# Complex Interactions between the Chaperonin 60 Molecular Chaperone and Dihydrofolate Reductase

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ABSTRACT: The spontaneous refolding of chemically denatured dihydrofolate reductase (DHFR) is completely arrested by chaperonin 60 (GroEL). This inhibition presumably results from the formation of a stable complex between chaperonin 60 and one or more intermediates in the folding pathway. While sequestered on chaperonin 60, DHFR is considerably more sensitive to proteolysis, suggesting a nonnative structure. Bound DHFR can be released from chaperonin 60 with ATP, and although chaperonin 10 (GroES) is not obligatory, it does potentiate the maximum effect of ATP. Hydrolysis of ATP is also not required for DHFR release since certain nonhydrolyzable analogues are capable of partial discharge. "Native" DHFR can also form a stable complex with chaperonin 60. However, in this case, complex formation is not instantaneous and can be prevented by the presence of DHFR substrates. This suggests that native DHFR exists in equilibrium with at least one conformer which is recognizable by chaperonin 60. Binding studies with $^{35}$S-labeled DHFR support these conclusions and further demonstrate that DHFR competes for a common saturable site with another protein (ribulose-1,5-bisphosphate carboxylase) known to interact with chaperonin 60.

Numerous in vitro studies on the folding pathways of chemically denatured proteins have demonstrated that many proteins successfully achieve their correct native structures by using information contained in the primary amino acid sequence [reviewed by Creighton (1990) and Jaenicke (1987)]. This has led to the general view that protein folding in vivo is also a spontaneous event. However, the cellular reality, for some proteins at least, may be quite different. In part this is due to a temporal element, whereby nascent polypeptides emerge from ribosomes in a vectorial fashion and are subject to the initiation of folding in the absence of the completed chain. A similar situation likely pertains to polypeptides which are translocated across biological membranes. To this must be added the chemical complexity of the cell, in which high concentrations of proteins in various states of folding, and with potentially interactive surfaces, must surely coexist. A class of proteins termed chaperonins (Hemmingsen et al., 1988) have been identified that affect the folding and subsequent assembly of proteins either in vivo (Georgopoulos et al., 1972; Takano & Kakefuda, 1972; Zweig & Cummings, 1973; Goloubinoff et al., 1989a), in isolated organelles (Barraclough & Ellis, 1980; Cannon et al., 1986; Gatenby et al., 1988; Cheng et al., 1989; Lubben et al., 1989; Ostermann et al., 1989; Prasad et al., 1990), or in cell extracts (Bochkareva et al., 1988).

In general, the isomerization of proteins from the unfolded state to the native state involves the transient formation of folding intermediates. Recently, in defined biochemical reactions using purified components, the chaperonin 60 (cpn60[1] or GroEL) and chaperonin 10 (cpn10 or GroES) proteins have been used to investigate the mechanism of chaperonin action (Goloubinoff et al., 1989b; Lecker et al., 1989; Viitanen et al., 1990; Laminet et al., 1990; Buchner et al., 1991). From these studies, at least one common characteristic has emerged. Thus, it is now apparent that certain protein folding intermediates are able to form extremely stable complexes with cpn60. The result of this interaction has two important consequences. For certain proteins, such as ribulose-1,5-bisphosphate carboxylase (Rubisco) and citrate synthase, a stable association with cpn60 protects the labile folding intermediates from undesirable side reactions which result in aggregation (Viitanen et al., 1990; Buchner et al., 1991). However, at the same time, a stable association with this chaperonin prevents the folding intermediates from progressing to their respective native states (Viitanen et al., 1990; Laminet et al., 1990).

While the formation of a stable cpn60 complex appears to be a common step for those proteins that interact with cpn60, subsequent events leading to the release of the bound polypeptides differ in important details. For example, at physiological temperatures, catalytically active Rubisco is only discharged from cpn60 in significant amounts when the cochaperonin cpn10 is also present, together with ATP and $K^+$ ions (Goloubinoff et al., 1989b; Viitanen et al., 1990). In contrast, liberation of pre-$\beta$-lactamase from cpn60 does not require cpn10, although the presence of ATP is again necessary (Laminet et al., 1990). Thus, with the sole exception of an ATP requirement, these limited examples appear to display a lack of unity in the mechanism by which bound polypeptides are released from this molecular chaperone. To obtain a more thorough understanding of the processes by which proteins are bound to and released from cpn60, we have investigated the interaction of mouse dihydrofolate reductase (DHFR) with cpn60 from *Escherichia coli*.

The choice of DHFR was favored by its small size (~20 kDa), simple monomeric structure, and extensive knowledge of the crystal structures and kinetics of the enzyme from

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[1] Abbreviations: AMP-PCP, adenosine 5′-($\beta$,$\gamma$-methylenetriphosphate); AMP-PNP, 5′-adenylyl imidodiphosphate; ATP$\gamma$S, adenosine 5′-*O*-(3-thiotriphosphate); cpn10, chaperonin 10; cpn60, chaperonin 60; cpn60$_{14}$, native tetradecameric form of cpn60; DHFR, dihydrofolate reductase; DN, native DHFR; DU, unfolded DHFR; DI, folding intermediates of DHFR; $H_2F$, dihydrofolate; Rubisco, ribulose-1,5-bisphosphate carboxylase.

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several species [reviewed by Kraut and Matthews (1987)]. In addition, mouse DHFR is frequently used for fundamental studies on the mechanisms of protein translocation (Van Steeg et al., 1986; Eilers & Schatz, 1986; Eilers et al., 1988; Rassow et al., 1989) and, more significantly, to probe the interaction of imported proteins with mitochondrial cpn60 (Cheng et al., 1989; Ostermann et al., 1989). Here we show that the in vitro refolding of chemically denatured mouse DHFR can be completely arrested by the presence of cpn60. As expected, this arrest results from the formation of a stable binary complex formed between these two proteins. While sequestered on cpn60, DHFR exhibits no enzymatic activity and appears to reside in a nonnative state. Active DHFR can be discharged from this complex in an ATP-dependent reaction. However, neither ATP hydrolysis nor cpn10 is obligatorily required. Finally, our results also demonstrate that "native" DHFR can form a stable association with cpn60, but only in the absence of DHFR ligands. This suggests that the native DHFR exists in equilibrium with at least one conformational state that can interact efficiently with cpn60.

## Experimental Procedures

*Materials.* pGEM-3Z, RNasin, and T7 RNA polymerase were from Promega; methotrexate–agarose, proteinase K, dihydrofolate, NADPH, ATP, AMP-PCP [adenosine 5′-(β,γ-methylenetriphosphate)], and AMP-PNP (5′-adenylyl imidodiphosphate) were from Sigma; ATPγS [adensine 5′-*O*-(3-thiotriphosphate)] was from Boehringer Mannheim; restriction enzymes and DNA ligase were from BRL; [$^{35}$S]-methionine and Enhance were from NEN; the HPLC-TSK sizing column and GpppG were from Pharmacia LKB Biotechnology. *E. coli* cpn60 and cpn10 and dimeric Rubisco from *Rhodospirillum rubrum* were purified as previously described (Viitanen et al., 1990). The concentration of cpn60 used in this study was determined by quantitative amino acid analysis.

*Plasmid Constructs.* Plasmid pDS5/3 (Stueber et al., 1984) directs synthesis of mouse DHFR in *E. coli*. To facilitate synthesis of radiolabeled DHFR in a wheat germ translation reaction, the DHFR gene was isolated from pDS5/3 as a *Bam*HI/*Hin*dIII fragment and cloned into the *Bam*HI and *Hin*dIII sites in the T7 expression vector pGEM-3Z. The resulting plasmid, pDHFR-3Z, positions the DHFR gene under the transcriptional control of the T7 promoter.

*Purification of Mouse DHFR. E. coli* JM101 cells, harboring the plasmid pDS5/3, were grown in LB medium with 100 μg/mL ampicillin in a 10-L fermenter to an $A_{600}$ of 7.0 and collected by centrifugation. Thirty-five grams of cells, in 70 mL of 75 mM $MgCl_2$, was disrupted by passage twice through a French pressure cell at 18 000 psi. Following centrifugation (26000*g*, 15 min), DHFR was isolated from the supernatant by zinc hydroxide and ammonium sulfate fractionation, followed by chromatography on methotrexate–agarose and hydroxyapatite columns, essentially as described for the chicken liver enzyme (Kaufman, 1974). Peak fractions from the final column were exchanged into 50 mM Tris-HCl (pH 7.6), 1 mM DTT, and 10% glycerol and concentrated to ~2.5 mg of protein/mL in a Centricon 10 (Amicon); aliquots were frozen in liquid $N_2$ and stored at −80 °C. Protein was determined by the Bio-Rad protein assay. Mouse DHFR was at least 90% pure as judged by gel electrophoresis (cf. Figure 4).

*Preparation and Purification of $^{35}$S-Labeled Mouse DHFR.* $^{35}$S-Labeled mouse DHFR was prepared by in vitro expression of the plasmid pDHFR-3Z. Transcription reactions were performed by using *Hin*dIII-linearized plasmid templates and T7 RNA polymerase essentially as described (Gatenby et al., 1988). RNA transcripts were translated in vitro by using a wheat germ translation system (Promega), essentially as recommended by the supplier. The reaction (final volume, 375 μL) contained 400 μCi of [$^{35}$S]methionine (800 Ci/mmol) and was incubated for 2 h at 25 °C. About 3% of the total [$^{35}$S]methionine was incorporated into intact mouse DHFR.

After translation, purified mouse DHFR (11.5 nmol) was added as carrier to yield a specific activity of ~1000 Ci mol$^{-1}$, and the sample was centrifuged (15000*g*, 15 min) to remove debris. The supernatant was passed through a PD-10 Sephadex G-25M column (Pharmacia) equilibrated with 50 mM potassium phosphate (pH 5.6) to remove unincorporated label. DHFR was then affinity-purified by using a 5-mL methotrexate-agarose column (Kaufman, 1974). Bound DHFR was eluted with 2 mM dihydrofolate, adjusted to 5% glycerol and 0.01% Tween-20, and concentrated in a Centricon 10 (Amicon). To remove dihydrofolate, the material was then rigorously washed and reconcentrated in the Centricon 10 using a solution containing 50 mM Tris-HCl (pH 7.7), 5% glycerol, and 0.01% Tween 20. Aliquots of affinity-purified $^{35}$S-labeled DHFR were frozen in liquid $N_2$ and stored at −80 °C until use. The overall recovery of in vitro synthesized DHFR was ~25%.

*Preparation of Unfolded DHFR.* For spectrophotometric DHFR assays, unfolded DHFR (DU) was prepared by denaturing native DHFR (DN) in 5 M guanidine hydrochloride, 0.1 M Tris-HCl, pH 7.7, and 1 mM NaEDTA, at a final concentration of 19.2 μM. Refolding reactions (Villafranca et al., 1986) were initiated by rapid dilution of the chaotrope, as detailed in the individual figure legends. To quantitate the degree of refolding in a particular experiment, an aliquot of DN was treated identically, except that the guanidine hydrochloride was omitted. For cpn60/DHFR binding studies, affinity-purified $^{35}$S-labeled DHFR was mixed with unlabeled DHFR to yield a specific activity of 90–120 Ci/mol and denatured in 4 M guanidine hydrochloride, 0.1 M Tris-HCl, pH 7.7, and 1 mM NaEDTA, at a final concentration of ~11.5 μM. Aliquots of $^{35}$S-labeled DN were treated identically, except that the guanidine hydrochloride was omitted. Unless otherwise noted, the denaturation step and all experiments described below were carried out at room temperature. To ensure rapid mixing during experiments, a small aliquot (5 μL) of DU (or DN) was added as a drop to the inside of the cap of a 1.5-mL microfuge tube, the bottom of which contained the other reaction ingredients. After the cap was replaced, reactions were initiated by rapidly inverting the tube and vigorously mixing its contents. The final concentration of guanidine hydrochloride never exceeded 0.08 M.

*DHFR Spectrophotometric Assays.* DHFR enzyme assays were conducted in quartz cuvettes with a 1-cm path length. Enzyme activity was assayed in the direction of NADPH oxidation in the presence of dihydrofolate. Oxidation of NADPH was monitored at 340 nm (over a 10-nm bandwidth), using a Hewlett-Packard 8450A diode-array spectrophotometer. Values plotted in figures (e.g., $\Delta A_{340}$) were arbitrarily normalized to a starting $A_{340}$ value of 1.0. Additional experimental details are given in the figure legends.

*Isolation of the Complex Formed between cpn60 and $^{35}$S-Labeled DHFR.* cpn60/DHFR binding reactions were performed in 1.5-mL microfuge tubes as specified in the figure legends. After a given incubation period, reaction aliquots (200 μL) were injected onto an HPLC-TSK sizing column (type G3000SW, 7.5 × 600 mm), equilibrated with buffer A [100 mM Tris-HCl (pH 7.6), 10 mM KCl, and 10 mM $MgCl_2$],

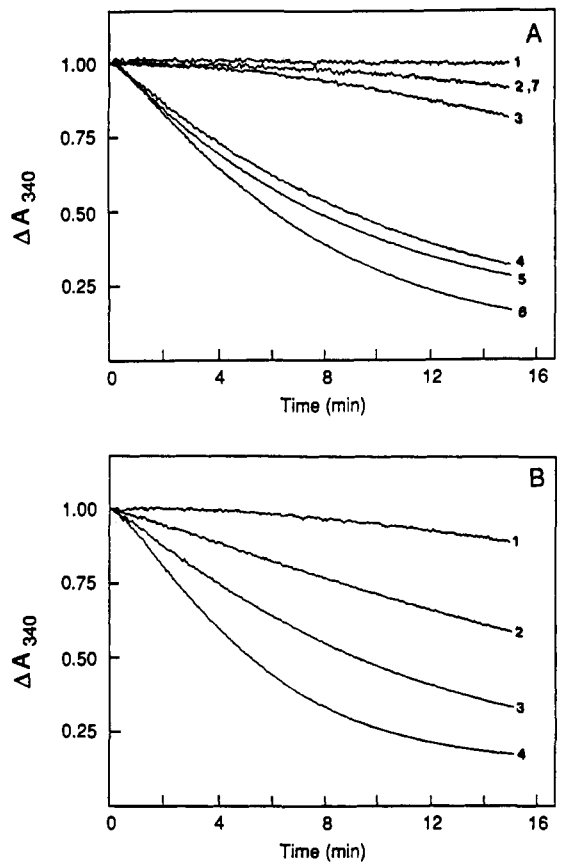



FIGURE 1: cpn60 suppresses the spontaneous refolding of chemically denatured DHFR. (A) Reactions (final volume, 300 μL) were constructed in 1.5-mL microfuge tubes, containing 0.05 M Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 3.3 mM KCl, 10 mM dithiothreitol, 0.1 mM $H_2F$, and 0.075 mM NADPH. Some reactions also contained $cpn60_{14}$ at 0.160 (trace 3), 0.638 (trace 7), or 1.27 μM (traces 2 and 4). The reaction shown in trace 1 also contained methotrexate (0.5 μM). At $t = -15$ s, reactions were initiated by the rapid addition of 5 μL of DN (traces 1, 4, and 5) or DU (traces 2, 3, 6, and 7) to a final concentration of 0.32 μM. After vigorous mixing, reactions were transferred to cuvettes, and $A_{340}$ was monitored ($t = 0$ min). (B) DU was rapidly diluted to a final concentration of 0.355 μM into a solution containing 0.05 M Tris-HCl (pH 7.7), 5.6 mM $MgCl_2$, 3.7 mM KCl, and 11.1 mM dithiothreitol, with (traces 1–3) or without (trace 4) $cpn60_{14}$ (0.71 μM). Some reactions (traces 2 and 3) were then provided with $H_2F$ (0.1 mM). Following a 1.5-h (trace 2) or 3-h (traces 1, 3, and 4) incubation period, all reactions were supplemented with $H_2F$ and NADPH to final concentrations of 0.10 and 0.075 mM, respectively. Reaction mixtures were then immediately transferred to cuvettes, and $A_{340}$ was monitored ($t = 0$ min).

with or without ATP (0.25 mM). Proteins were eluted at room temperature at a flow rate of 1.0 mL $min^{-1}$.

*Other Techniques.* SDS/PAGE, fluorography, autoradiography, and the quantitation of radioactive gel bands were carried out essentially as described (Gatenby et al., 1988).

## RESULTS

*In Vitro Refolding of DHFR and Its Suppression by cpn60.* In preliminary experiments, measurements of circular dichroism indicated that mouse DHFR is esesntially devoid of secondary structure when incubated at 25 °C in either 4 or 5 M guanidine hydrochloride (not shown). This unfolded material was used as the substrate for refolding experiments. As shown in Figure 1A, when unfolded DHFR is diluted 60-fold into a cuvette containing its natural substrates, enzymatic activity is observed almost immediately (trace 6). Thus, following a brief lag period, the oxidation of NADPH, in the presence of dihydrofolate ($H_2F$), reaches a maximal rate which is comparable to that exhibited by the native enzyme (trace 5). Clearly, the spontaneous refolding of DHFR occurs very rapidly under the conditions employed. As shown, greater enzymatic activity was frequently obtained upon the unfolding and refolding of DHFR, indicating the presence of some inactive molecules in the original "native" enzyme preparation.

Very different results were obtained when cpn60 was also present during refolding. As shown in Figure 1A, even a substoichiometric amount of the cpn60 oligomer ($cpn60_{14}$) greatly retarded the regain of DHFR activity (trace 3), and a nearly complete arrest of refolding was observed at higher molar ratios (traces 2 and 7). The ability to inhibit DHFR refolding was quite specific for cpn60 since several other proteins (Rubisco, bovine serum albumin, yeast hsp70, *E. coli* dnaK, and *E. coli* cpn10) had virtually no effect even at concentrations greater than 1 μM (not shown). Importantly, under the conditions described, the enzymatic activity of native DHFR was not significantly influenced by the presence of even the largest concentration of cpn60 tested (trace 4). These results imply that cpn60 forms a complex with either the unfolded or the partially folded DHFR and, as a consequence, bound DHFR is prevented from obtaining its active native state. Hence, refolding is arrested.

During the spectrophotometric assay (Figure 1A), it was apparent that the complex formed between cpn60 and denatured DHFR is not entirely stable. Thus, even in the presence of a maximally inhibitory concentration of cpn60 (trace 2), the slope of NADPH oxidation gradually increased with time, indicating a slow breakdown of the complex to yield active enzyme. This initially suggested that the cpn60·DHFR complex is rather labile, at least in the presence of substrates. In Figure 1B, the stability of this complex was examined in greater detail. However, for this experiment, unfolded DHFR was permitted to form a complex with cpn60 in the complete absence of substrates. Subsequently, the reaction mixtures were incubated for various times, in the presence or absence of $H_2F$, prior to being assayed for DHFR activity.

In the absence of $H_2F$ (trace 1), the cpn60·DHFR complex remained stable even after 3 h at room temperature. However, once again, a slow dissociation of the complex was observed during the spectrophotometric assay. In contrast, a significant dissociation of the complex occurred during the preincubation periods with $H_2F$ (traces 2 and 3). This dissociation occurred in a slow, time-dependent manner, such that by 3 h, most of the complex had broken down to yield active DHFR. Similar results were obtained when preformed cpn60·DHFR complexes were preincubated in the presence of NADPH (not shown). These results show, that in the absence of DHFR ligands, the cpn60·DHFR complex is extremely stable and that either substrate (NADPH or $H_2F$) can alter the observed equilibrium between bound and free DHFR.

*Native DHFR Can Also Interact with cpn60.* In view of the above results, we reevaluated whether or not a cpn60·DHFR complex could be formed starting from the "native" enzyme. As shown in Figure 2, when native DHFR is preincubated, in the *absence* of substrates, with a 2-fold molar excess of $cpn60_{14}$, a slow time-dependent loss of DHFR activity is subsequently observed (traces 2–5). As indicated below, this loss of DHFR activity is fully reversible upon the addition of ATP and, more importantly, is not observed when native DHFR is added to identical preincubation mixtures containing either NADPH or $H_2F$ (not shown). As suggested for the arrest of spontaneous refolding (Figure 1), this loss of native enzyme activity presumably results from a physical interaction between cpn60 and DHFR. However, the fact that in this case the $t_{1/2}$ for formation of the inhibitory complex is on the order

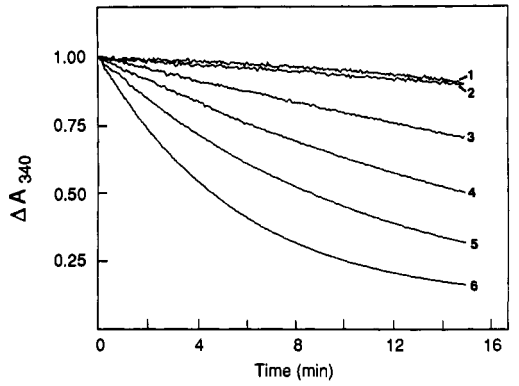
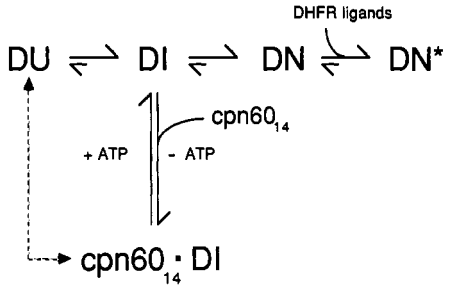



FIGURE 2: "Native" DHFR also interacts with cpn60, but only in the absence of substrates. Reactions (final volume, 300 μL) were constructed in 1.5-mL microfuge tubes, containing 0.05 M Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 3.3 mM KCl, 10 mM dithiothreitol, and $cpn60_{14}$ (0.66 μM), in the presence (traces 1 and 6) or absence (traces 2–5) of $H_2F$ (0.1 mM) and NADPH (0.075 mM). Reactions were initiated by the rapid addition of DU (trace 1) or DN (traces 2–6) to a final concentration of 0.32 μM. After this addition, the reactions shown in traces 1 and 6 were immediately transferred to cuvettes, and $A_{340}$ was monitored ($t$ = 0 min). The remaining reactions were incubated for either 0.5 (trace 5), 2.0 (trace 4), 7.5 (trace 3), or 15 min (trace 2) prior to their supplementation with $H_2F$ (0.1 mM) and NADPH (0.075 mM). After this addition, these reactions were immediately transferred to cuvettes, and $A_{340}$ was monitored ($t$ = 0 min). Note: all reactions received the same final concentration of guanidine hydrochloride (0.08 M) regardless of whether DU or DN was used.

Scheme I: Upon Dilution from Guanidine Hydrochloride, Unfolded DHFR (DU) Collapses to Folding Intermediates (DI) Which Can either Isomerize to Native DHFR (DN) or Interact with $cpn60_{14}$[a]




[a] DN exists in conformational equilibrium with a species which can also form a stable complex with $cpn60_{14}$. Although this species is also designated as DI, it is not implied that it is identical with the folding intermediate which interacts with the chaperonin. DHFR ligands (dihydrofolate or NADPH) stabilize DN in a conformation (DN*) which cannot interact with $cpn60_{14}$. The cpn60·DI complex can be discharged by ATP to yield DN; however, the exact species released from this complex is unknown. The dotted line acknowledges the possibility that cpn60 might interact directly with the fully unfolded species, DU.

of minutes and can be prevented by DHFR ligands suggests that cpn60 does not interact directly with a native DHFR per se.

It seems more likely that, similar to DHFR from other sources (Blakley, 1984; Kraut & Matthews, 1987), the mouse enzyme exists in slow equilibrium with a number of conformational states, at least one of which can efficiently interact with cpn60. According to this notion, in the absence of DHFR ligands, by mass action alone, eventually all of the DHFR can be sequestered on the chaperonin in an enzymatically inactive form (see Scheme I). It also follows that DHFR ligands must somehow prevent the conformational interconversions which are necessary for DHFR to assume a stable association with cpn60. Interestingly, DHFR ligands are unable to prevent *unfolded* DHFR from interacting with cpn60 upon dilution from guanidine hydrochloride [Figure 1A (trace 2); Figure 2 (trace 1)]. This implies that under these conditions a

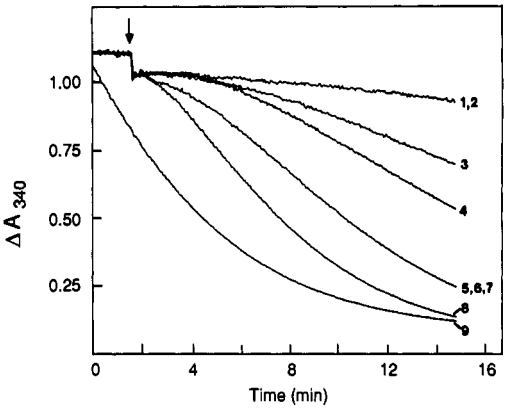



FIGURE 3: Effect of cpn10, ATP, and ATP analogues on the cpn60-induced folding arrest of DHFR. Reactions (270 μL) were constructed in 1.5-mL microfuge tubes, containing 0.05 M Tris-HCl (pH 7.7), 5.4 mM $MgCl_2$, 3.5 mM KCl, 10.7 mM dithiothreitol, 0.11 mM $H_2F$, and 0.08 mM NADPH, in the presence (traces 1–8) or absence (trace 9) of $cpn60_{14}$ (0.70 μM). The reaction shown in trace 8 also contained cpn10 (21 μM protomer). At $t$ = −15 s, reactions were initiated by the rapid addition of 5 μL of DU to a final concentration of 0.34 μM. After vigorous mixing, reactions were transferred to cuvettes, and $A_{340}$ was monitored ($t$ = 0 min). One hundred seconds later (downward arrow), the following additions were made: trace 1, 15 μL of 50 mM Tris-HCl (pH 7.7); trace 2, 15 μL of 118 mM AMP-PCP; trace 3, 15 μL of 56.5 mM AMP-PNP; trace 4, 15 μL of 113 mM AMP-PNP; trace 5, 15 μL of 55 mM ATPγS; trace 6, 15 μL of 25 mM ATP; traces 7 and 8, 15 μL of 50 mM ATP; trace 9, no addition.

nonnative DHFR species, perhaps a folding intermediate, is captured by cpn60 prior to its isomerization to the native state.

*Discharge of the cpn60·DHFR Complex Requires ATP but Not cpn10.* A number of partially folded proteins form stable complexes with purified bacterial cpn60 (Goloubinoff et al., 1989b; Lecker et al., 1989; Viitanen et al., 1990; Laminet et al., 1990; Buchner et al., 1991). Of these, the best studied is the dimeric Rubisco from *Rhodospirillum rubrum*. At 25 °C, the efficient discharge of active Rubisco from the cpn60·Rubisco complex requires ATP hydrolysis as well as the participation of the co-chaperonin cpn10. In marked constrast, although the maximal rate of release of DHFR from the cpn60·DHFR complex (Figure 3) requires *both* ATP and cpn10 (trace 8), ATP alone is at least partially effective (traces 6 and 7). Furthermore, the release of DHFR is also triggered by relatively low concentrations of ATPγS (trace 5) and higher concentrations of the nonhydrolyzable analogue AMP-PNP (traces 3 and 4). Parallel experiments confirmed that, as previously reported (Goloubinoff et al., 1989b), neither ATPγS nor AMP-PNP was effective (e.g., <5%) in discharging active Rubisco from the cpn60·Rubisco complex (not shown), ruling out the possibility of significant ATP contamination in the analogues tested.

The ATP-dependent discharge of the cpn60·DHFR complex therefore does not require cpn10 or even the hydrolysis of ATP. Instead, the release of DHFR is mediated in part through the binding of ATP to cpn60. Apparently, nucleotide binding imparts a conformational change in the chaperonin which sufficiently weakens its interaction with DHFR. As a consequence, DHFR is liberated from cpn60 and allowed to assume its native state. In a number of ways, this situation resembles the interaction reported for cpn60 and pre-β-lactamase (Laminet et al., 1990). The cpn60·pre-β-lactamase complex could also be formed starting from either the unfolded or the "native" protein and was discharged by ATP in a manner which was potentiated by cpn10, but not dependent upon it. Furthermore, in contrast to Rubisco, both pre-β-lactamase and DHFR are relatively small monomeric proteins

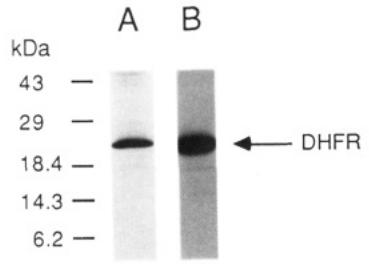



FIGURE 4: Radiochemical purity of $^{35}$S-labeled mouse DHFR. Purified in vitro synthesized $^{35}$S-labeled mouse DHFR (41 000 dpm) was mixed with purified unlabeled mouse DHFR (2.9 $\mu$g) and analyzed by SDS/PAGE on a 15% gel. Lane A shows the Coomassie blue stained gel, and lane B shows a picture of a fluorograph of the gel.

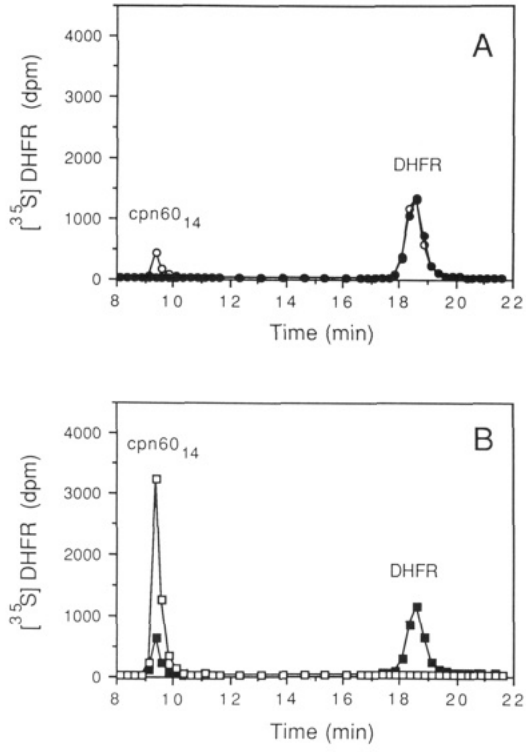



FIGURE 5: Chemically denatured DHFR forms a stable isolatable complex with the purified bacterial cpn60. $^{35}$S-Labeled DU or DN ($\sim$11.5 $\mu$M, 90 Ci/mol) was prepared as described under Experimental Procedures. (A) Five microliters of $^{35}$S-labeled DN was diluted 50-fold into a solution containing 0.09 M Tris-HCl (pH 7.7), 7.8 mM $MgCl_2$, 7.8 mM KCl, 0.12 mM $H_2F$, and 4.8 $\mu$M BSA, in the presence (○) or absence (●) of cpn60$_{14}$ (0.76 $\mu$M). After a 1-min incubation, aliquots (200 $\mu$L) were injected onto an HPLC-TSK sizing column, equilibrated with buffer A. Proteins were eluted at 1.0 mL min$^{-1}$, and fractions (0.25 mL) were collected. Radioactivity was quantitated by liquid scintillation after mixing 0.2 mL of each fraction with 0.5 mL of $H_2O$ and 10 mL of Ready Safe (Beckman). Radioactivity is plotted as a function of column retention time. The positions where authentic cpn60$_{14}$ and mouse DHFR elute are indicated. Note: on this column cpn60$_{14}$ elutes in the void volume. (B) Five microliters of $^{35}$S-labeled DU was diluted 50-fold into a solution containing 0.09 M Tris-HCl (pH 7.7), 7.8 mM $MgCl_2$, 7.8 mM KCl, 0.12 mM $H_2F$, and 4.8 $\mu$M BSA, in the presence (□) or absence (■) of cpn60$_{14}$ (0.76 $\mu$M). Reactions were then treated as follows: (□) after a 1-min incubation, a 200-$\mu$L aliquot was injected onto the column and processed as above. (■) After a 3-min incubation (to allow for DHFR refolding), cpn60$_{14}$ was added to a concentration of 0.76 $\mu$M, and the mixture was allowed to incubate for 1 additional min, prior to injection onto the column.

which readily refold spontaneously at 25 °C. It will be of interest to see whether ATP hydrolysis is required for a significant discharge of the cpn60·pre-$\beta$-lactamase complex.

*Isolation of a Stable cpn60·DHFR Complex.* The ability of cpn60 to arrest the spontaneous refolding of DHFR (Figures 1 and 3) suggested that a physical complex was formed between these two proteins. Such a complex has been reported for imported mouse DHFR and mitochondrial cpn60 (Ostermann et al., 1989). To demonstrate a similar complex with the bacterial cpn60, $^{35}$S-labeled mouse DHFR was prepared by in vitro transcription/translation and affinity-purified to radiochemical and electrophoretic homogeneity (Figure 4).

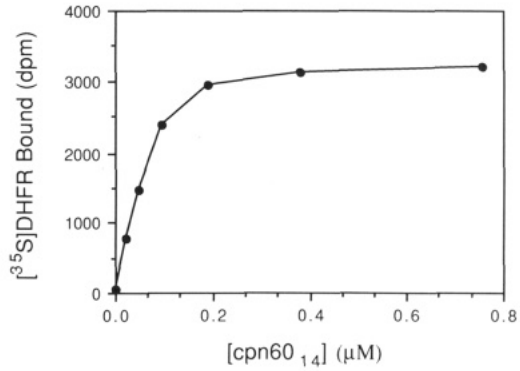



FIGURE 6: High-affinity binding of nonnative DHFR to cpn60$_{14}$. Reactions were initiated by diluting 5 $\mu$L of $^{35}$S-labeled DU ($\sim$11.5 $\mu$M, 97 Ci/mol) 50-fold into solutions containing 0.09 M Tris-HCl (pH 7.7), 7.8 mM $MgCl_2$, 7.8 mM KCl, and cpn60$_{14}$ at concentrations ranging from 0 to 0.76 $\mu$M. After a 1-min incubation, aliquots (200 $\mu$L) were injected onto an HPLC-TSK sizing column, equilibrated with buffer A. Proteins were eluted at 1 mL min$^{-1}$, and the eluate emerging between 8.5 and 10.5 min (e.g., corresponding to cpn60$_{14}$) was collected in tubes which contained 50 $\mu$L of BSA (10 mg/mL). Radioactivity was quantitated by liquid scintillation; half of each sample was counted.

When radiolabeled *native* DHFR ($^{35}$S-labeled DN) was injected onto an HPLC-TSK sizing column (Figure 5A), all of the radioactivity emerged between 17.5 and 19.5 min (●). This region corresponds to where authentic mouse DHFR elutes. Only $\sim$60–70% of the DHFR is recovered from the column, presumably due to nonspecific adsorption. Regardless, when $^{35}$S-labeled DN was incubated for 1 min in the simultaneous presence of $H_2F$ and a molar excess of cpn60$_{14}$ (○), $\sim$10% of the radioactivity now eluted between 8.5 and 10.5 min. This shift in retention time indicates the formation of a complex between DHFR and the bacterial chaperonin, since this is the region where cpn60$_{14}$ elutes. However, most of the radioactivity still cofractionated with free DHFR, confirming that only a small amount of this complex can be formed when folded DHFR is incubated with cpn60 in the presence of its ligand $H_2F$.

In marked contrast, when an identical experiment (Figure 5B) was performed using *chemically denatured* DHFR ($^{35}$S-labeled DU), virtually all of the radioactivity now coeluted with cpn60$_{14}$ (□). For example, in the experiment shown, $\sim$75% of $^{35}$S-labeled DU emerged from the column as a stable complex with cpn60, and no *free* DHFR was detected. This occurred despite the fact that $H_2F$ was present during complex formation. As anticipated, only a small amount of the cpn60·DHFR complex was formed when $^{35}$S-labeled DU was allowed to refold spontaneously, for 3 min, prior to the addition of cpn60 (■). Thus, the DHFR species which is recognized by cpn60 during the refolding reaction is not stable and exists only transiently.

As shown in Figure 6, extremely low concentrations of the cpn60 oligomer are able to efficiently capture this unstable species before it partitions to its native state. A constant amount of $^{35}$S-labeled DU (0.23 $\mu$M) was added to reaction mixtures containing various amounts of cpn60$_{14}$, and the resulting radioactive binary complexes were quantitated. Half-maximal binding of DHFR occurred at a concentration

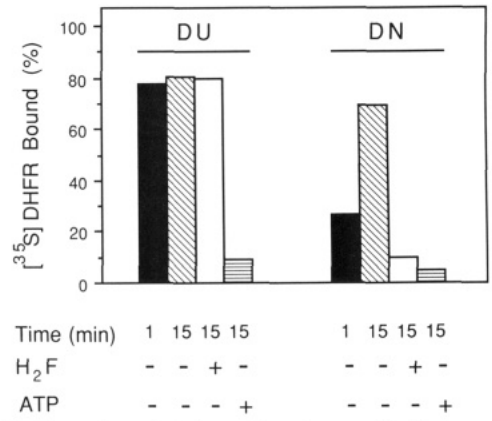



FIGURE 7: Time-dependent formation of a complex between $cpn60_{14}$ and "native" DHFR. Binding experiments were performed with either $^{35}S$-labeled DU (bar graphs at left) or $^{35}S$-labeled DN (bar graphs at right), prepared as described in the legend to Figure 5. (Black bars) Reactions were initiated by diluting 5 $\mu$L of $^{35}S$-labeled DU (or DN, as indicated) 50-fold into a solution containing 0.09 M Tris-HCl (pH 7.7), 7.8 mM $MgCl_2$, 7.8 mM KCl, 4.8 $\mu$M BSA, and $cpn60_{14}$ (0.76 $\mu$M). After a 1-min incubation, aliquots (200 $\mu$L) were injected onto a TSK sizing column, equilibrated with buffer A. As described in the legend to Figure 6, the eluent emerging between 8.5 and 10.5 min was collected, and radioactivity was quantified by liquid scintillation. (Hatched bars) Reactions were performed as described above, but were incubated for 15 min prior to injection onto the column. (White bars) $^{35}S$-Labeled DU (or DN) was diluted into the solution described above which also contained 0.12 mM $H_2F$. Reactions were incubated for 15 min prior to injection onto the column. (Horizontally striped bars) $^{35}S$-Labeled DU (or DN) was diluted into a solution containing 0.09 M Tris-HCl (pH 7.7), 7.8 mM $MgCl_2$, 7.8 mM KCl, 4.8 $\mu$M BSA, and $cpn60_{14}$ (0.76 $\mu$M). After 1 min, reactions were supplemented with ATP (3.0 mM) and were allowed to incubate for 14 additional min prior to injection onto the column. In this case, the column elution buffer also contained 0.25 mM ATP. Bar graphs depict the percentage of total injected $^{35}S$-labeled DHFR found associated with $cpn60_{14}$. Note: all reactions received the same final concentration of guanidine hydrochloride (0.08 M) regardless of whether DU or DN was used.

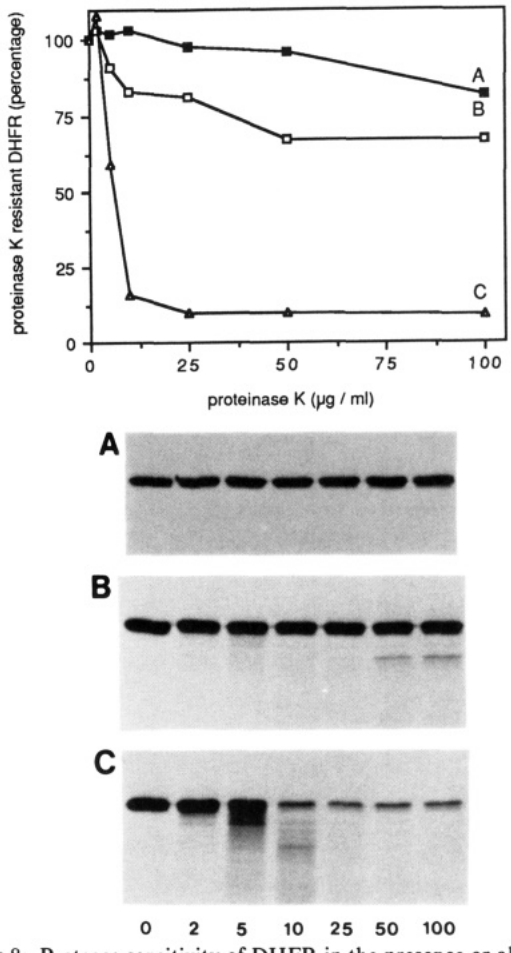



FIGURE 8: Protease sensitivity of DHFR in the presence or absence of cpn60. For reactions B (□) and C (△), $^{35}S$-labeled DHFR (~700 Ci $mol^{-1}$) was concentrated by precipitation with 80% acetone and dissolved in 4 M guanidine hydrochloride to yield $^{35}S$-labeled DU at a concentration of ~70 $\mu$M. This $^{35}S$-labeled DU was rapidly diluted 66-fold into a solution containing 100 mM Tris-HCl (pH 7.7), 10 mM KCl, 10 mM $MgCl_2$, 0.14 mM $H_2F$, and $cpn60_{14}$ (1.2 $\mu$M), and the mixture was then incubated for 15 min at 23 °C in either the presence (B, □) or the absence (C, △) of 5 mM ATP. Subsequently, small aliquots (15 $\mu$L) of each reaction were treated with proteinase K (10 min at 0 °C) at concentrations ranging from 0 to 100 $\mu$g $mL^{-1}$. Proteolysis was terminated with 10 mM phenylmethanesulfonyl fluoride, and samples were analyzed by SDS–PAGE/fluorography using 15% gels. (■) represents an experiment performed with $^{35}S$-labeled DN (native DHFR) in the absence of cpn60 and ATP. Radioactive bands corresponding to intact DHFR have been quantitated in the upper part of the figure, and the original fluorographs are shown below.

of about 50 nM $cpn60_{14}$. While this experiment was not intended to address the important issue of substrate binding stoichiometry, this preliminary result opens up the possibility that each cpn60 oligomer may be able to accommodate more than one DHFR molecule. Assuming our determinations of protein concentrations are correct, a similar conclusion is reached from the data shown in Figure 1A (trace 3), where the spontaneous refolding of 0.32 $\mu$M DHFR was nearly arrested by a concentration of 0.160 $\mu$M $cpn60_{14}$. Additional experiments have indicated that DHFR and Rubisco compete for a common saturable site on bacterial cpn60 (P. V. Viitanen, unpublished observations). For example, when cpn60 is presaturated with Rubisco in its unfolded or partially folded state, it no longer arrests the spontaneous refolding of DHFR, and subsequent binding of $^{35}S$-labeled DU is virtually abolished (>85%). This does not occur when identical experiments are performed with *native* Rubisco.

*Comparison of the cpn60·DHFR Complex Formed from Unfolded vs Native DHFR.* The results shown in Figure 2 suggest that it should be possible to form significant amounts of the cpn60·DHFR complex starting from native DHFR. This prediction is borne out in the experiment shown in Figure 7 where complexes were permitted to form in the complete absence of DHFR ligands. However, in contrast to DU, which immediately formed a complex with cpn60 (black bars), complete formation of the complex starting from DN required at least several minutes of incubation (hatched bars). As expected, the presence of dihydrofolate largely prevented formation of the complex starting from DN (white bars) but had no effect when the complex was formed from DU. This observation supports the notion that DHFR ligands stabilize a conformation of DHFR which is poorly recognized by cpn60 (Scheme I). Moreover, that complex formation starting from DU is refractory to the presence of $H_2F$ (Figures 1A and 7) might suggest that either the unfolded protein or rather early folding intermediates, unable to interact with DHFR ligands, are the species which are captured by the chaperonin during the refolding reaction. Regardless, whether cpn60·DHFR complexes were formed starting from folded or unfolded DHFR (Figure 7), incubation with ATP resulted in their substantial dissociation (horizontally striped bars).

*DHFR Shows Enhanced Sensitivity to Proteolysis When Bound to Bacterial cpn60.* Upon import into isolated mitochondria, mouse DHFR stably associates with mitochondrial cpn60 and exhibits enhanced sensitivity to digestion by proteinase K (Ostermann et al., 1989). This suggested that DHFR resides on the chaperonin in an "unfolded or loosely

folded conformation". As shown (Figure 8), mouse DHFR also exhibits enhanced sensitivity to proteinase K while associated with bacterial cpn60. Binary complexes were formed starting from DU, and these were preincubated with or without ATP prior to treatment with proteinase K. In contrast to free native DHFR (A, ■), largely resistant to 100 $\mu g\ mL^{-1}$ proteinase K, DHFR bound to cpn60 (C, △) was readily digested by a 10-fold lower concentration. The small amount of DHFR which remained resistant to proteinase K (~10%) presumably represents material which was not bound to cpn60 or a protease-resistant subpopulation of the cpn60-associated enzyme.

It was expected that ATP would discharge DHFR from its complex with cpn60 and that this would be accompanied by the recovery of resistance to proteinase K. Qualitatively, this result was obtained (B, □). However, under the conditions described, the discharge of DHFR does not appear to have been complete, and presumably a small amount of enzyme remained bound and thus sensitive to low concentrations of protease. This incomplete release might relate to the low temperature at which the protease digestions were performed or to a direct effect of proteinase K on the chaperonin. Nevertheless, these observations support the proposal that DHFR resides on cpn60 in a "nonnative" conformation (Ostermann et al., 1989) and offer an explanation for the observed lack of enzyme activity exhibited by the cpn60-DHFR complex (Figure 1–3).

## Discussion

It now seems apparent that cpn60 from prokaryotes, mitochondria, and chloroplasts has evolved to interact and form stable complexes with a wide variety of proteins in their nonnative states. The exact binding forces responsible for these interactions are not known. However, they could relate to some fundamental feature, common to most proteins in a partially folded or unfolded state, such as hydrophobicity or a shared, ubiquitous structural element. For Rubisco (Goloubinoff et al. 1989b; Viitanen et al., 1990) and citrate synthase (Buchner et al., 1991), complex formation with cpn60 prevents nonproductive, off-pathway folding reactions that would otherwise result in aggregation. Yet at the same time, this stable interaction prevents the target polypeptide from isomerizing to its native state. Thus, the spontaneous refolding of chemically denatured Rubisco (Viitanen et al., 1990), pre-β-lactamase (Laminet et al., 1990), and now mouse DHFR is completely arrested in the presence of purified cpn60.

To some extent, all three of these polypeptides could be discharged from their respective cpn60 complexes by the mere addition of ATP. However, it should be appreciated, that in each case, this ATP-dependent discharge was potentiated by the co-chaperonin cpn10. cpn10 is therefore not necessarily required for the release process in vitro, but instead acts to increase its efficiency, as previously suggested (Laminet et al., 1990). The extent to which cpn10 potentiates ATP varies from one target protein to the next and is likely to depend on the experimental conditions employed. For example, at 25 °C, cpn10 stimulates the maximal rate of the ATP-dependent discharge of DHFR only 2-fold (Figure 3), in contrast to the 50-fold stimulation observed for the discharge of active dimeric Rubisco (Goloubinoff et al., 1989b; Viitanen et al., 1990). These cpn10-enhanced rates of release, while not essential to obtain the desired product in vitro, may be significant to the physiology of cells and account for the simultaneous requirement of both cpn60 and cpn10 for cell viability (Fayet et al., 1989).

The previous assumption that ATP hydrolysis is required for the discharge of proteins from cpn60 is challenged by the observation that a nonhydrolyzable analogue (AMP-PNP) was partially able to liberate DHFR from its inactive complex with cpn60. Moreover, ATPγS, which competitively inhibits but cannot support the chaperonin-mediated refolding of Rubisco (Goloubinoff et al., 1989b), was as effective as ATP in releasing the bound DHFR. This argues that in vitro, discharge of the cpn60·DHFR complex is mediated in part through the binding of ATP to cpn60. Thus, in the presence of ATP, or a nonhydrolyzable analogue capable of producing a similar conformational change, there is a drastic reduction in the affinity of cpn60 for the target protein DHFR. This shifts the equilibrium towards *free* enzyme, and spontaneous folding resumes. Interestingly, ATPγS and AMP-PNP were also partially effective in the ATP-dependent, self-assembly of the cpn60 tetradecamer (Lissin et al., 1990).

Imported mouse DHFR forms a stable complex with mitochondrial cpn60 (Ostermann et al., 1989). However, the addition of ATP alone could not liberate DHFR from the isolated complex. Instead, it caused a structural change in the bound DHFR moiety. This structural change (e.g., increased resistance to proteinase K digestion) presumably resulted from an ATP-dependent conformational change in cpn60, which apparently was insufficient to alter the observed equilibrium between bound and free DHFR. Nevertheless, in agreement with our results, the nonhydrolyzable ATP analogue AMP-PNP was at least partially effective in eliciting the change in DHFR structure which occurred on mitochondrial cpn60 [cf. Figure 4C of Ostermann et al. (1989)].

NADPH and dihydrofolate also appear to liberate DHFR from its complex with cpn60 (Figure 1B). This process is slow, however, requiring several hours. While we cannot formally exclude the possibility that DHFR ligands interact directly with chaperonin-bound DHFR, we think that this is unlikely considering its lack of catalytic activity (Figures 1–3) and nonnative state (Figure 8). Alternatively, these ligands bind to free DHFR, as it slowly dissociates from the complex, and effectively trap it in a conformation, unable to interact with cpn60 (Scheme I). It has been reported, however, that some folding intermediates of DHFR from *E. coli* are able to bind ligands (Frieden, 1990) and this may also be true for the partially folded mouse enzyme while associated with cpn60.

The most interesting result is the finding that native DHFR coexists with at least one conformer which is able to interact with cpn60. As a result, most of the native enzyme can eventually be sequestered on the chaperonin in an inactive form. This might imply that cpn60 can recognize certain protein species which possess considerable secondary and perhaps even tertiary structure. Superficially, complex formed between cpn60 and native DHFR appears to be identical with that formed from the denatured protein. However, formation of the complex with native DHFR requires several minutes and can be prevented by DHFR ligands, which "lock" the enzyme in a conformation which cannot interact with cpn60. Whether or not other differences exist between these two types of complexes remains to be determined.

It is still not known whether cpn60 functions in vivo as a catalyst of protein folding or merely acts as a "molecular chaperone". Strictly speaking, a catalyst enhances the rate of a reaction and is capable of multiple turnovers. Undoubtedly, cpn60 undergoes multiple turnovers in vivo, although this has not been demonstrated using purified components. With regard to a rate enhancement, the limited number of in vitro studies suggest that cpn60 does not necessarily function as a catalyst. Mouse DHFR actually folds more slowly in the presence of the complete chaperonin system than in its absence

(Figure 3). Little or no rate enhancements were observed for denatured pre-β-lactamase (Laminet et al., 1989) or citrate synthase (Buchner et al., 1991). In contrast, a 5–10-fold rate enhancement was observed for the chaperonin-assisted refolding of Rubisco at 15 °C (Viitanen et al., 1990).

Assuming that one important function of cpn60 is the formation of transient complexes with nonnative proteins, protecting them from factors which interfere with proper folding, it is not surprising that these same interactions might also retard a progression to the native state. Alternatively, without invoking catalysis, a small rate enhancement might result from populating a species of the folding intermediate on the chaperonin which is more directly in line to the native state, or immediately precedes its transition state. Whether or not chaperonins can function in vivo as catalysts of protein folding in the complex milieu of the cell must await further experimentation.

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## References

Barraclough, R., & Ellis, R. J. (1980) *Biochim. Biophys. Acta 608*, 19–31.

Blakely, R. L. (1984) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.) Vol. 1, pp 191–253, John Wiley and Sons, New York.

Bochkareva, E. S., Lissin, N. M., & Girshovich, A. S. (1988) *Nature 336*, 254–257.

Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F. X., & Kiefhaber, T. (1991) *Biochemistry 30*, 1586–1591.

Cannon, S., Wang, P., & Roy, H. (1986) *J. Cell Biol. 103*, 1327–1335.

Cheng, M. Y., Hartl, F.-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L., & Horwich, A. L. (1989) *Nature 337*, 620–625.

Creighton, T. E. (1990) *Biochem. J. 270*, 1–16.

Eilers, M., & Schatz, G. (1986) *Nature 322*, 228–232.

Eilers, M., Hwang, S., & Schatz, G. (1988) *EMBO J. 7*, 1139–1145.

Frieden, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4413–4416.

Gatenby, A. A., Lubben, T. H., Ahlquist, P., & Keegstra, K. (1988) *EMBO J. 7*, 1307–1314.

Georgopoulos, C. P., Hendrix, R. W., Kaiser, A. D., & Wood, W. B. (1972) *Nature 239*, 38–42.

Goloubinoff, P., Gatenby, A. A., & Lorimer, G. H. (1989a) *Nature 337*, 44–47.

Goloubinoff, P., Christeller, J. T., Gatenby, A. A., & Lorimer, G. H. (1989b) *Nature 342*, 884–889.

Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., & Ellis, R. J. (1988) *Nature 333*, 330–334.

Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117–237.

Kaufman, B. T. (1974) *Methods Enzymol. 34*, 272–281.

Kraut, J., & Matthews, D. A. (1987) in *Biological Macromolecules and Assemblies* (Jurnak, F. A., & McPherson, A., Eds.) Vol. 3, pp 1–71, John Wiley and Sons, New York.

Laminet, A. A., Ziegelhoffer, T., Georgopoulos, C., & Plückthun, A. (1990) *EMBO J. 9*, 2315–2319.

Lecker, S., Lill, R., Ziegelhoffer, T., Georgopoulos, C., Bassford, P. J., Kumamoto, C. A., & Wickner, W. (1989) *EMBO J. 8*, 2703–2709.

Lissin, N. M., Venyaminov, S. Yu, & Girshovich, A. S. (1990) *Nature 348*, 339–342.

Lubben, T. H., Donaldson, G. K., Viitanen, P. V., & Gatenby, A. A. (1989) *Plant Cell 1*, 1223–1230.

Ostermann, J., Horwich, A. L., Neupert, W., & Hartl, F.-U. (1989) *Nature 341*, 125–130.

Prasad, T. K., Hack, E., & Hallberg, R. L. (1990) *Mol. Cell. Biol. 10*, 3979–3986.

Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F.-U., & Neupert, W., (1989) *J. Cell Biol. 109*, 1421–1428.

Stueber, D., Ibrahimi, I., Cutler, D., Dobberstein, B., & Bujard, H. (1984) *EMBO J. 3*, 3143–3148.

Takano, T., & Kakefuda, T. (1972) *Nature 239*, 34–37.

Van Steeg, H., Oudshoorn, P., Van Hell, B., Polman, J. E. M., & Grivell, L. A. (1986) *EMBO J. 5*, 3643–3650.

Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, D. P., & Lorimer, G. (1990) *Biochemistry 29*, 5665–5671.

Villafranca, J. E., Howell, E. E., Oatley, S. J., Xuong, N., & Kraut, J. (1986) *Biochemistry 26*, 2182–2189.

Zweig, M., & Cummings, R. J. (1973) *J. Mol. Biol. 80*, 505–518.