Partitioning of Rhodanese onto GroEL

CHAPERONIN BINDS A REVERSIBLY OXIDIZED FORM DERIVED FROM THE NATIVE PROTEIN*

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The mammalian mitochondrial enzyme, rhodanese, can form stable complexes with the Escherichia coli chaperonin GroEL if it is either refolded from 8 M urea in the presence of chaperonin or is simply added to the chaperonin as the folded conformer at 37 °C. In the presence of GroEL, the kinetic profile of the inactivation of native rhodanese followed a single exponential decay. Initially, the inactivation rates showed a dependence on the chaperonin concentration but reached a constant maximum value as the GroEL concentration increased. Over the same time period, in the absence of GroEL, native rhodanese showed only a small decline in activity. The addition of a non-denaturing concentration of urea accelerated the inactivation and partitioning of rhodanese onto GroEL. These results suggest that the GroEL chaperonin may facilitate protein unfolding indirectly by interacting with intermediates that exist in equilibrium with native rhodanese. The activity of GroEL-bound rhodanese can be completely recovered upon addition of GroES and ATP. The reactivation kinetics and commitment rates for GroEL-rhodanese complexes prepared from either unfolded or native rhodanese were identical. However, when rhodanese was allowed to inactivate spontaneously in the absence of GroEL, no recovery of activity was observed upon addition of GroEL, GroES, and ATP. Interestingly, the partitioning of rhodanese and its subsequent inactivation did not occur when native rhodanese and GroEL were incubated under anaerobic conditions. Thus, our results strongly suggest that the inactive intermediate that partitions onto GroEL is the reversibly oxidized form of rhodanese.

The chaperonin GroEL together with the co-chaperonin GroES, has been shown to facilitate protein folding in vitro and in vivo (1). GroEL functions as a tetradecamer of identical subunits and can bind a wide variety of unfolded or partially folded proteins (for review, see Fenton and Horwich (2)). In the presence of GroES and ATP, the partially folded proteins dissociate from GroEL and can continue to fold either sequestered in the cavity of the transient GroEL-GroES complex or released as an intermediate committed to fold to the native state in solution (3). It is important to note that the folding reaction does not necessarily have to be completed before the protein exits the GroEL-GroES complex. As the protein substrate folds, the lifetime of the GroEL-GroES complex determines whether the protein completes its folding reaction inside the GroEL-GroES cavity or outside in bulk solution. During the folding reaction, if the protein has not acquired a native state, it can either aggregate, misfold or rebind to the chaperonin complex (3–7). It is becoming clear that the lifetime of the interaction between folding protein and GroEL depends on the nature of the substrate protein. Different substrate proteins may either require very brief interaction with GroEl (glutamine synthetase, ornithine decarboxylase) or may undergo numerous rounds of rebinding and release from GroEL throughout the folding reaction (rhodanese, mitochondrial malate dehydrogenase) (5, 8, 9).

The extensive research on different chaperonin substrates has demonstrated that substrate interactions with the chaperonin are highly variable. This may be related to the variability in the initial conformations of the binding substrate species. Since the protein folding intermediates that bind to the chaperonin are diverse, the chaperonin mechanism observed with one protein substrate may be different with other substrates. During the initial interactions between substrates and the chaperonin, it has been suggested that GroEL recognizes the “molten globule” state of the protein, a compact folding intermediate with significant amount of secondary structure and an ill-defined tertiary structure (4). However, other investigators have shown that for some substrates, unfolded early intermediates tend to bind more tightly to the chaperonin than do more compact late folding intermediates (10–14). Kuwajima and coworkers argue that the α-lactalbumin molten globule intermediates that contain primarily non-native disulfide rearrangements and bind tightly to GroEL, may not be “molten globules” in the strict sense (15). Likewise, Schmid and co-workers have demonstrated that misfolded RNase T is a good substrate for GroEL (16). Recently, two groups have provided evidence suggesting that there is a substantial amount of native-like folding contacts that can be resolved in stable GroEL-dihydrofolate reductase (DHFR)3 complexes (17, 18). From the accumulated data, it is apparent that the nature of intermediate polypeptides that can bind to GroEL is substrate dependent.

Although a majority of the initial protein substrates used to bind to GroEL in vitro are initially unfolded by denaturants, Viitanen et al. were the first to show that the chaperonins can form complexes with DHFR when this protein was initially present in its native form (19). Because the complex formation was slow, they speculated that a rate-limiting step occurs between the native conformer and a DHFR intermediate that subsequently interacts with the chaperonin. A kinetic analysis by Clark and Frieden (20) indicated that the GroEL-bound DHFR conformers derived from either unfolded or the native state were the same. Recently, it was found that GroEL can
form a stable complex with rhodanese when this protein was also present in its native form (7, 21). Once the GroEL-rhodanese complex is formed, rhodanese still requires GroES and ATP or ADP for reactivation, and thus remains a stringent chaperonin substrate (22).

Our previous work with rhodanese indicated that the denaturation conditions may influence the aggregation propensity of this substrate after its release from GroEL. These results suggest that different unfolded rhodanese intermediates could initially bind to GroEL (7). Data presented in this report indicate that some of the intermediates that can be recognized and bound by GroEL are reversibly oxidized rhodanese species that exist in equilibrium with the native protein. In the presence of reducing agents, the reactivation and commitment rates of the GroEL-bound oxidized conformers are identical to those of GroEL-bound protein species derived from the urea-unfolded rhodanese.

**EXPERIMENTAL PROCEDURES**

**Materials**—Methyl viologen, titanium chloride, sodium thiosulfate, sodium cyanide, ATP, and DT were purchased from Sigma. Urea, >99% purity was from ICN Biomedicals (Aurora, OH). Bovine liver rhodanese was purchased from Sigma. All the other chemicals were of the highest purity available.

The *Escherichia coli* chaperonins, GroEL and GroES, were isolated from lysates of cells containing the appropriate overexpression plasmids (gift of Dr. George Lorimer and Dr. Edward Eisenstein) and were purified as described earlier (23, 24). Because GroEL or GroES do not contain tryptophan, the loss of the tryptophan indole absorption, assayed by second derivative analysis of absorption spectra, was used as a criterion for purity of the preparations, in addition to analysis by SDS-polyacrylamide gel electrophoresis (25). In the final purified forms of GroEL and GroES, no contribution from tryptophan-containing contaminants were detected.

**Unfolding and Refolding of Rhodanese**—Rhodanese was denatured in 50 mM Tris-Cl (pH 7.5), 8 M urea, 5 mM EDTA, 10 mM DTT, and 50 mM sodium thiosulfate for 2–4 h at 0 °C. The rhodanese-GroEL complexes were formed at 37 °C after denatured rhodanese was rapidly diluted 100-fold into 50 mM Tris-Cl (pH 7.5), 0.5 mM EDTA, 10 mM MgCl₂, 50 mM KC1, 10 mM DTT, and 50 mM sodium thiosulfate (buffer A) containing 1 μM GroEL and 2 μM GroES. The final rhodanese concentration in all experiments was 1 μM. The complex was incubated at 37 °C for 15 to 120 min before 5 mM ATP was added to initiate refolding. Rhodanese reactivation was followed by monitoring the formation of a stable ferric-thiocyanate color complex (absorbance at 460 nm) after 10 min at 25 °C (26). The concentration of rhodanese was determined from its absorbance spectra after correction for light scattering using an extinction coefficient of 1.75 for a 0.1% solution at 280 nm (27).

**Partitioning of Native Rhodanese onto GroEL**—Native rhodanese was solubilized in a small volume of buffer A and then diluted into the same buffer to a final concentration of 1 μM. The measurements of the inactivation rates were initiated by addition of 0.1–50 μM GroEL and/or 0.2–100 μM GroES followed by incubation for up to 24 h at 37 °C. The rhodanese activity was determined at regular time intervals. The presence of GroEL did not affect inactivation kinetics (data not shown). For some experiments, the inactivation of rhodanese was performed in the absence of DTT and sodium thiosulfate. The reactivation of rhodanese was initiated by adding ATP to the final concentration of 5 mM. To measure the commitment rates, the activity of released rhodanese was assayed after GroEL was rapidly removed from the folding mixture by immunoprecipitation with anti-GroEL antibody (5). All incubations were carried out in a 37 °C warm air incubator to minimize problems associated with condensation of solution on the sides of the enclosed tubes.

Anaerobic solution conditions were achieved by subjecting all concentrated stock solutions to a series of degas/purge cycles with argon in an enclosed argon atmosphere. In addition, all solution buffers were repeatedly degassed and purged with oxygen-free argon. To ensure that anaerobic conditions are maintained, titanium chloride-reduced methyl viologen was used as both an oxygen indicator and an oxygen scrubber. Final sample mixtures were degassed briefly, placed into thick-walled glass tubes and overlaid with argon-purged mineral oil to maintain anaerobic conditions and prevent the diffusion of atmospheric oxygen into the solution. No substantial change in sample volumes was observed during the multiple rounds of degassing and purging.

**RESULTS**

**Inactivation of Rhodanese Is Reversible Only if It Occurs in the Presence of GroEL**—In our previous work we have demonstrated that native rhodanese partitions onto GroEL and can be recovered when GroES and ATP is added to GroEL-rhodanese complex (7). Here, we compared GroEL-induced and spontaneous inactivation of rhodanese and the effect of DTT and sodium thiosulfate on this inactivation. The thiosulfate anion is a substrate of rhodanese and like DTT, its presence prevents oxidative modification of rhodanese (28).

In the presence of DTT and sodium thiosulfate, GroEL dramatically enhanced the inactivation of rhodanese (Fig. 1). When incubations were performed without DTT and sodium thiosulfate, the rates of both the spontaneous and GroEL-induced inactivation were significantly increased with the latter inactivation rate remaining the fastest (Fig. 1). However, regardless of the presence of DTT and sodium thiosulfate during inactivation, only the samples that initially included GroEL recovered full activity upon addition of GroES and ATP (Fig. 1). Solution conditions remained “nonpermissive,” i.e. both GroES and ATP were still required for renaturation of rhodanese (data not shown). No significant recovery of activity was detected in the rhodanese samples that were inactivated in the absence of DTT and sodium thiosulfate even though these samples were incubated with 10 μM GroEL for 3 h prior to addition of GroES and ATP (Fig. 1). It appears that the irreversibly inactivated rhodanese either does not interact with or cannot be released from the chaperonin suggesting that GroEL-induced inactivation involves a rhodanese intermediate different from those produced by spontaneous inactivation. It is noteworthy that an increase in native rhodanese activity observed within 30 min of incubation was due to reactivation of lypophilized enzyme upon solvation. In subsequent experiments, solubilized native rhodanese was incubated at 37 °C for 30 min prior to the addition of GroEL to achieve a maximum level of reactivation.

**The Rates of Rhodanese Inactivation Are Dependent on the Chaperonin Concentration**—At 37 °C, in the presence of a molar excess of GroEL, the inactivation reaction proceeded at a defined rate showing an optimal fit to a single exponential
decay (Fig. 2, inset). The rates of inactivation were dependent on the GroEL concentration and reached a constant value of approximately $1.7 \times 10^5 \text{ min}^{-1}$ (Fig. 2).

To test whether a small shift in the unfolding equilibrium may be sufficient to effect the inactivation rates, a nondenaturing concentration of urea (1 M) was included in the inactivation reaction. This addition resulted in an increased rate of GroEL-dependent inactivation of rhodanese (Fig. 2). A similar increase in the inactivation rates was observed when the incubation temperature was raised from 37 °C to 45 °C (data not shown). It appears that GroEL interacts with an intermediate of rhodanese that exists in equilibrium with the native state and that this interaction becomes more prominent under conditions that increase intermediate conformations of the protein.

Renaturation and Committed Refolding of Rhodanese from GroEL Complexes Formed with Unfolded or Native Protein—Since different folding intermediates may bind to the chaperonin, it is conceivable that the GroEL-bound rhodanese derived from the native and from the urea-unfolded protein have different conformations. In this case, different rates of renaturation may be observed. Using similar strategies employed by Buchner and co-workers (12, 29), the rates of rhodanese refolding were examined at various times after either native or urea-denatured rhodanese were mixed with nucleotide-free GroEL and GroES at 37 °C. After the formation of the complex, ATP was added at various times to release and reactivate GroEL-bound rhodanese. The reactivation data were fit to a single exponential function. The reactivation rates remained constant throughout the inactivation time course (Fig. 3A).

Although the reactivation rates from the urea denatured or GroEL-inactivated native rhodanese were identical, the rate at which rhodanese is released from GroEL in a state committed to fold to a native protein (i.e., no longer requires the chaperonin) still may depend on the structure of the bound intermediates. Since multiple release and rebinding steps accompany GroEL-dependent rhodanese refolding, it is possible that an intermediate that can commit to the native state may rebind to the chaperonin. These prolonged interactions with the chaperonin will inhibit the folding rate (19, 30). Thus, the commitment rates reflect the properties of protein folding intermediates and their turnover rates with chaperonin (5, 31). Following the rapid removal of GroEL from the folding mixture by precipitation with anti-GroEL antibody (5), the amount of protein that commits to a native folded state was assayed. The commitment rates for GroEL-rhodanese complexes formed from the native state mirrored their respective renaturation rates (Fig. 4). Similar data were previously obtained for the urea-unfolded rhodanese (5).

Incubation of Native Rhodanese with GroEL under Anaerobic Conditions—To further investigate the nature of the rhodanase intermediate that partitions onto GroEL, we have performed experiments in the absence of oxygen. Horowitz and Criscimagna (32) have shown that rhodanase is susceptible to numerous oxidative inactivation reactions. Since oxidized rhodanase is more hydrophobic than the native form, the oxidized conformer may preferentially partition onto GroEL. Under anaerobic conditions, a partitioning and inactivation of rhodanase by GroEL no longer occurred after several hours of incubation (Fig. 5). Even after 18 h of incubation, rhodanese exhibited 80–90% of its initial activity (data not shown). These data
strongly suggest that the rhodanese species partitioning onto GroEL is an oxidized form of rhodanese.

DISCUSSION

We have demonstrated that the rhodanese intermediate that interacts with GroEL under non-denaturing conditions is an oxidized form of rhodanese. This oxidized intermediate exists in equilibrium with native protein and binds to chaperonin even in the presence of stabilizers of native rhodanese such as DTT and sodium thiosulfate (28). It has been demonstrated that oxidation of rhodanese involves two steps, a fast and reversible formation of single disulfide bond and/or sulfenyl group and a kinetically slower process of formation of a second disulfide and/or further oxidation of sulfenyl group. The second step is practically irreversible even in the presence of reducing agents (33, 34). Our data suggest that GroEL is capable of recognizing the product of the first oxidation step. After partitioning onto chaperonin, rhodanese is protected from further oxidation and regains its full native activity upon release from GroEL into reducing environment. However, if oxidation of rhodanese is allowed to proceed further and GroEL is added after a significant delay, no activity could be recovered.

The identical reactivation and commitment rates observed with either oxidized or unfolded forms of rhodanese suggest a structural similarity of these GroEL-bound forms. However, comparable reactivation and commitment rates may also result from the same kinetic barriers to the formation to the native state. In this latter situation, different intermediates may become bound to GroEL but the rate-limiting step would be the same for a wide variety of different folding intermediates. It is interesting, though, that GroES still remains an absolute requirement for chaperonin-assisted folding regardless of property of the initial state (unfolded or oxidized) of rhodanese. Buchner and co-workers found a correlation between the extent of unfolding and the requirement for GroES to facilitate folding of different oxidation states of Fab antibody fragments with GroEL (12, 35). By these criteria, we suggest that the overall structure of the GroEL-bound intermediate derived from the oxidation of native rhodanese is at least as unfolded as the urea-derived intermediate.

Previously, Horowitz and co-workers observed that rhodanese inactivation rates increased with temperature but inclusion of GroEL in its high affinity form (no nucleotide) protected the protein from irreversible inactivation (36). However, the observed decrease in activity was primarily attributed to thermal inactivation of rhodanese (36). Our studies have conclusively shown that partitioning of native rhodanese onto GroEL is triggered by an oxidation event. Although there appears to be an increase in rhodanese inactivation with either a temperature increase or in 1 M urea, no GroEL-induced inactivation can be observed at 37 °C when oxygen is removed. Because the oxidation of native rhodanese is required prior to formation of the GroEL-rhodanese complex, the maximal inactivation rate is predicted to be sensitive to the amount of reducing agent (DTT and sodium thiosulfate) present. Indeed, removal of these components results in a much faster decline in the observed rhodanese activity.

Our study suggests that oxidized rhodanese undergoes a conformational change resulting in its binding to GroEL. Although the nature of this conformational change has not been identified, it is well known that oxidized proteins in general are more sensitive to thermal denaturation (37–40). Thus, it is possible that oxidation results in a substantial shift in the folding-unfolding equilibrium toward the unfolded species which, in turn, provides the increased source of conformers

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**FIG. 4.** For chaperonin-rhodanese complexes formed from native rhodanese, the commitment rates of refolding mirror the reactivation rates. Rhodanese (1 μM) was incubated with 1 μM GroEL. After 3 and 20 h at 37 °C, GroES and ATP were added to the samples containing GroEL, and reactivation kinetics were measured (■). In separate aliquots, the activity of rhodanese was assayed after a rapid removal of GroEL from the folding mixture by precipitation with anti-GroEL antibody followed by incubation for 1 h at 37 °C (○). The identical reactivation and commitment rates observed with either oxidized or unfolded forms of rhodanese suggest a structural similarity of these GroEL-bound forms. However, comparable reactivation and commitment rates may also result from the same kinetic barriers to the formation to the native state. In this latter situation, different intermediates may become bound to GroEL but the rate-limiting step would be the same for a wide variety of different folding intermediates. It is interesting, though, that GroES still remains an absolute requirement for chaperonin-assisted folding regardless of property of the initial state (unfolded or oxidized) of rhodanese. Buchner and co-workers found a correlation between the extent of unfolding and the requirement for GroES to facilitate folding of different oxidation states of Fab antibody fragments with GroEL (12, 35). By these criteria, we suggest that the overall structure of the GroEL-bound intermediate derived from the oxidation of native rhodanese is at least as unfolded as the urea-derived intermediate.

**FIG. 6.** A model of partitioning of rhodanese onto GroEL (see text for details). N is the native state, $I_{rd}$ and $I_{ox}$ are the reversibly and irreversibly oxidized intermediates, $[I_{rd1} \ldots I_{rdx}]$ represent the population of reduced folding intermediates, and $U_{ox}$ and $U_{rd}$ are the oxidized and reduced unfolded states.
capable of partitioning onto GroEL. This partitioning may be thermodynamic in origin. Rather than cause an acceleration in unfolding rates, the chaperonins may interact with partially folded intermediates or unfolded states that exist in equilibrium with the native state (i.e., oxidized state in case of rhodanese) to form an energetically favorable substrate-chaperonin complex. Indeed, Schmid and co-workers have recently demonstrated that partitioning of modified Rnase T (which cannot fold to a native state) onto GroEL does not change the rate constants of unfolding (16).

From our data and previous data by Horowitz and co-workers, we propose a model illustrated in Fig. 6. In the presence of sodium thiosulfate and DTT, increasing concentrations of the chaperonin GroEL will compete against the reactivation reaction and bind a small fraction of reversibly oxidized rhodanese ($I_{red}$). After the oxidized protein binds to the chaperonin (EL-$I_{red}$), the reactivation reaction may become essentially irreversible within our experimental time frame because an additional dissociation step is now required. Alternatively, following the reduction, the dissociation of the reduced intermediate (EL-$I_{red}$) may be slow. Since the inactivation rate approaches a constant maximum value at high GroEL concentrations, we suggest that the rate-limiting step in the inactivation reaction is the oxygen-dependent oxidation of native rhodanese ($N \rightarrow I_{ox}$). Eventually, the amount of native rhodanese decreases due to mass action effects and results in the observed inactivation rates of rhodanese in the presence of GroEL. Thus, in the presence of sodium thiosulfate and DTT, continuous oxidative inactivation of native rhodanese in the presence of GroEL accelerates the GroEL-dependent inactivation reaction and bind a small fraction of reversibly oxidized rhodanese (EL-$I_{red}$). The GroEL and oxidized rhodanese is faster than the re-reduction and the accompanying conformational change. The GroEL and oxidized rhodanese is faster than the re-reduction and the accompanying conformational change.

Our observations carry a cautionary note. Because oxidative damage to proteins can easily occur during protein isolation and general unfolding reactions, intermediates generated by oxidation may contribute to the interactions between protein folding intermediates and GroEL. If appropriate measures are not taken, oxidative modification may also complicate the identification of protein substrates that become bound to the chaperonin following isolation of GroEL-protein substrate complexes. Therefore, in cases where substrate proteins are susceptible to oxidative damage, care must be taken to rule out complicating covalent modifications.

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