Chaperones of Thermus thermophilus cooperate in reactivation of heat-inactivated proteins. The protein, inactivated at a high temperature in a TDNAKJ-GrpE set, recovered its activity during subsequent incubation with TCPB at moderate temperature (Motohashi, K., Watanabe, Y., Yokda, M., and Yoshida, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7184–7189). Here, we report that the addition of chaperonin (Tcpn) at moderate temperature improves the yield of the TDNAKJ-GrpE-ClpB-dependent reactivation. The trap-Tcpn, which binds substrate protein but does not release it, inhibits reactivation severely. Maximum recovery is gained at stoichiometric amounts of each component of TDNAKJ, TGrpE, and TCPB relative to the substrate monomer. These observations indicate that, driven by ATP hydrolysis, TDNAKJ-GrpE-ClpB chaperones catalytically cooperate and release heat-inactivated protein as a non-native, chaperonin-recognizable folding intermediate.

Recently, the functional chaperone cooperation between Hsp70 and Hsp104 in reactivation of heat-inactivated proteins has been noted. In eukaryotes, this cooperation enables yeast to recover from heat stress in vivo (1, 2) and reactivate proteins that had been chemically denatured and allowed to aggregate in vitro (3). In prokaryotes, we have demonstrated that heat-inactivated proteins were rescued by the cooperation between DnaK (Hsp70)-DnaJ-GrpE and ClpB (Hsp104) from a thermophilic eubacteria, Thermus thermophilus (4). Such a cooperation was also reported for Escherichia coli (5). Here, we report the role of each component in this cooperation and relationship to Hsp60 (GroEL, chaperonin).

E. coli DnaK, together with its co-chaperones, DnaJ and GrpE, can assist in the refolding of some proteins (6–8). The binding and release of substrate proteins are regulated by ATP/ADP state of DnaK, which is under tight control with sus ATP-binding sequence motifs, and forms a homohexameric complex (18–21). Like E. coli ClpB, TCPB (97 kDa) has an ATPase activity that is stimulated by the addition of a model denatured protein (4, 22). TCPB shows chaperone activity only when combined with TDNAKJ complex and TGrpE. When thermophilic lactate dehydrogenase (LDH) is heat-inactivated at high temperature in the presence of the KJE set and ATP, the enzyme activity of LDH is recovered during subsequent incubation with TCPB at a moderate temperature (4).

Chaperonin (Hsp60) is another chaperone that plays a critical role in cells, even under condition without stress (23). E. coli chaperonin, GroEL, is composed of 14 identical subunits forming two heptamer rings stacked back to back (24). GroEL binds nonnative protein and sequesters it into the central cavity surrounded by the inside wall of a heptamer ring and capped by GroES, a co-chaperonin that also has a heptameric structure (25–27). ATP hydrolysis provides a timer to give the encapsulated protein several seconds to fold without fear of aggregation, and then GroES dissociates from GroEL, allowing substrate protein to escape from the cavity (28). Unlike GroEL of E. coli, GroEL of T. thermophilus is purified as a complex of GroEL (tetradecamer) and GroES (heptamer) (Tcpn) (29). It can protect substrate proteins from irreversible heat denaturation at high temperatures and reactivate them at moderate temperatures by the addition of ATP (30).

To obtain further insight into the DnaKJ-GrpE-ClpB (KJE-B)-dependent reactivation of heat-inactivated protein, we introduced Tcpn into the reaction and changed the concentrations of each chaperone component. The results indicate that KJE set and TCPB cooperate and release the substrate protein into the medium as a chaperonin-recognizable folding intermediate.
**EXPERIMENTAL PROCEDURES**

**Expression of Tcpn—**Plasmid containing genes for GroEL and GroES of *T. thermophilus* in pET21c was constructed as follows. A Pet1-KpnI fragment of pRCC29 coding the C-terminal half of TGroE (31) and a Kpn1/BamHI fragment of pRCC29 spanning the C-terminal region of TGroE to the center of TGroE were ligated into Pet1-BamHI-digested pRCC70 (31). Plasmid pRCC50, obtained thereby, was further digested with Ndel-BamHI. The fragment was subcloned into the corresponding Ndel-BamHI sites in pET21c, and it was named the pRCC501. *E. coli* BL21 (DE3) bearing pRCC501 was cultured at 37 °C in 2× YT culture medium containing 50 μg/ml ampicillin. Production of Tcpn was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration, 1 mM).

**Isolation of proteins—**TDnAJ complex, TGrpE, TClpB, and Tcpn were expressed in *E. coli* carrying pMKJ6 (TDnAJ complex), pMGE3 (TGrpE), pMCB1 (TClpB), and pRCC501 (Tcpn), respectively, and purified as described (4, 14, 15, 32). Throughout this paper, concentrations of substrate proteins are expressed as monomers and those of the *T. thermophilus* chaperones as trigonal complex (TDnAJ complex), dimer (TGrpE), hexamer (TClpB), and TGROE14-TGroE57 complex (Tcpn).

**Trap-Tcpn Preparation—**Glutaraldehyde-cross-linked Tcpn was prepared as follows. Tcpn was solubilized at a final concentration of 0.5 mg/ml in buffer A (50 mM MOPS-NaOH, pH 7.5, 150 mM KCl, 5 mM MgCl₂) containing 1.5% glacial acetic acid and incubated at 25 °C for 45 min. Glutaraldehyde was removed by incubation with 40 mM sodium borohydride for 40 min, and the solution was dialyzed to buffer A.

**Rotation of Inactivated Proteins—**Glucose-6-phosphate dehydrogenase (G6PDH, 53 kDa, purchased from Sigma) and α-glucosidase (55 kDa, purchased from Sigma) from *Bacillus stetherophilus* were used as substrate proteins. Two substrate proteins (0.2 mM) were dissolved in 250 μl of the reaction mixture (50 mM MOPS-NaOH, pH 7.5, 5 mM MgCl₂, 150 mM KCl, 1 mM diethiothreitol) containing chaperones and ATP (3 mM) as indicated. The reaction mixture was incubated at 72 °C for 8 min for G6PDH or at 73 °C for 10 min for α-glucosidase. Then, the temperature was shifted to 55 °C, and other indicated components were immediately added to the solutions (0 min in the abscissa). Incubation at 55 °C continued, and the recovered enzyme activity levels were measured at the indicated times. The final concentrations of added components were 0.2 μM (Tcpn) and 3 mM (ATP). Note that components added at 72 or 73 °C remained present after the temperature shift. The order of the additions is shown in the figure. Activities of G6PDH and α-glucosidase are expressed as percent before heat inactivation. B1 and B2, reactivation by the KJE-B system. The experimental procedures were the same as described in A1 and A2 except for the indicated components. Final concentrations of added components were 0.4 μM (TDnAJ complex), 0.8 μM (TGrpE), 0.4 μM (TClpB), and 3 mM (ATP). KJ, E, and B represent TDnAJK complex, TGrpE, and TClpB, respectively.

**RESULTS**

**Tcpn-mediated Reactivation of Heat-inactivated Protein—**We previously demonstrated that Tcpn was capable of protecting several proteins from irreversible heat denaturation at high temperatures (30). We tested whether G6PDH and α-glucosidase were also protected by Tcpn (Fig. 1, A1 and A2). Throughout this paper, the experiments consisted of two steps: incubation at a high temperature and subsequent incubation at a moderate temperature. G6PDH and α-glucosidase were incubated at 72 °C for 8 min and at 73 °C for 10 min, respectively, in the presence of indicated components (inactivation period). Their enzymatic activities were lost almost completely by this heat treatment. None of chaperones could prevent this inactivation. Then, the solution was transferred to a 55 °C incubation bath, and the indicated components were added immediately. The incubation at 55 °C continued, and the recovered enzymatic activities were measured at the indicated times (reactivation period). All of the chaperones used in the experiments were stable at 73 °C, and native G6PDH and α-glucosidase were stable and active at 55 °C. It should be noted that the

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1 We had used *B. stetherophilus* lactate dehydrogenase as a substrate protein in the previous paper (4). However, Unitika stopped the production and sale of this enzyme, and it was no longer available. LDH from *B. stetherophilus* purchased from Sigma was much more stable than the Unitika one and not suitable for the experiment.
tion with all chaperones, with a 7% net recovery yield for both G6PDH and α-glucosidase (Fig. 1, B1 and B2, open and closed squares). To summarize, for G6PDH and α-glucosidase, the presence of TDnaKJ complex during the heat inactivation period is critical to keep them in a retrievable state. These results are in contrast to the previous observation that LDH heat-inactivated without any chaperone recovered some activity during next incubation at moderate temperature in the presence of all the chaperones (4). Thus, the requirement for chaperones at inactivation periods to keep them in a retrievable state differs from one substrate protein to another. In contrast to Tcpn-mediated reactivations, which saturated at 30 min after temperature shift, the KJE-B-mediated reactivations, especially in the case of G6PDH, continued slowly even after 30 min.

Tcpn Improves the Yield of KJE-B-mediated Reactivation—We investigated the effect of Tcpn on KJE-B-mediated reactivation (Fig. 2). G6PDH and α-glucosidase heated with KJE set were scarcely reactivated by subsequent incubation with Tcpn at 55 °C (bar 1 in Fig. 2, A and B), and thus Tcpn cannot substitute in the role of TCpB as shown previously for LDH (4). Combination of TCpB at 72 °C (or 73 °C) and Tcpn at 55 °C also failed to recover the activity (bar 2). When substrate proteins were heated in the absence of any chaperones, only very small amount of reactivation (1–12%) was observed during the next 55 °C incubation with KJE-B chaperones and/or Tcpn (bars 3–5). As mentioned, G6PDH and α-glucosidase heated with Tcpn were reactivated efficiently by adding ATP at the reactivation period (bar 6). The yield was slightly increased by the further addition of KJE-B chaperones at the reactivation period (bar 7). A similar effect of KJE-B chaperones was observed for the substrate proteins inactivated in the presence of Tcpn and ATP, although the yields were much lower (bars 8 and 9). These increments of recoveries caused by adding KJE-B chaperones to the proteins heated with Tcpn were nearly the same as the levels of recovery caused by adding KJE-B chaperones to the proteins heated without Tcpn (bar 4), suggesting that KJE-B chaperones had only little effect, if any, on Tcpn-mediated recovery. On the other hand, the yield of KJE-B-mediated reactivation was significantly increased by adding Tcpn at a moderate temperature, especially in the case of G6PDH (bars 10 and 11). The improvement of the yield by Tcpn was also observed when TCpB was added from the beginning of the heat inactivation period (bars 12 and 13). The extent of these increments was too great to be attributed to an independent action of Tcpn, apart from KJE-B chaperones.

Trap-Tcpn Inhibits KJE-B-mediated Reactivation—The straightforward interpretation of the above result was that KJE-B chaperone released the substrate protein into the medium, which then bound to Tcpn and was assisted in folding. To examine this explanation, we generated “trap”-Tcpn, which can bind nonnative protein but cannot release it even in the presence of ATP. It has been found that glutaraldehyde-treated chaperonin from E. coli (GroEL) acts as a trap chaperonin (34). Tcpn treated with glutaraldehyde showed properties of a trap; refolding of acid denatured green fluorescent protein diluted into neutral buffer was arrested by glutaraldehyde-treated Tcpn even in the presence of ATP, whereas native Tcpn assisted refolding by 100% with the same procedures (not shown). As mentioned above, substrate proteins heated with KJE set and ATP were reactivated during subsequent incubation at 55 °C with TCpB (Fig. 3, A and B, open circles), and the yields were further increased by the addition of Tcpn at the reactivation period (closed circles). However, the yields were drastically decreased in the experiment in which trap-Tcpn was added instead of intact Tcpn (Fig. 3, A and B, open triangles). Recovery of α-glucosidase was decreased down to 13% by the addition of a double amount of trap-Tcpn (not shown). Recovery of G6PDH reached up to 42% at 30 min and continued to increase up to 57% at 90 min. The addition of trap-Tcpn at 30 min stopped the recovery immediately, and further reactivation was not observed. These observations indicate that the KJE-B chaperones release the substrate protein in a chaperonin-recognizable conformational form.

Effect of Chaperone Concentrations on Recovery—The concentrations of chaperones were changed, and the yield of recovery was measured (Fig. 4, open circles). Concentrations of the substrate protein were always fixed at 0.2 μM. For simplicity, all chaperones and ATP were added from the beginning of the heat inactivation period, and the enzymatic activity at 90 min after the temperature shift to 55 °C was measured. First, the concentrations of TDnaKJ complex and TGrpE were changed by keeping the ratio of TDnaKJ complex to TGrpE at 2:1. The result showed that recoveries of both G6PDH and α-glucosidase were saturated at ~0.2 μM TDnaKJ complex, a concentration equal to that of the substrate (Fig. 4, A1 and B1). Next, the TGrpE concentration was changed with fixed TDnaKJ and

![Fig. 2. Functional interaction between KJE-B chaperones and Tcpn in the reactivation of proteins.](https://example.com/fig2.png)
**FIG. 4.** The effect of concentrations of individual components of KJE-B chaperones on the recovery yield from heat inactivation. The experimental procedures were the same as in Fig. 1. Indicated concentrations of KJE-B chaperone components and ATP (3 mM) were added at the beginning of the inactivation period. The recovery rates of enzyme activities were measured at 90 min after the temperature shift to 55 °C. The concentrations of substrate proteins were 0.2 μM in all experiments. Tcnp (0.2 μM) was supplemented (closed circles) or not (open circles) at 55 °C. A1–A3, recovery of G6PDH. B1–B3, recovery of α-glucosidase.

TCPB concentrations (both 0.2 μM) (Fig. 4, A2 and B2). Interestingly, 0.1 μM TGrpE, one-half of the concentration of substrate protein and TDnaKJ complex, was enough to attain the maximum recovery yield. The yield started decreasing as concentrations of TGrpE increased beyond 0.1 μM. Therefore, TGrpE was recycled in the interaction with TDnaKJ complex. An inhibitory effect of excess GrpE on DnaK-DnaJ-dependent refolding was also reported for E. coli preparations (7, 8, 35) and might be general. Finally, the concentration of TCPB was changed (Fig. 4, A3 and B3). The yields of recovery of G6PDH and α-glucosidase were saturated at a remarkably low concentration of TCPB, 0.02 μM, one-tenth of the substrate concentration. Such sub-stoichiometry relative to both the substrate proteins and TDnaKJ complex means that TCPB acts as a catalyst in a cycling reaction.

The presence of Tcnp (0.2 μM) at the reactivation period improved the recovery in two instances (Fig. 4, closed circles); at sub-optimum concentrations of TDnaKJ complex for the G6PDH (Fig. 4, A1) and at excess concentrations of TGrpE for both enzymes (Fig. 4, A2 and B2). Because Tcpn existed only at 55 °C, it did not affect the population of potentially retrievable proteins after the heat treatment. Therefore, it was then clear that the low yields of recovery observed in the absence of Tcpn in the two instances above were attributable not to the low population of retrievable proteins but to unsuccessful folding, which Tcpn could have helped to make successful. TCPB concentration-recovery profiles were not affected by Tcpn when optimum concentrations of TGrpE (0.1 μM) were present (Fig. 4, A3 and B3).

**DISCUSSION**

**KJE-B Chaperones Release Substrate Protein in a Chaperonin-recognizable Form**—Heat-inactivated proteins can be reactivated through two independent chaperone pathways; first, through the Tcpn pathway and the second, through the KJE-B pathway (Fig. 1). A major finding of this report is that Tcpn can improve the yield of KJE-B-mediated reactivation, but KJE-B chaperones can do very little to improve Tcpn-mediated reactivation (Fig. 2). In other words, although the substrate released from Tcpn is not manageable for KJE-B chaperones, the substrate released from KJE-B chaperones is recognized and bound by Tcpn. This contention was strongly supported by the experiment using trap-Tcpn (Fig. 3). The effect of Tcpn on reactivation of α-glucosidase was less obvious than that of G6PDH, probably because the molecular size of α-glucosidase (65 kDa) is almost beyond the upper size limit of what is able to be accommodated in the cavity of chaperonin (27). Thus, KJE-B chaperones release the substrate protein in a non-native state that is well recognized by chaperonin. The protein in this state has a potential to fold by itself, but chaperonin can improve the folding yield. Because the transfer of substrate protein from E. coli DnaK-DnaJ to GroEL has been reported (36–38) but the transfer from ClpB to GroEL has not, our finding favors, if not proves, the contention that the chaperone that finally releases the substrate protein into medium in KJE-B system is TDnaKJ complex rather than TCPB.

**Recycling of Chaperones in the KJE-B System**—In a previous paper, we used molar excess KJE-B chaperones over the substrate protein to observe recovery from the heat-inactivated state. However, dose-recovery experiments (Fig. 4) revealed that each chaperone component of KJE-B system gives the maximum yield of recovery at sub-stoichiometric amount. As for TDnaKJ complex, although a 1:1 stoichiometry ratio of TDnaKJ complex to the substrate protein appeared to be necessary for the maximum recovery, the inclusion of Tcpn decreased the stoichiometry down to 0.5:1 in the case of G6PDH. It appears that chaperones in the KJE-B system participate in the recovery reaction as catalysts. A recent report also showed the catalytic role of ClpB (39). The dynamic nature of chaperone-substrate interaction in the KJE-B system has also been suggested by the fact that any attempt to isolate the TDnaKJ-substrate complex from the inactivation mixture as yet has been unsuccessful.

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Heat-inactivated Proteins Managed by DnaKJ-GrpE-ClpB Chaperones Are Released as a Chaperonin-recognizable Non-native Form
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