAPDH folding intermediates, cross-linking was used. We added DSS to the TF-assisted GAPDH refolding system after 3 h of incubation at 25 °C, after which the reactivation yield of GAPDH does not change further (Fig. 1, *insert*) (17). The cross-linked product was then analyzed by gradient SDS-PAGE. When native GAPDH (36 kDa monomeric molecular mass) or TF (48 kDa monomeric molecular mass) was incubated independently with DSS, they each showed bands consistent with a mixture of monomeric and dimeric species (Fig. 2*A*, *lanes 3* and *4*). In addition, TF showed bands of a size unable to enter the gel matrix, corresponding to higher order cross-linked oligomers. However, it is clear that under the conditions used, individual cross-linking of either TF or GAPDH does not result in formation of tetrameric cross-linked species (Fig. 2*A*, *lanes 3* and *4*). When native GAPDH was incubated with TF, the bands observed were consistent with a mixture of the same monomeric and dimeric TF and GAPDH species (Fig. 2*A*, *lane 7*). However, when denatured GAPDH was refolded in the presence of TF, a new band with molecular mass of ~200 kDa was observed (Fig. 2*A*, *lanes 1* and *2*), suggesting formation of a complex between TF and refolding intermediates of GAPDH. Earlier studies detected a dimeric folding intermediate of GAPDH under the same conditions as used here (30). Thus, the 200-kDa species is likely to correspond to the complex between dimeric TF and dimeric GAPDH. Other cross-linked complexes involving different ratios of TF and GAPDH may also be present but would co-migrate with the broad bands for the other oligomeric species.

The involvement of TF in the ~200-kDa cross-linked complex was confirmed by Western blot using an anti-TF polyclonal antibody (Fig. 2*B*). To confirm the involvement of GAPDH, DTSP, a cleavable analog of DSS, was used as the cross-linker. The electrophoresis pattern of the products cross-linked by DTSP was identical to those cross-linked with DSS under the same conditions (data not shown). Subsequent reduction of the disulfide bond of DTSP allows separation of the DTSP cross-linked products. Therefore, the composition of the DTSP cross-linked ~200-kDa strip could be further analyzed by re-electrophoresis on reducing SDS-PAGE. The result showed clearly that the ~200-kDa cross-linked product contains both TF and GAPDH (Fig. 2*C*). This provides direct evidence that tight complexes form between folding intermediates of GAPDH and TF.

[2] C.-P. Liu, Z.-Y. Li, G. C. Huang, S. Perrett, and J.-M. Zhou, unpublished data.

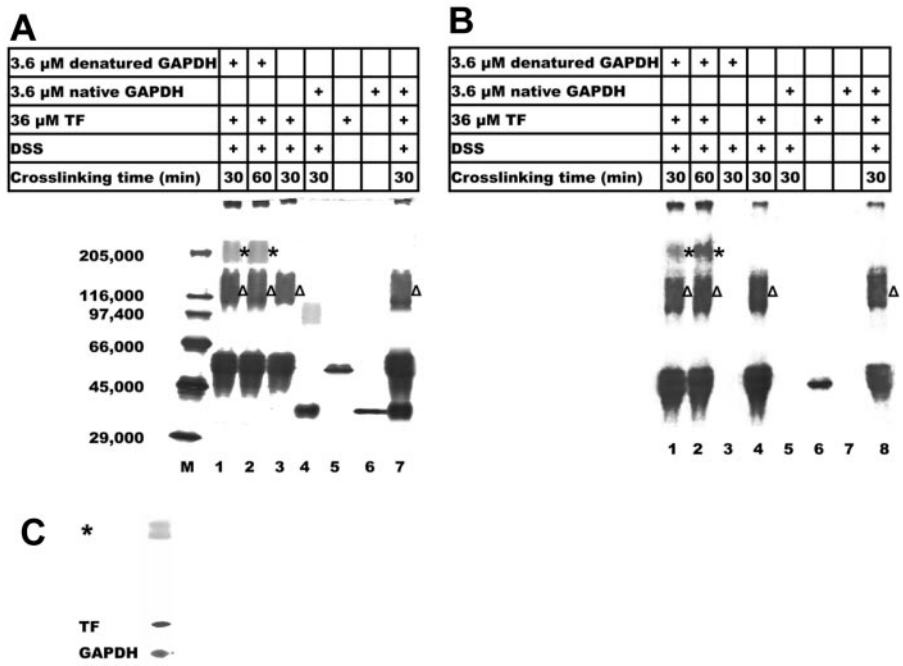



FIG. 2. **Cross-linking of trigger factor with GAPDH.** GAPDH was refolded in the presence of TF (details as indicated or as in Fig. 1). Proteins were then cross-linked with DSS (see "Experimental Procedures"). Samples were separated by gradient SDS-PAGE and detected by silver staining (*A*) or Western blot using anti-TF polyclonal antibody (*B*). The sizes of the molecular mass markers (*M*) and the positions of the bands corresponding to TF dimer (Δ) and the ~200-kDa TF·GAPDH complex (*) are indicated. *C*, re-electrophoresis of the DTSP cross-linked ~200-kDa strip on reducing SDS-PAGE (see "Experimental Procedures") detected by silver staining.

*TF-bound GAPDH Intermediates Could Be Specifically Rescued by the KJE System*—DnaK-DnaJ-GrpE is known to act as an ATP-dependent downstream chaperone system that can promote non-native proteins held by "binding" chaperones, such as the small Hsps, to refold to the native state (31–35). Therefore, if the formation of a TF·GAPDH complex represents a binding chaperone function of TF, then a similar mechanism for release and rescue of the substrate from TF should exist, and the KJE system is a likely candidate for this role. To test this, GAPDH was refolded in buffer containing 30 μM TF. Under these conditions, the reactivation yield of the TF·GAPDH folding mixture reaches only 15% after 2 h of incubation at 25 °C, and no further change was observed after longer incubation times (Fig. 1, *inset*; Fig. 3*A*, *No dilution*). If after 2 h of incubation, the refolding mixture was diluted 1:1 with buffer containing the complete KJE+ATP system, a marked increase in the reactivation yield was observed (Fig. 3*A*, *KJE+ATP*). As shown previously (17), dilution has also been observed to allow a degree of recovery of the GAPDH reactivation yield after arrest of folding by high concentrations of TF. However, the increase in the reactivation yield that could be attributed to the dilution effect (Fig. 3*A*, *Buffer*) was much lower than the effect of KJE (Fig. 3*A*, *KJE+ATP*). Further, when the KJE system was added in the absence of ATP (Fig. 3*A*, *KJE−ATP*), no increase in the reactivation yield was observed and the activity remained the same as for the undiluted sample (Fig. 3*A*, *No dilution*). These experiments illustrate that the KJE system is able to interact with the TF·GAPDH complex and support subsequent refolding of GAPDH intermediates in an ATP-dependent manner. However, in the absence of ATP, the KJE system contributes to the arrest of GAPDH refolding by TF.

We also investigated the effect of the timing of addition of KJE to the folding mixture. As shown above, addition of KJE after the reactivation of GAPDH has already reached a plateau (Fig. 1, *inset*, *2 h*) results in a marked increase in the refolding yield (Fig. 3*A*, *KJE+ATP*). When we decreased the delay in the addition time of KJE, a further increase in reactivation yield was observed (Fig. 3*B*, *filled squares*). The much smaller increase in the spontaneous refolding yield produced by an equivalent degree of dilution also increased steadily with decreased delay of addition (Fig. 3*B*, *empty squares*), although the effect was less pronounced than in the presence of KJE. These results imply that after dilution and preincubation of denatured GAPDH at 4 °C in the presence of a high concentration of TF, the majority of GAPDH molecules are initially in a conformation that is competent to be recognized and refolded by the KJE system. However, during prolonged incubation with TF, GAPDH continues to undergo conformational changes, either while bound to TF or during binding-release cycles, resulting in a decrease in the proportion of GAPDH molecules that are competent to refold.

*Dimeric Rather Than Monomeric TF Binds and Holds GAPDH Intermediates*—The effect of TF concentration on the efficiency of GAPDH reactivation by the KJE system was investigated (Fig. 4). Denatured GAPDH was diluted into buffer containing 0, 6, or 30 μM TF at 4 °C, and the samples were preincubated for 30 min. The samples were then diluted 1:1 with buffer (Fig. 4*A*, *1:1 Buffer*) or buffer containing the complete KJE system (Fig. 4*A*, *1:1 KJE*) and transferred to 25 °C to allow refolding. In a parallel experiment, the effect of TF concentration on the relative population of dimeric TF or stable TF·GAPDH complexes in the 4 °C preincubation mixture was also determined (Fig. 4*B*).

In the absence of TF, the low spontaneous reactivation yield of GAPDH (Fig. 4*A*, *No dilution*) was not affected by 1:1 dilution with buffer (Fig. 4*A*, *1:1 Buffer*) and was increased only slightly by the addition of the complete KJE system (Fig. 4*A*, *1:1 KJE*). This implies that the KJE system alone is unable to efficiently capture GAPDH folding intermediates or to prevent GAPDH misfolding and aggregation under the conditions used here.

When GAPDH was refolded in the presence of 6 μM TF, the reactivation yield of GAPDH increased to 60% (Fig. 4*A*, *No dilution*). In this case, 1:1 dilution with either buffer alone or buffer containing the KJE system gave no further improvement in the reactivation yield. Under these conditions, TF is predominantly monomeric and no stable complex between TF and GAPDH intermediates could be detected in the preincubation mixture (Fig. 4*B*, *lane 3*), suggesting that interaction between monomeric TF and GAPDH folding intermediates is highly dynamic.

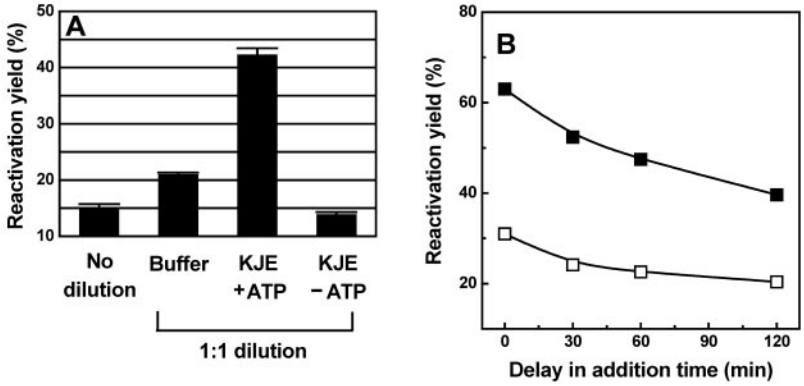
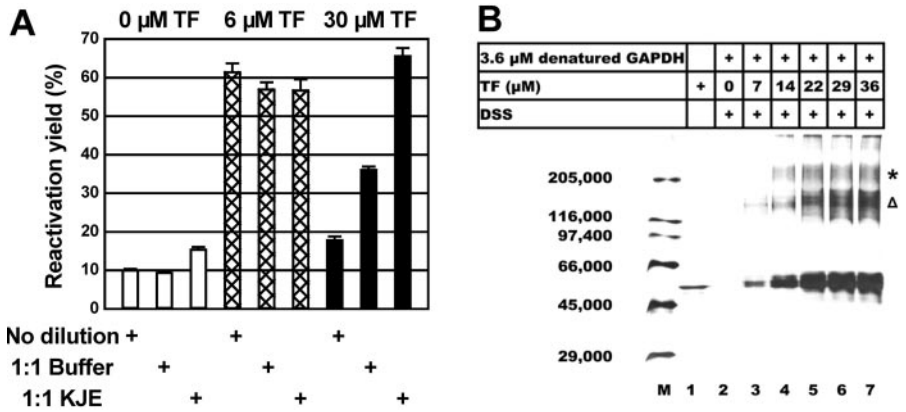



FIG. 3. **Cooperation of TF and the KJE chaperone system.** *A*, denatured GAPDH was refolded in the presence of 30 $\mu$M TF and incubated for 2 h at 25 °C (other details as in Fig. 1). The refolding mixture was then diluted 1:1 with buffer containing the KJE system with or without ATP. Refolding was allowed to proceed for a further 5 h before assay of GAPDH activity. The final concentrations of TF, DnaK, DnaJ, GrpE, and ATP were 15, 15, 3, and 1.5 $\mu$M and 5 mM, respectively. Samples diluted 1:1 with buffer, or with no dilution, were included as controls. *B*, effect of the time of addition of the KJE system (*filled squares*) or buffer (*empty squares*) on the reactivation yield of GAPDH. The time indicated is the delay between transfer of the refolding mixture to 25 °C and addition of the KJE system. Other details are as in *panel A*).

FIG. 4. *A*, effect of the KJE system on GAPDH reactivation in the presence of different concentrations of TF. Denatured GAPDH was refolded in buffer containing 0, 6, or 30 $\mu$M TF and preincubated at 4 °C for 30 min before 1:1 dilution with buffer containing the complete KJE system. The reaction mixture was then transferred to 25 °C and incubated for a further 5 h before assay of GAPDH activity. Other details are as in Fig. 3. *B*, TF concentration dependence of the cross-linked products formed after dilution of denatured GAPDH in the presence of TF and preincubation at 4 °C for 30 min (other details as in Fig. 2).




When GAPDH was refolded in the presence of 30 $\mu$M TF, the observed arrest of reactivation (Fig. 4*A*, *No dilution*) coincides with increased population of both TF dimer (Fig. 4*B*, Δ) and the stable TF·GAPDH complex (Fig. 4*B*, *) in both the 4 °C preincubation mixture (Fig. 4*B*) and after prolonged reactivation times at 25 °C (Fig. 2). In this case, 1:1 dilution with the KJE system resulted in a dramatic increase in the reactivation yield of GAPDH to over 60% (Fig. 4*A*, *1:1 KJE*). 1:1 dilution with buffer alone (Fig. 4*A*, *1:1 Buffer*) gave a lower increase in the reactivation yield to 35%. Interestingly, this is lower than the 50% yield obtained when denatured GAPDH is diluted in the presence of 15 $\mu$M TF. This implies that monomeric TF may act on earlier folding intermediates of GAPDH than are recognized and bound by dimeric GAPDH.

Taken together, these results indicate that it is dimeric rather than monomeric TF that can bind and hold folding intermediates of GAPDH. Further, the KJE system is able to interact with the TF·GAPDH complex and assist refolding of the stably bound intermediates in an ATP-dependent manner.

## DISCUSSION

Earlier studies have shown that TF and DnaK possess overlapping substrate pools and binding specificities (14, 19, 36) that contribute to the efficient transfer of the nascent peptide from ribosome-associated TF to the Hsp70 (KJE) system (12, 13, 22). Here, we investigated whether TF might work in concert with KJE in a function distinct from its ribosome-associated role, which could shed light on the physiological significance of the high cytosolic concentration of TF (37), particularly in its dimeric form (20).

We were prompted to investigate the possibility of a distinct functional role for dimeric TF by the observation of an unusual dependence on TF concentration in the assisted folding of two substrates commonly used in chaperone studies, namely GAPDH (17, 38) and carbonic anhydrase II (23). GAPDH, which is a homotetramer with monomeric molecular mass of 36 kDa, is widely used as a substrate to study the function of molecular chaperones *in vitro*, and with most chaperones, including GroEL (39), DsbC (27), and PDI (28), the reactivation yield of GAPDH increases and then reaches a plateau with increasing chaperone concentration. However, in the case of TF-assisted GAPDH refolding, the reactivation yield is found to increase but then decrease with further increase in the TF concentration (17, 38), even though aggregation is completely suppressed (Fig. 1) (17). We suggested previously that this might be due to formation of a stable complex between TF and GAPDH folding intermediates. Consistent with this view, the GAPDH refolding yield can be partially restored by addition of reduced $\alpha$-lactalbumin as a competitor for TF binding, by further diluting the refolding mixture, or by changing the nature of the solvent to disrupt hydrophobic interactions in the complex (17). In another study, reactivation of the monomeric substrate carbonic anhydrase II was found to plateau at moderate TF concentrations (above 12 $\mu$M), whereas further increase in the TF concentration (up to 30 $\mu$M) continued to decrease the degree of aggregation due to formation of a stable complex between dimeric TF and an off-pathway aggregation-prone intermediate of carbonic anhydrase II (23). These results led us to speculate that dimeric TF might have a distinct ability to stably bind partially denatured states.

To investigate this further, we first determined the compo-

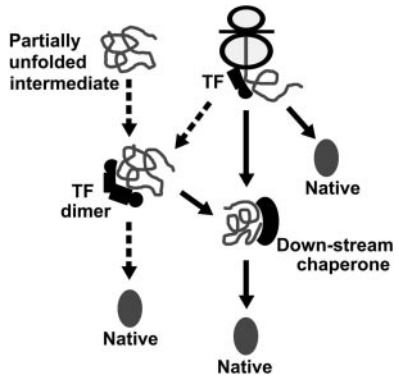



FIG. 5. **The possible role of dimeric TF in post-translational folding in the cytosol.** Upon emerging from the exit of the peptide tunnel in the ribosome, nascent polypeptides generally interact with ribosome-associated TF and most small proteins fold rapidly without further assistance. Longer chains interact subsequently with the downstream chaperones, such as DnaK-DnaJ-GrpE and GroEL-ES, allowing completion of folding. In certain cases, folding intermediates may be bound and held by dimeric TF in the cytosol. These polypeptides may then be delivered to downstream chaperones such as DnaK-DnaJ-GrpE.

sition of the species formed when GAPDH is refolded in the presence of high concentrations of TF. We found that the arrest of GAPDH reactivation at high TF concentrations is due to formation of a stable complex between TF and GAPDH. The size of this complex (Fig. 2) and the TF concentration dependence of its appearance (Fig. 4*B*) are consistent with it containing dimeric TF.

An important aspect of binding chaperone function is the ability to cooperate with other chaperone systems to trigger release of the substrate (2). Without this type of release mechanism, the stable interaction would represent suicide for the chaperone and wastage of the substrate. As it is already well established that TF cooperates closely with Hsp70 in a number of contexts in the cell (12, 13, 22), then if dimeric TF does form stable substrate complexes *in vivo*, the Hsp70 system would be the main candidate to play the release and rescue role. We therefore investigated the ability of TF to cooperate with KJE in the reactivation of GAPDH. Interestingly, the KJE system was found to be extremely inefficient at assisting refolding of GAPDH in the absence of TF (Fig. 4*A*), suggesting that KJE alone is unable to rescue aggregation-prone intermediates of GAPDH. In addition, KJE did not contribute further to the TF-assisted refolding yield of GAPDH at low concentrations of TF (Fig. 4*A*) where TF is predominantly monomeric and interaction with GAPDH intermediates is dynamic (Fig. 4*B*). However, folding-competent intermediates that were trapped and held at high concentrations of TF were efficiently released and refolded by KJE in an ATP-dependent manner (Figs. 3*A* and 4*A*). This then presents a plausible physiological scenario in which dimeric TF could bind and hold denatured or aggregation-prone protein states, for example under stress conditions, only releasing the substrate when the following conditions are met: 1) permissive folding conditions have been restored, 2) ATP levels are sufficient, and 3) Hsp70 is available to assist refolding. In the absence of any one of these conditions, the substrate would remain safely bound.

It has been suggested that the TF monomer-dimer equilibrium is of physiological relevance, either because dimeric TF represents a storage form ensuring saturation of ribosomes with TF or because the monomeric and dimeric forms have distinct functions (20). The results presented here show that the dimeric form of TF is able to form stable complexes with partially folded species and cooperate specifically with the KJE system to facilitate their refolding. The significantly stronger binding energy provided by a dimeric molecule, with two substrate binding sites, compared with the individual monomers (40), could account for distinct functional properties of dimeric and monomeric (or ribosome-bound) TF. This would be consistent with a distinct functional role for dimeric TF. The question then is whether tight substrate binding by dimeric TF is an extension of its role in nascent peptide folding or whether TF may, in fact, play a more diverse range of roles in the cell than previously thought (Fig. 5).

It was recently shown that ribosome-associated TF can cooperate with KJE to improve the folding yield of larger nascent chains by binding to the substrate co-translationally and delaying the folding process to involve a post-translational component (22). This involves recruitment of additional TF molecules to the ribosome, although no dependence on TF concentration was detected over the range 5–15 $\mu$M. It will be interesting to determine whether there is any direct involvement of dimeric TF in this process.

Surprisingly, deletion of the TF gene actually increases the rate of protein export in *E. coli*, which is attributed to the ability of TF to bind nascent chains for a prolonged period (41). Further, it is the presence of TF that gives rise to the requirement for the accessory chaperone SecB to promote protein export (41), and DnaK can at least partially substitute for SecB (42). The fact that the rate of protein export in *E. coli* is found to be inversely proportional to the TF concentration (41) raises the intriguing possibility that dimeric TF might be involved.

A role that has not previously been ascribed to TF is in post-translational or post-stress folding and quality control. The ability of dimeric TF to hold aggregation-prone intermediates in a folding competent state and to cooperate with the Hsp70 system to allow release and refolding is highly reminiscent of the properties described for the small heat shock proteins. These binding chaperones appear to have limited ability to contribute to the refolding process but provide an efficient reservoir for partially denatured states and facilitate their delivery to Hsp70 (2, 31–35). Deletion of the TF gene is nonlethal, even under heat shock conditions, which has been ascribed to its redundancy of function with the Hsp70 system in co-translational folding (12–14). Similarly, if TF dimer plays a role as a binding chaperone during the stress response, this function is likely to overlap with that of the bacterial small Hsps IbpA/B (33). Nevertheless, the results presented here indicate that the role of dimeric TF *in vivo* is an area worthy of further investigation.

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