Chaperonin cpn60 from *Escherichia coli* Protects the Mitochondrial Enzyme Rhodanese against Heat Inactivation and Supports Folding at Elevated Temperatures*

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The chaperonin protein cpn60 from *Escherichia coli* protects the monomeric, mitochondrial enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) against heat inactivation. The thermal inactivation of rhodanese was studied for four different states of the enzyme: native, refolded, bound to cpn60 in the form of a binary complex formed from unfolded rhodanese, and a thermally perturbed state. Thermal stabilization is observed in a range of temperatures from 25 to 48 °C. Rhodanese that had been inactivated by incubation at 48 °C, in the presence of cpn60 can be reactivated at 25 °C, upon addition of cpn10, K⁺, and MgATP. A recovery of about 80% was achieved after 1 h of the addition of those components. Thus, the enzyme is protected against heat inactivation and kept in a reactivatable form if inactivation is attempted using the binary complex formed between rhodanese folding intermediate(s) and cpn60. The chaperonin-assisted refolding of urea-denatured rhodanese is dependent on the temperature of the refolding reaction. However, optimal chaperonin assisted refolding of rhodanese observed at 25 °C, which is achieved upon addition of cpn10 and ATP to the cpn60-rhodanese complex, is independent of the temperature of preincubation of the complex, that was formed previously at low temperature. The results are in agreement with a model in which the chaperonin cpn60 interacts with partly folded intermediates by forming a binary complex which is stable to elevated temperatures. In addition, it appears that native rhodanese can be thermally perturbed to produce a state different from that achieved by denaturation that can interact with cpn60.

The first and common step to all enzymes during their thermoinactivation is the partial unfolding of the molecule which is usually a reversible process. The subsequent steps are generally specific for individual enzymes and are responsible for the irreversibility of the thermoinactivation process. After initial unfolding, the enzyme may be chemically altered, aggregated, or incorrectly fold as the result of covalent or noncovalent changes during thermal inactivation (1). Aggregation can result from association of hydrophobic surfaces that become exposed after the initial partial unfolding of the enzyme similar to the commonly observed association process during the refolding of polypeptides in *vitro* after unfolding in denaturants.

It has been suggested that *in vivo* proteins serve the function of preventing such aggregation processes of newly synthesized polypeptides by keeping them in a conformation that allows interaction with other components of the folding and transport machinery of the cell, but prevents self-association due to exposed hydrophobic surfaces (2).

A group of such proteins has been termed chaperonins. The best studied chaperonin protein is cpn60, a group of such proteins has been termed chaperonins. The best studied chaperonin protein is cpn60, which is encoded by the groEL gene of *Escherichia coli* (3). This protein is a tetradecamer (14-mer) of 60-kDa subunits and has been reported to promote the folding and/or assembly of the enzyme ribulose-5-phosphate oxidase (4), pre-β-lactamase (5), citrate synthase (6), dihydrofolate reductase (7, 8), cpn60 itself (9), and rhodanese from unfolded polypeptides (8, 10).

The enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) is a mitochondrial enzyme which *in vitro* catalyzes the transfer of the outer sulfur of thiosulfate to the nucleophlic acceptor, cyanide. Rhodanese has been a valuable model for studying protein folding (8, 10–14) and the functional role of protein dynamics and domain interactions (15, 16). The enzyme has been reported to be very sensitive to thermal inactivation in an apparently irreversible way leading to rapid precipitation even at very low protein concentrations. Reversible thermal denaturation of rhodanese was possible only after covalent coupling of the enzyme to an insoluble support to prevent the association of the protein (17).

The first step in the *in vitro* chaperonin-assisted refolding of rhodanese is the formation of the cpn60-rhodanese complex (10). In addition to cpn60, refolding requires MgATP, K⁺, and a second protein, cpn10 (groES), which forms an oligomer composed of 7 subunits, each with a molecular mass of 10 kDa. The details of the mechanism of action of these proteins are still unclear. It has been suggested that partially folded polypeptides are stabilized by cpn60, preventing them from aggregating, perhaps by hydrophobic interactions between hydrophobic surfaces on cpn60 and exposed hydrophobic surfaces of folding intermediates (18). Consistent with that suggestion, we have previously reported the detection of hydrophobic regions on cpn60 (10). We have also reported that by forming the cpn60-rhodanese complex at temperatures where aggregation of unfolded rhodanese is minimized, it is possible

*The abbreviations used are: cpn60, chaperonin 60 or groEL; cpn10, chaperonin 10 or groES; 5ME, β-mercaptoethanol.
to optimize the conditions for the formation of the complex (19). Here, we report that cpn60 can protect native rhodanese against heat inactivation by reducing the rate of the inactivation process, presumably by interacting with a form of the enzyme that is active in the standard assay. In addition, rhodanese that had been incubated at 48 °C in the presence of cpn60, conditions that would irreversibly inactivate rhodanese in the absence of chaperonins, can be reactivated at 25 °C upon addition of cpn10, K⁺, and MgATP. It is also reported that the chaperonins support refolding of rhodanese at high temperature and that the recovery of active enzyme is independent of the temperature of incubation of the cpn60-rhodanese complex when this is previously formed at low temperature.

MATERIALS AND METHODS

Reagents and Proteins—All the reagents used were of analytical grade. Rhodanese was prepared as previously described (20) and stored at −70 °C as a crystalline suspension in 1.8 M ammonium sulfate. Rhodanese concentration was determined using a value of A₂₈₀nm = 1.75 (21) and a molecular mass of 33 kDa (22). The E. coli chaperonins cpn60 and cpn10 were purified from lysates of cells bearing the multicopy plasmid pGroESL (23). After purification, the chaperonins were dialyzed against 50 mM Tris-HCl, pH 7.5, and 1 mM dithiothreitol, then made 10% (v/v) in glycerol, rapidly frozen in liquid nitrogen, and stored at −70 °C. The protector concentration of cpn60 for the chaperonins were measured at 280 nm with extinction coefficients determined by quantitative amino acid analyses: 3340 M⁻¹ cm⁻¹ for cpn10 and 23,800 M⁻¹ cm⁻¹ for cpn60 (24), and assuming molar masses of 10 and 60 kDa, respectively.

Rhodanese Assay—Rhodanese activity was measured by a colorimetric method based on the absorbance at 460 nm of the complex formed between ferric ions and the reaction product, thiocyanate (21). The assay was initiated by adding microgram quantities of the enzyme, and the reaction was stopped by adding formaldehyde.

Kinetics of Thermoinactivation—The time course of thermoinactivation of rhodanese was measured by incubating the enzyme at the desired temperature in 50 mM Tris-HCl buffer, pH 7.8, in the absence or presence of cpn60 and periodically removing samples and assaying them for enzymatic activity at 25 °C.

Unfolding and Refolding—(a) In the refolding experiments, rhodanese at 90 μg/ml was unfolded in 8 M urea for at least 30 min. After unfolding, the protein was diluted to 3.6 μg/ml in a buffer containing cpn60 (2.5 μM, protomer), cpn10 (2.5 μM, protomer), 50 mM Tris-HCl, pH 7.8, 200 mM βME, 50 mM sodium thiostiofate, 10 mM MgCl₂, 10 mM KCl, and 2 mM ATP at 25 or 42 °C. (b) In the refolding experiments after initial formation of the cpn60-rhodanese complex, rhodanese was unfolded as above and diluted to 3.6 μg/ml in a buffer containing cpn60 (2.5 μM, protomer), 50 mM Tris-HCl, pH 7.8, 200 mM βME, 50 mM sodium thiostiofate, 10 mM MgCl₂, and 10 mM KCl at 10 °C. After incubation for 30 min at that temperature, the mixture was transferred to a water bath and incubated at the desired temperature. After 30 min, the samples were transferred to 25 °C and supplemented with cpn10 (2.4 μM protomer) and 2 mM ATP and incubated for 60 min. To assess the regain of rhodanese activity, a 40-μl aliquot of the incubation mixture was withdrawn and added to 1 ml of the standard assay mix (above), and the product was determined after 10 min. Controls using native enzyme that had been similarly diluted and incubated were also prepared. Additional experimental details are presented in the text and figures.

RESULTS

Native Rhodanese Is Protected against Thermoinactivation by cpn60—Rhodanese is very sensitive to thermal inactivation. This process appears to be irreversible as a consequence of thermally induced structural transitions in the protein that lead to precipitation of soluble enzyme (17). When native rhodanese was incubated at 3.6 μg/ml in buffer, in the absence of its substrate which stabilizes the native active structure of the enzyme, the enzymatic activity was gradually lost even at 25 °C (Fig. 1A, open circles). The inactivation process was faster at 37 °C (Fig. 1B, open circles), and at 48 °C (Fig. 1C, open circles) the enzymatic activity was almost completely lost after only 10 min of incubation. However, when the enzyme was incubated at the same temperatures, but in the presence of the chaperonin cpn60, the inactivation process appeared to be substantially retarded. As shown in Fig. 1A, at 25 °C, the amount of active enzyme that remained after 20 min of incubation was about 70% in the presence (filled circles) of cpn60, while there was only 30% of active enzyme in its absence (open circles). In contrast, at 37 °C (Fig. 1B) the enzyme activity was virtually lost after 45 min of incubation in the absence (open circles) of cpn60, while in its presence (filled circles) the amount of active enzyme that remained was about 45%. The protection effect by the chaperonin on the thermal inactivation of rhodanese was also observed at 48 °C (Fig. 1C). At this temperature, the enzyme activity was lost after only 10 min in the absence (open circles) of cpn60, while in its presence (filled circles) it took over 60 min. Thus, the protection effect appeared to be dramatically increased by increasing the temperature of incubation. However, even in the presence of cpn60 the enzyme eventually lost its activity at all temperatures.

Intermediates Captured by cpn60 during Thermal Perturbation of Native Rhodanene Are Efficiently Reactivated—The thermally inactivated enzyme in the presence of cpn60 (Fig. 1C, filled circles) was kept in a reactive form even when it was inactivated at 48 °C, since upon addition of cpn10, K⁺, and MgATP to the sample and incubation at 25 °C for 1 h, high recovery (about 80%) of active enzyme was obtained. Thus, the binary cpn60-rhodanese complex can be formed starting with the native protein during the thermal unfolding
of rhodanese. In addition, by the formation of the complex, rhodanese can be kept in a refolding form at elevated temperatures. In the absence of cpn60 (open circles), however, the thermally inactivated enzyme is not reactivatable.

Chaperonin-assisted Refolding of Rhodanese Is Temperature-dependent—As shown in Fig. 2, the refolding process is strongly dependent on the temperature of the refolding reaction. At 25 °C (filled triangles), a significant recovery of active enzyme was obtained after 1 h of refolding, as was previously observed (10). During the chaperonin-assisted refolding of rhodanese, the formation of the cpn60-rhodanese complex competes kinetically with irreversible aggregation, a process which is strongly favored at higher temperatures. Thus, when the chaperonin-assisted refolding of rhodanese was attempted at 42 °C (filled circles), the chaperonins are still able to support the refolding of active rhodanese. The extent of recovery of active enzyme was substantially reduced, however.

As previously reported, there is little (3%) spontaneous refolding of rhodanese at 42 °C because of the formation of protein aggregates (13).

The Chaperonin-assisted Refolding of Rhodanese Is Independent of the Temperature of Incubation of the cpn60-Rhodanese Complex If This Is Preformed at Low Temperature—When the binary cpn60-rhodanese complex is formed, active enzyme can be released upon the addition of the other components required for the chaperonin system. Fig. 3 shows the chaperonin-assisted refolding of rhodanese at 25 °C (open bars) after having incubated the cpn60-rhodanese complex at the indicated temperatures for 30 min, before the addition of the components needed for the release of active enzyme. In all cases, the binary complex had been previously formed by diluting unfolded rhodanese into a buffer containing cpn60, at 10 °C. The complex appears to be able to recover after preincubation at all temperatures since active rhodanese can be recovered upon the addition of cpn10 and MgATP. The enzyme also appears to be protected against heat inactivation in the form of the binary complex since the amount of active enzyme that can be recovered is independent of the temperature at which the complex was incubated after its formation at low temperature. When unfolded rhodanese was replaced by native enzyme (wide diagonal bars), a systematic decrease in the amount of active enzyme was obtained by increasing the incubation temperature of the mixture of cpn60 and native enzyme. When unfolded rhodanese was incubated in the absence of cpn60, but treated similarly (narrow diagonal bars), the recovery of active enzyme that was refolded at low temperature was dependent on the incubation temperature of the protein solution. Thus, the higher the incubation temperature, the lower the recovery that was obtained. However, here the loss of enzymatic activity appears to be more dramatic because the solution lacks cpn60.

**DISCUSSION**

We report here a thermoprotection effect by the chaperonin cpn60 on the inactivation of the enzyme rhodanese. Our results indicate that during the thermal inactivation process, the chaperonin cpn60 interacts with a rhodanese conformation that is either active or can recover in the standard assay. It has been proposed that the chaperonins may recognize incompletely folded polypeptides, and at least part of the interaction may be via hydrophobic interactions. Consistent with this view, we have previously detected (10) hydrophobic surfaces on cpn60 from *E. coli*, which could be related to the mechanism of action of this chaperonin.

The enzyme rhodanese is a monomeric enzyme composed of two equal sized domains with no amino acid homology between them. The enzyme active site resides in a hydrophobic cleft between the two domains. This extensive hydrophobic interface could be a potential contact surface with cpn60. However, the opening of the active structure with the concomitant exposure of hydrophobic surfaces to the solvent could lead to inactivation because of the disruption of the active site. It has also been proposed that the amphiphilic α-helix present in mitochondrial targeting sequences could be a recognition target for the chaperonins (25, 26). The α-helix in the putative targeting signal of rhodanese can be seen in the x-ray structure to be formed from amino acid residues 11–23. It might be possible that during the thermal unfolding of rhodanese an initial event would be the disruption of the non-covalent interactions between the N-terminal sequence and the rest of the protein. The interaction with the chaperonin of the hydrophobic side of the helix or of the hydrophobic surface on the protein that would be left exposed would not necessarily cause any perturbation of the domain-domain interactions, and an active conformation would be preserved or could be reestablished on dilution into the assay.

Unfolding of the enzyme into an inactive conformation, however, is not prevented by cpn60 since incubation of the native, active enzyme in the presence of the chaperonin would

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**FIG. 2.** Temperature dependence of the chaperonin-assisted refolding of rhodanese. Urea unfolded rhodanese was diluted into a buffer containing cpn60 (2.5 μM, protomer), cpn10 (2.5 μM, protomer), and all of the other components required for the chaperonin-assisted system at 25 °C (filled triangles) or 42 °C (filled circles). The final concentration of rhodanese in the refolding reaction was 3.6 μg/ml in a final volume of 250 μl. The samples were assayed for rhodanese activity at the indicated times.

**FIG. 3.** Chaperonin-assisted refolding of active rhodanese after initial low temperature formation of the cpn60-rhodanese complex and its subsequent incubation at several temperatures. Open bars represent refolding experiments in which unfolded rhodanese was diluted in a buffer containing cpn60 at 10 °C. After incubation for 30 min, the mixtures were incubated for an additional 30-min period at the indicated temperature and then transferred to 25 °C and supplemented with cpn10 and ATP. Rhodanese activity was then determined after incubation for 60 min prior to assay. Wide diagonal bars represent native rhodanese that was similarly diluted and incubated. Narrow diagonal bars represent refolding experiments in which unfolded rhodanese was diluted into a buffer in the absence of the chaperonins at the indicated temperature. Rhodanese activity was also determined after incubation for 60 min. For other experimental details see “Materials and Methods.”
lead eventually to an inactive protein. However, the "trapping" of rhodanese by cpn60 while the enzyme is still in an active form would prevent the enzyme from becoming aggregated even at high temperatures. In fact, we observe that, upon addition of the components needed for discharge of the complex, the efficient refolding of rhodanese is observed at 25 °C. These results also suggest that in later steps of the thermal unfolding of rhodanese other sequences of the protein may be necessary for the interaction with the chaperonin, which lead eventually to the formation of a thermally stable binary complex between cpn60 and rhodanese. This view appears to be consistent with a proposed model of the chaperonin having multiple binding sites for a single polypeptide chain (27). The multiple contacts between the two proteins are perhaps needed to prevent incorrect intra- or intermolecular interactions that would otherwise lead to irreversibly inactive enzyme. The result is the formation of a very stable binary complex that keeps the enzyme in a reactivable state. The complex appears to be stable at temperatures as high as 48 °C. The refolding of urea-unfolded rhodanese is possible under conditions, such as low temperatures, that suppress the aggregation of folding intermediates. At low temperatures, the chaperonins do not support the refolding, but the binary complex can still be formed (14). Our results indicate that when the binary complex is formed at low temperatures and then incubated at higher temperatures, the amount of active enzyme that can ultimately be recovered at 25 °C becomes independent of the temperature at which the complex was incubated. Therefore, cpn60 protects rhodanese in the form of a thermally stable binary complex from aggregation at elevated temperatures.

We have previously shown (13) that spontaneous refolding of urea-unfolded rhodanese is possible under conditions, such as low temperatures, that suppress the aggregation of folding intermediates. At low temperatures, the chaperonins do not support the refolding, but the binary complex can still be formed (14). Our results indicate that when the binary complex is formed at low temperatures and then incubated at higher temperatures, the amount of active enzyme that can ultimately be recovered at 25 °C becomes independent of the temperature at which the complex was incubated. Therefore, cpn60 protects rhodanese in the form of a thermally stable binary complex from aggregation at elevated temperatures.

A model can be proposed, based on these results, that accounts for the interaction of several forms of rhodanese with cpn60 during the thermal unfolding of the enzyme in the presence of cpn60.

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\begin{align*}
\text{Aggregation} \\
N &\rightarrow N' & \rightarrow \text{l} &\rightarrow \text{l'} &\rightarrow D \\
\text{cpn}10/\text{ATP} &\downarrow & \downarrow & \downarrow & \downarrow \\
\text{cpn}60-N' &\rightarrow \text{cpn}60-1 \\
\text{cpn}10/\text{ATP} &
\end{align*}
\]

A major feature of this model is that intermediates interact with cpn60 at different stages in the course of unfolding/refolding. Native rhodanese (N) passes through intermediates N', I, and I' in the process of denaturation to the state D. In the unassisted unfolding/refolding of rhodanese, activity was lost at an early stage with small structural changes (13). In contrast, in the presence of cpn60 an intermediate (N') is formed relatively fast, which by interacting with cpn60 in a reversible step becomes protected from losing its activity while bound to the chaperonin or losing its ability to recover during the standard assay. Transition from N' to I occurs with loss of activity, directly as for the unassisted process, in a transition that involves substantial structural changes and seems to be the rate-limiting step. The transition can also be via binding to cpn60 indicating an interaction of cpn60 with a different form of rhodanese. This would be in agreement with the report of multiple states of rhodanese interacting with cpn60 during its folding (8). Intermediates I and I' contain exposed hydrophobic surfaces that make them labile to irreversible aggregation through self-interactions. In contrast, intermediates bound to cpn60 are protected from aggregation in the form of a stable complex (cpn60-I). The steps that lead to discharge of the cpn60-I complex are those that require cpn60 and ATP.

The results conform to the hypothesis that partially folded polypeptides are prevented from aggregation by cpn60. They also support the suggestion that cpn60 may interact with more than one form of rhodanese and that folding could occur on the surface of the chaperonin.

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