Determination of Regions in the Dihydrofolate Reductase Structure That Interact with the Molecular Chaperonin GroEL†

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ABSTRACT: Dihydrofolate reductase (DHFR) from *Escherichia coli* does not interact with the molecular chaperonin GroEL regardless of whether the interaction is initiated from the native or the unfolded state. In contrast, murine DHFR shows a strong interaction with GroEL. Using the structure of human DHFR as a model for the murine protein, a superimposition of the two structures shows that there are three distinct external loops in the eukaryotic DHFR that are not present in the *E. coli* protein. Removal of one loop (residues 99−108) from the eukaryotic murine DHFR has no effect on the interaction with GroEL. On the basis of the differences in structures, we inserted either of two surface loops of murine DHFR into the corresponding regions of *E. coli* DHFR. In the first mutant (EcDHFR-i$_9$36), residues 36 and 37 (L-N) of *E. coli* DHFR were replaced with the nine amino acid sequence T-T-S-S-V-E-G-K-Q. In the second mutant (EcDHFR-i$_7$136), residues 136−139 (V-F-S-E) of *E. coli* DHFR were replaced with the seven amino acid sequence L-P-E-Y-P-G-V. Both *E. coli* DHFR mutants formed a complex with GroEL starting from either the native or the unfolded states of DHFR. The binding was specific since the presence of MgATP caused the release of the proteins from GroEL. As with murine DHFR, nonnative conformations of EcDHFR-i$_9$36 and EcDHFR-i$_7$136 are bound to GroEL. Fluorescence titration techniques were used to quantitate the interaction between GroEL and these proteins. A simple chromatographic procedure was developed to remove contaminating tryptophan containing peptides from GroEL samples. The mutant EcDHFR-i$_7$136 binds to GroEL with a stoichiometry of 4−5 mol of DHFR per mol of GroEL tetradecamer, while murine DHFR binds to GroEL with a stoichiometry of 2 mol of DHFR per mol of GroEL tetradecamer. Both murine DHFR and EcDHFR-i$_7$136 bind to GroEL very tightly, with equilibrium dissociation constants of less than 85 nM.

Although Anfinsen (1973) demonstrated that the biologically active, three-dimensional structure of a protein can be determined by the linear sequence of amino acids, conditions *in vivo* may not be conducive to spontaneous folding or may give rise to off-pathway processes that result in insoluble protein aggregates. A mechanism of protein folding *in vivo* must kinetically partition folding intermediates away from off-pathway reactions and thus cannot be based solely on thermodynamic considerations.

Molecular chaperones are large multisubunit proteins that interact with nonnative conformations of other proteins (Gething & Sambrook, 1992; Jaenicke, 1993). They are of biological importance in preventing incorrect interactions between polypeptide chains during *de novo* protein synthesis and protecting pre-existing proteins from denaturation under cellular stress. The chaperonins of the cpn60 class are highly conserved throughout evolution (McMullin & Hallberg, 1988; Horovitz et al., 1993), with the corresponding prokaryotic cpn60 being GroEL. The GroEL complex has been shown by electron microscopy and X-ray crystallography to be a tetradecamer of identical subunits, each with a M_r of 57 300 (Braig et al., 1994, 1995; Hendrix, 1979; Ishii et al., 1992). The subunits are arranged in two stacks of heptameric rings around a central cavity. A putative peptide binding site is disordered in the crystal, but several residues important for peptide binding have been identified in mutagenesis studies (Fenton et al., 1994). GroEL has weak ATPase activity (Hendrix, 1979; Viitanen et al., 1990) and associates with the co-chaperonin GroES (cpn10) to form either an asymmetric complex, in which one GroES is bound to one toroid of the GroEL structure (Langer et al., 1992; Jackson et al., 1993), or a symmetric complex (Azem et al., 1994; Schmidt et al., 1994), in which one GroES is bound to each end of the GroEL double toroid. Viitanen et al. (1992) have shown that approximately half of the soluble proteins in *Escherichia coli* in their unfolded or partially folded states form stable binary complexes with GroEL and hypothesize that the folding of many proteins in *E. coli* takes place while associated with molecular chaperones rather than spontaneously in solution. In most cases the protein substrate is released from GroEL upon binding of GroES and ATP (Bochkareva & Girshovich, 1992; Todd et al., 1994), although in some cases the presence of GroES is not required for release (Hayer et al., 1994; Lilie & Buchner, 1995; Viitanen et al., 1991). This cycle does not accelerate "on-pathway" folding but rather prevents off-pathway processes such as aggregation, thereby increasing the final yield of active protein (Buchner et al., 1991; Holl et al., 1991; Mendoza et al., 1991).

Dihydrofolate reductase from mouse (Viitanen et al., 1991), casein (Martin et al., 1991), and pre-β-lactamase (Zahn et al., 1994) have been shown to interact with GroEL from the "native" states of these proteins. For most proteins,

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however, the interaction with GroEL must be initiated from the unfolded state. Hartl and co-workers have suggested that GroEL interacts with a molten globule state (Martin et al., 1991) and have shown that collapsed states of α-lactalbumin with various arrangements of disulfide bonds bind differentially to GroEL (Hayer et al., 1994).

Little is known about the substrate binding site on GroEL or the sequences or structures recognized in the substrate proteins since very few target structures have been identified in an intact protein. Landry and Gierasch (1991) have shown that a peptide with a propensity to form an amphipathic α-helix binds to GroEL, but the chaperonin also interacts with the Fab fragment of a monoclonal antibody, an all *β*-sheet protein (Schmidt & Buchner, 1992). Proteolysis studies of rhodanese have defined two fragments of the protein thought to be responsible for binding to GroEL (Hlodan et al., 1995), and studies of truncated eosinophil cationic protein demonstrated a 17 amino acid region responsible for binding (Rosenberg et al., 1993). There is increasing evidence for the involvement of hydrophobic interactions between GroEL and the substrate protein (Hayer et al., 1994; Landry & Gierasch, 1991; Zahn et al., 1994; Zahn & Pluckthun, 1994), although the importance of a specific sequence of charged groups on the target protein also has been discussed (Gray et al., 1993). Buchner has suggested that the surface properties, especially the hydrophobic properties, accessible to GroEL may be more important than a specific sequence of residues (Lilie & Buchner, 1995). Obviously, the determinants for binding to GroEL have not been clearly defined.

Because of these considerations, we have studied the interactions of dihydrofolate reductase from mouse (MuDHFR)[1] and *E. coli* (EcDHFR) with the molecular chaperonin GroEL. There are several advantages to using DHFR in these studies. MuDHFR and EcDHFR are small, monomeric proteins with M_r of 21 446 and 17 680, respectively. The enzymatic mechanism of DHFR from mouse (Thillet et al., 1990) and *E. coli* (Fierke et al., 1987; Penner & Frieden, 1987) has been studied extensively. The equilibrium folding of EcDHFR has been shown to be consistent with a two-state transition while kinetic studies have shown the presence of several transient intermediates during refolding (Touchette et al., 1986; Ahrweiler & Frieden, 1991; Frieden, 1990; Jennings et al., 1993). The amino acid sequences of DHFRs from eukaryotes are highly conserved, with 73−93% sequence identity among protein from vertebrates (Prendergast et al., 1988). Although the sequence identity is much lower between prokaryotic and eukaryotic DHFRs, the crystal structures of DHFRs from several sources, including *E. coli* and human, demonstrate that the native conformation has been evolutionarily conserved (Bolin et al., 1982; Davies et al., 1990).

We show here that EcDHFR does not interact with GroEL, regardless of whether the interaction is initiated from the native or the unfolded state. In contrast, the structurally homologous MuDHFR shows a strong interaction with GroEL. A comparison of the structure of EcDHFR with that of human DHFR (a model for the MuDHFR) demonstrates that the native conformations differ primarily by three surface loops present on the human DHFR (Figure 1). A mutant of MuDHFR in which residues 99−108 (loop 2 in Figure 1) were replaced with the four amino acid sequence A-S-G-D demonstrated an interaction with GroEL, indicating that the residues in this loop were not responsible for the interaction (Hugo and Frieden, unpublished data). Two of the loops from MuDHFR (loops 1 and 3 in Figure 1) were inserted into the corresponding regions of EcDHFR in an attempt to define the structural determinants responsible for the interaction of MuDHFR with GroEL. Both mutants of EcDHFR (EcDHFR-$i_9$36 and EcDHFR-$i_7$136) interact with GroEL, regardless of whether complex formation is initiated from the native or the unfolded state. The mutant EcDHFR-$i_7$136 binds to GroEL with a stoichiometry of 4−5 mol of DHFR per mol of GroEL tetradecamer, and MuDHFR binds with a stoichiometry of 2 mol of DHFR per mol of GroEL tetradecamer. The equilibrium dissociation constants for both MuDHFR and EcDHFR-$i_7$136 are estimated to be less than 85 nM, demonstrating that the binding affinity for both proteins to GroEL is very high.

MATERIALS AND METHODS

Materials. Methotrexate (MTX), NADPH, MTX-agarose, Reactive Red 120 agarose (type 3000-CL), fast-flow DEAE-Sepharose, Bis-tris, Tris, and Na_2ATP were from Sigma. Bio-Gel A5M was from Bio-Rad. Ultrapure urea was from ICN. Magnesium acetate was from Mallinckrodt. Magnesium chloride was from Fisher. Restriction enzymes *Eco*RI and *Ksp*632I were from Boehringer Mannheim, *Bsa*HI was from New England Biolabs, *Hind*III was from IBI, *Sal*I was from Pharmacia, and T4 DNA polymerase, *Nco*I, and *Xba*I were from BRL. Site-directed mutagenesis was carried out using the Sculptor In Vitro Mutagenesis system, and dideoxy sequencing was done using the Sequenase 2.0 kit, both from Amersham.

Plasmid Construction and Site-Directed Mutagenesis. Plasmid pHOG1 was constructed from plasmids pGroESL (Goloubinoff et al., 1989) and pJMB100A (Buzan and Frieden, unpublished data). The coding regions for the *E. coli* groES and groEL genes were excised as a single band from plasmid pGroESL. The plasmid was treated with the restriction endonuclease *Bsa*HI, and a blunt end was created by treatment with T4 DNA polymerase. The plasmid was then digested with *Hind*III. This fragment was inserted into plasmid pJMB100A that had been digested with *Sal*I, treated with T4 DNA polymerase to create a blunt end, and then digested with *Hind*III.

Plasmid pMUD1 was constructed by inserting the gene encoding the murine DHFR, amplified by PCR from plasmid pLTRdhfr26 (Murray et al., 1983), into plasmid pJMB100A. The PCR primers introduced a unique *Nco*I site at the 5′ end of the gene and a unique *Xba*I site at the 3′ end of the gene. The PCR amplified DNA was digested with *Nco*I and *Hind*III and ligated into plasmid pJMB100A digested with the same enzymes.

[1] Abbreviations: MuDHFR and EcDHFR, dihydrofolate reductase from mouse and *Escherichia coli*, respectively; NADPH, reduced nicotinamide adenine dinucleotide phosphate; H_2F, dihydrofolate; MTX, methotrexate; Bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; the single-letter code is used for the amino acids; EcDHFR-$i_9$36, *E. coli* DHFR in which residues 36 and 37 have been replaced with the nine amino acid sequence TTSSVEGKQ; EcDHFR-$i_7$136, *E. coli* DHFR in which residues 136−139 have been replaced with the seven amino acid sequence LPEYPGV.

Plasmids pMONDHFR-$i_9$36 and pMONDHFR-$i_7$136 were produced by site directed mutagenesis of the gene for wild-type *E. coli* DHFR encoded on the plasmid pMONDHFR (Hoeltzli & Frieden, 1994). For plasmid pMONDHFR-i_9-36, an oligomer of 57 bases was used. The mutagenesis reaction resulted in the deletion of amino acid residues 36 and 37 (L-N) and insertion of 9 amino acids at position 36: T-T-S-S-V-E-G-K-Q. For plasmid pMONDHFR-$i_7$136, an oligomer of 63 bases was used. The mutagenesis reaction resulted in the deletion of amino acid residues 136−139 (V-F-S-E) and insertion of 7 amino acids at position 136: L-P-E-Y-P-G-V. Clones for each mutant were screened initially by restriction endonuclease digestions; the mutation for pMONDHFR-$i_9$36 resulted in a new *Ksp*632I site, and the mutation for pMONDHFR-$i_7$136 resulted in the loss of one *Eco*RI site. The mutations were confirmed by sequencing the entire gene.

Protein Purification. Wild-type *E. coli* DHFR, the mutants EcDHFR-$i_9$36 and EcDHFR-$i_7$136, and wild-type murine DHFR were purified as described previously (Hoeltzli & Frieden, 1994; Ahrweiler & Frieden, 1991). *E. coli* cells which contained the plasmids pMONDHFR-$i_9$36 and pMONDHFR-$i_7$136 also contained the plasmid pGroESL. It should be noted that the murine DHFR elutes in the wash of the fast-flow DEAE-Sepharose column. Protein was stored at either −20 or −80 °C.

GroEL was purified from *E. coli* BL21 containing the plasmid pHOG1. Cells were grown in Terrific medium (Sambrook et al., 1989) at 37 °C to an A_{600} of approximately 4. GroESL production was induced by the addition of nalidixic acid to a final concentration of 50 μg/mL, and the cells were harvested after 3 h of induction. Cells were lysed (on ice) in a buffer of 50 mM potassium phosphate, pH 7.2, 1 mM EDTA, 1 mM PMSF, and 2 mM DTT using a French pressure cell. After centrifugation to remove cell debris, GroEL and GroES were fractionated between 30% and 65% saturation ammonium sulfate. The pellet was dissolved in gel filtration buffer (50 mM Tris-HCl, pH 7.6, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM Na_2ATP) and dialyzed against the same buffer. The sample was loaded onto a Bio-Gel A5M column (96 cm × 6 cm) equilibrated with gel filtration buffer. Following elution from this column, the fractions containing GroEL were identified by SDS−PAGE, pooled, concentrated, and dialyzed against a buffer of 20 mM Bis-tris, pH 6.5, 100 mM KCl, 1 mM EDTA, and 2 mM DTT. The sample was loaded onto a fast-flow DEAE-sepharose column (33.5 cm × 2.6 cm), equilibrated with the same buffer, and eluted with a linear salt gradient of 0.1−1.2 M KCl. The fractions containing GroEL were again identified by SDS−PAGE, pooled, and concentrated. All procedures were carried out at 4 °C except for the Bio-Gel A5M and fast-flow DEAE-Sepharose columns, which were run at room temperature. Following elution from these columns, GroEL appeared >95% pure as judged by SDS−PAGE and Coomassie staining. However, a fluorescence emission scan (excitation at 295 nm) demonstrated a peak at approximately 335 nm, indicating the presence of tryptophan containing contaminating peptides. This contaminating fluorescence was completely removed by elution from a Reactive Red 120 agarose (type 3000-CL) column. The column (33.5 cm × 5 cm) was equilibrated with a buffer of 20 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$ at 4 °C. The protein (dialyzed against the same buffer) was added at a concentration of approximately 2−10 mg/mL and was allowed to equilibrate on the column for 15 min prior to elution with the same buffer. The fractions containing GroEL were identified by absorbance at 280 nm, and the fluorescence emission (excitation at 295 nm) was monitored in order to determine the fractions to be combined. The protein was concentrated and stored at −80 °C as described (Mendoza & Horowitz, 1994). In addition to the GroEL purified by this method, some GroEL used in these studies was a kind gift of Dr. Paul Horowitz (University of Texas, San Antonio).

The concentration of GroEL was determined using $\epsilon_{280} = 12\,200\ M^{-1}\ cm^{-1}$ (Fisher, 1992) and was confirmed by Bradford analysis. The concentrations shown here are those of the 14-mer. The ϵ_{280} of murine DHFR was determined by the method of Edelhoch (1967) to be $25\,180\ M^{-1}\ cm^{-1}$.

Enzymatic Activity Assays and Fluorescence Scans. Stock solutions of DHFR and GroEL were added to 2 mL siliconized Eppendorf tubes (National Scientific Supply) containing a buffer of 50 mM Bis-tris, pH 7.2, 100 mM KCl, and 1 mM DTT. The solutions were incubated between 20 min and 4 h at 22 °C to allow for equilibration. The solutions were then transferred to a quartz cuvette, NADPH and H_2F were added to the final concentrations indicated in the figures, and the activity was monitored as a change in absorbance at 340 nm. The final volume in the cuvette was 1 mL. The protein concentrations shown in the figures account for the dilution upon addition of substrates.

For refolding assays, DHFR was incubated for a minimum of 20 min at 22 °C in a buffer of 50 mM Bis-tris, pH 7.2, 100 mM KCl, and 1 mM DTT that was 5 M in urea. A dilution of 50-fold was made into the same buffer minus urea to give a final urea concentration of 0.1 M and a final DHFR concentration of 0.5 μM. In some experiments, the buffer also contained GroEL at a concentration of 2.5 μM. In separate experiments, some solutions which contained GroEL also contained 10 mM magnesium acetate and 1 mM Na_2ATP. After dilution of unfolded DHFR into buffer, the solutions were incubated at 22 °C for 10 min and then diluted 2-fold. Substrates were added to the final concentrations shown in the figures, and the A_{340} was monitored. The protein concentrations shown in the figures account for the dilution upon addition of buffer and substrates.

For fluorescence scans, stock solutions of DHFR and GroEL were added to 2 mL siliconized Eppendorf tubes containing a buffer of 50 mM Bis-tris, pH 7.2, 100 mM KCl, and 1 mM DTT. The solutions were incubated between 20 min and 4 h at 22 °C to allow for equilibration and then were transferred to a fluorescence cuvette. The excitation wavelength was 295 nm, and the fluorescence emission was collected from 310 to 400 nm (PTI Alpha scan spectrofluorometer, Photon Technologies, Inc.). All data were corrected for background fluorescence.

Fluorescence titrations were performed in a buffer of 50 mM Bis-tris, pH 7.2, 100 mM KCl, and 1 mM DTT at 22 °C as described previously (Lohman & Bujalowski, 1991; Lohman & Mascotti, 1992). The concentration of DHFR was 1 μM, and the concentrations of GroEL were as indicated in the figure. The samples were incubated for 5 min after each addition of GroEL. The initial volume in the cuvette was 2.5 mL and changed less than 5% during the titration. The excitation wavelength was 295 nm, and fluorescence emission was measured at 338 nm. The observed fluores-

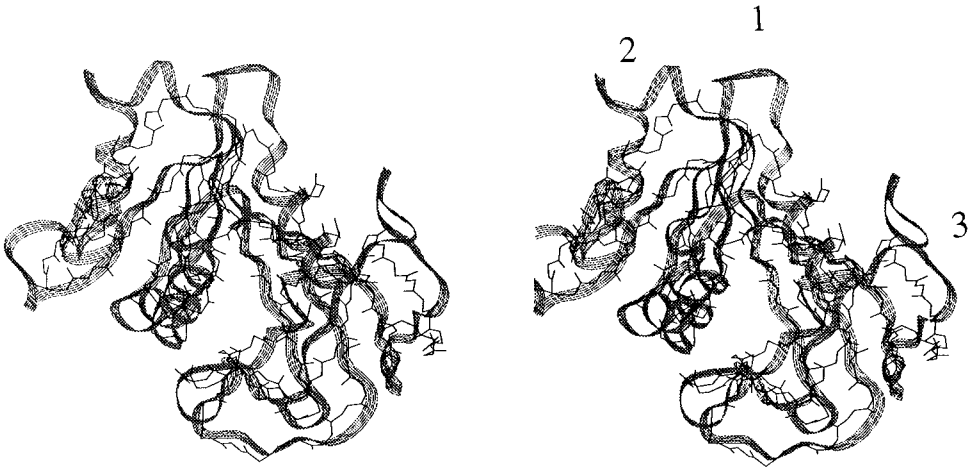

FIGURE 1: Stereoview of the structure of human DHFR superimposed onto the structure of *E. coli* DHFR. The EcDHFR backbone is represented by the thin solid line. The human DHFR backbone is represented as a ribbon structure. The loops in human DHFR which do not superimpose with the EcDHFR structure are identified as follows: Loop 1 corresponds to murine DHFR residues 39−47, loop 2 corresponds to murine DHFR residues 100−108, and loop 3 corresponds to murine DHFR residues 159−165.

cence was corrected for background fluorescence and for change in volume.

RESULTS

Comparison of EcDHFR and MuDHFR Structures. In a subsequent section we show that *E. coli* DHFR does not interact with GroEL whereas murine DHFR interacts strongly. A comparison of the structures of EcDHFR and human DHFR (a model for MuDHFR) demonstrates that the primary differences between the two proteins reside in three large loops present in the human DHFR that are absent in the *E. coli* protein (Figure 1). Two of these loops are located in turns of the EcDHFR: residues 36−37 (L-N) and residues 86−88 (G-D-V). The third loop is located in a region of the β-sheet (βG strand) in which there is a β-bulge present in EcDHFR (residues 136−139) (Bolin et al., 1982).

In order to test whether these surface loops were responsible for the interaction with GroEL, mutants of MuDHFR were constructed in which each of the three loops was truncated (Hugo and Frieden, unpublished data). Only one of the MuDHFR mutants, in which MuDHFR residues 99−108 (loop 2 in Figure 1) were replaced with the four-residue sequence A-S-G-D, was soluble when expressed in *E. coli*. This MuDHFR mutant was purified and tested for an interaction with GroEL. The enzymatic activity of this mutant was monitored in the presence or absence of GroEL (data not shown) and demonstrated a decrease in activity when incubated with GroEL, as did the wild-type MuDHFR. This indicated that the residues in this loop were not responsible for the interaction of MuDHFR with GroEL.

The two other MuDHFR mutants (truncations of loops 1 and 3 shown in Figure 1) were produced in inclusion bodies in *E. coli* and did not refold in sufficient quantities when isolated from the inclusion bodies. Therefore, in order to determine whether either of these two loops was responsible for the interaction of MuDHFR with GroEL, each loop was inserted, separately, into the corresponding regions of EcDHFR. These mutations are summarized in Figure 2. In the first EcDHFR mutant (EcDHFR-$i_9$36), residues 36−37 (L-N) of EcDHFR were replaced with the nine amino acid sequence, T-T-S-S-V-E-G-K-Q. This mutation resulted in

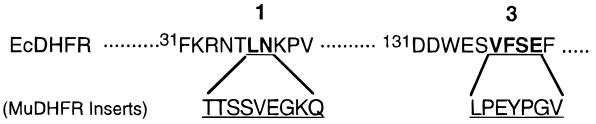

FIGURE 2: Summary of *E. coli* DHFR mutations. The *E. coli* DHFR residues shown in bold were replaced with the murine DHFR residues shown below each region of mutation. The numbers above each region of mutation correspond to the numbers of the loops shown in Figure 1. The *E. coli* DHFR mutant EcDHFR-$i_9$36 was created by the deletion of residues 36 and 37 (L-N) and insertion of the nine amino acid sequence TTSSVEGKQ. The *E. coli* DHFR mutant EcDHFR-$i_7$136 was created by the deletion of residues 136−139 (V-F-S-E) and insertion of the seven amino acid sequence LPEYPGV.

the insertion of a large loop into a tight turn region (loop 1 in Figure 1). In the second EcDHFR mutant (EcDHFR-i_7-136), residues 136−139 (V-F-S-E) of EcDHFR were replaced with the seven amino acid sequence, L-P-E-Y-P-G-V. The insertion of these amino acids at position 136 disrupts the β-sheet (loop 3 in Figure 1).

A third mutant of EcDHFR was constructed in which both MuDHFR sequences (loops 1 and 3) were inserted, together, into the corresponding EcDHFR regions. This double mutant was produced in inclusion bodies in *E. coli* and did not refold in sufficient quantities to use in the studies described here.

Interaction of MuDHFR but Not EcDHFR with GroEL. Viitanen et al. (1991) have shown that murine DHFR interacts with GroEL when the native protein is incubated with the chaperonin. When we measured the activity of *E. coli* DHFR after incubation with up to a 20-fold molar excess of GroEL, we found little or no change in activity (Figure 3A). Note that the curve to the right in Figure 3A is the EcDHFR activity in the absence of GroEL. A small amount of EcDHFR adsorbs to the cuvette in the absence of GroEL, thus the activity is slightly lower. The activity of MuDHFR over the same range of GroEL concentration is shown for comparison (Figure 3B).

These data suggest that either complex formation of EcDHFR with GroEL does not occur when the reaction is initiated from the native conformation of EcDHFR, as it does for MuDHFR, or that EcDHFR does not interact with GroEL. The latter idea was particularly intriguing because the two proteins are structurally homologous (see Figure 1). In

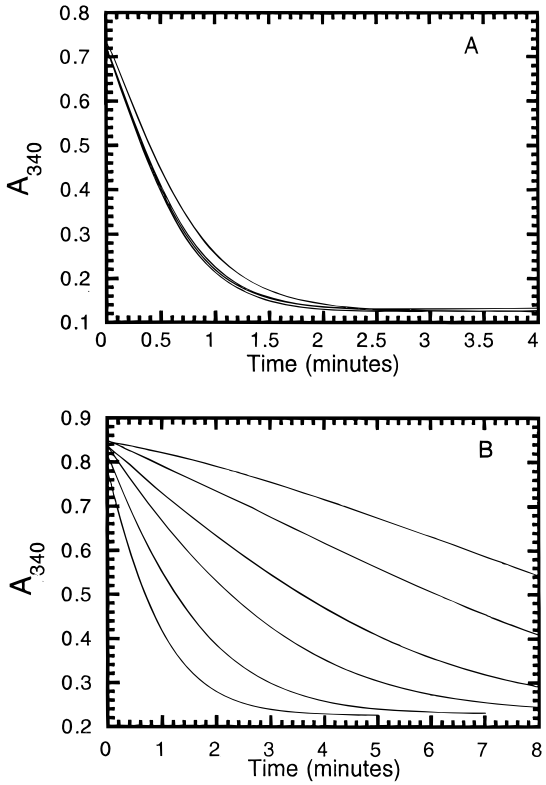

FIGURE 3: Activity of native wild-type EcDHFR and MuDHFR in the absence or presence of GroEL. Wild-type EcDHFR (A) and MuDHFR (B) were incubated with varying concentrations of GroEL for up to 4 h, and then substrates were added and assays performed as described in Materials and Methods. The final DHFR concentration in each case was 0.27 μM. For panel A, the final concentrations of GroEL were 0, 0.6, 3.0, and 6.0 μM. Note that for EcDHFR the curve to the right is the activity in the absence of GroEL. For panel B, the concentrations of GroEL were (from left to right) 0, 0.3, 0.6, 0.9, 2.0, and 6.0 μM. For panels A and B, the concentrations of NADPH and H_2F were 50 μM.

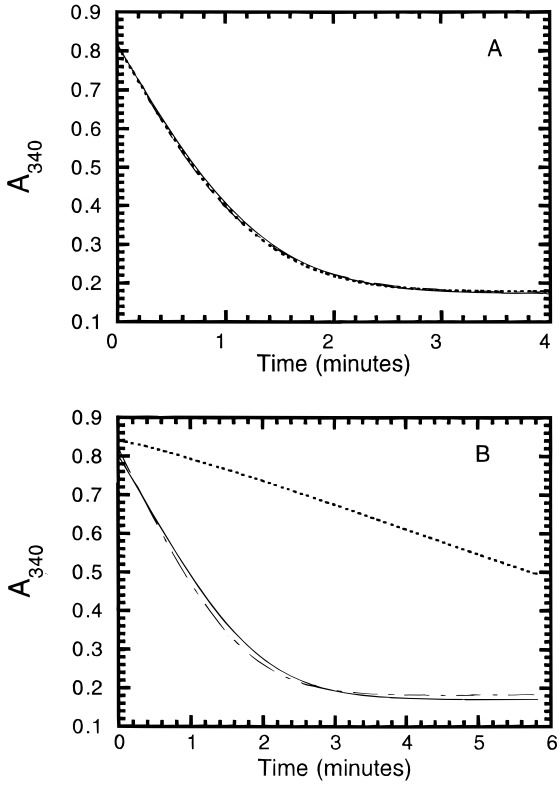

FIGURE 4: Activity of refolded wild-type EcDHFR and MuDHFR in the absence or presence of GroEL. Wild-type EcDHFR (A) and MuDHFR (B) were initially unfolded in urea and refolded as described in Materials and Methods. For panels A and B, the solid line represents the activity of DHFR after refolding in the absence of GroEL, the short-dashed line represents the activity of DHFR after refolding in the presence of GroEL, and the long-dashed line represents the activity of DHFR after refolding in the presence of GroEL, Mg^{2+}, and ATP. The final DHFR concentrations in the assays were 0.225 μM, and the final concentrations of NADPH and H_2F were 50 μM.

separate experiments, EcDHFR and MuDHFR, both of which had been unfolded in 5 M urea, were refolded in the absence or presence of GroEL (Figure 4). The activity of EcDHFR was unchanged in the presence of GroEL (Figure 4A). However, MuDHFR showed a marked decrease in activity when the protein was refolded in the presence of GroEL (Figure 4B, short-dashed line), demonstrating the formation of a stable complex. The MuDHFR bound to GroEL could be separated from unbound MuDHFR by gel filtration (Sephacryl S300HR) (data not shown). The peak in activity shifted from the elution volume of the low molecular weight MuDHFR species to the elution volume of the high molecular weight GroEL·MuDHFR complex. In contrast, all of the EcDHFR activity eluted within the EcDHFR elution volume, even in the presence of GroEL. These data demonstrate that the EcDHFR does not interact with GroEL, even during refolding.

Interaction of EcDHFR Mutants with GroEL. Both EcDHFR mutants (EcDHFR-$i_9$36 and EcDHFR-$i_7$136) were soluble in *E. coli* and expressed in sufficient quantities to study interactions with GroEL *in vitro.* When the native mutant proteins were incubated with GroEL, the activity of both mutants decreased as a function of GroEL concentration (Figure 5), with EcDHFR-$i_7$136 showing the largest decrease in activity (Figure 5B). Likewise, when the mutants were refolded from 5 M urea, the activity of both mutants was decreased when refolded in the presence of GroEL (Figure 6), although the change was much smaller than when the system was allowed to reach equilibrium (Figure 5).

Viitanen et al. (1991) demonstrated that in the absence of GroES, the addition of MgATP was sufficient to cause the release of MuDHFR from GroEL (see also Figure 4B). The same is true for EcDHFR-$i_9$36 and EcDHFR-$i_7$136. When these proteins were refolded in the presence of GroEL, Mg^{2+}, and ATP, the resulting activity curves were superimposable with the activity in the absence of GroEL (Figure 6, panels A and B, long-dashed line). This demonstrates that the binding of the EcDHFR mutants to GroEL is specific, as for MuDHFR.

Fluorescence Changes upon Binding to GroEL. GroEL contains no tryptophans in its primary sequence, while EcDHFR and MuDHFR contain five and three tryptophans, respectively. This allows for the study of fluorescence changes that occur in the substrate when bound to GroEL. The fluorescence emission spectrum of the wild-type EcDHFR shows essentially no difference in the presence or absence of GroEL (Figure 7A). The emission maximum remains at 338 nm, and there is little difference in the fluorescence quantum yield. The fluorescence emission spectrum of MuDHFR shows a red-shift from a maximum at 320 nm to a maximum at 335 nm and a slightly greater quantum yield when bound to GroEL (Figure 7B). The fluorescence emission maxima for the mutants EcDHFR-$i_9$36 and EcDHFR-$i_7$136 are also at 338 nm, as for wild-type EcDHFR;

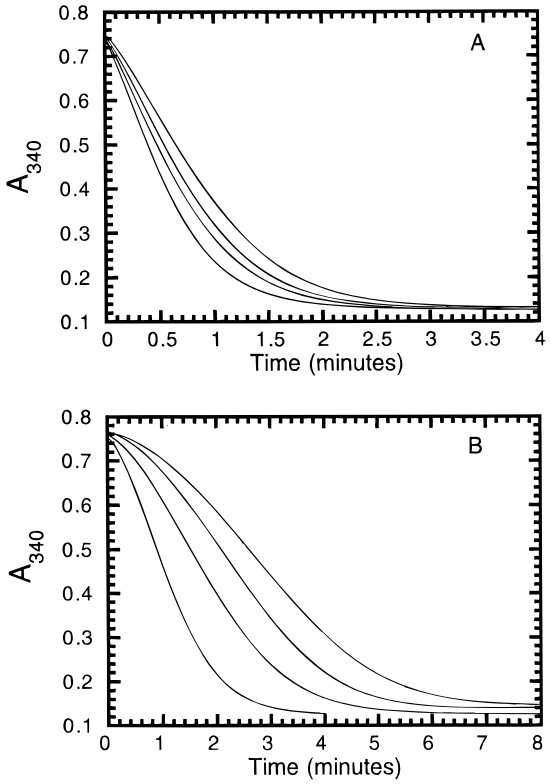

FIGURE 5: Activity of native EcDHFR-$i_9$36, and EcDHFR-$i_7$136 in the absence or presence of GroEL. EcDHFR-$i_9$36 (A) and EcDHFR-$i_7$136 (B) were incubated with varying concentrations of GroEL, and assays were performed as described in Materials and Methods. The final DHFR concentration in each case was 0.27 μM. The final concentrations of GroEL were (from left to right) 0, 0.6, 3.0, and 6.0 μM. For panels A and B, the concentrations of NADPH and H_2F were 50 μM.

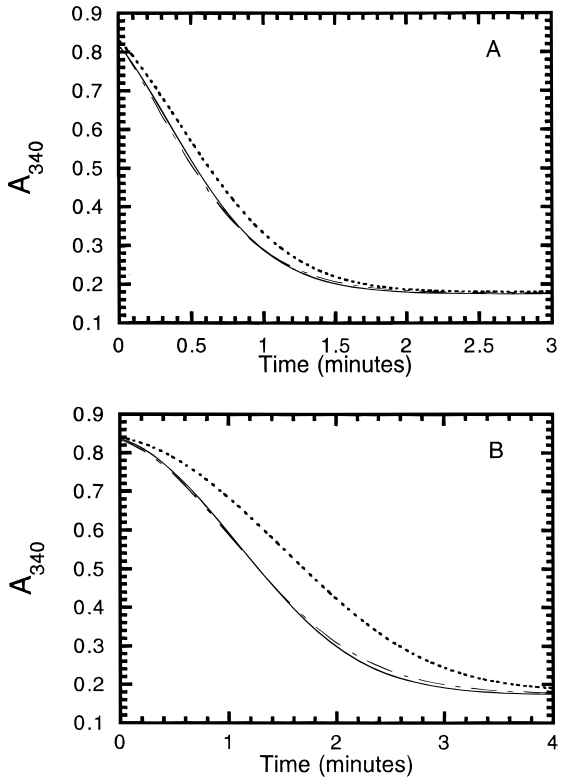

FIGURE 6: Activity of refolded EcDHFR-$i_9$36 and EcDHFR-$i_7$136 in the absence or presence of GroEL. EcDHFR-$i_9$36 (A) and EcDHFR-$i_7$136 (B) were initially unfolded in urea and refolded as described in Materials and Methods. For panels A and B, the solid line represents the activity of DHFR after refolding in the absence of GroEL, the short-dashed line represents the activity of DHFR after refolding in the presence of GroEL, and the long-dashed line represents the activity of DHFR after refolding in the presence of GroEL, Mg^{2+}, and ATP. The final DHFR concentrations in the assays were 0.225 μM, and the final concentrations of NADPH and H_2F were 50 μM.

however, in the presence of GroEL the emission maxima are blue-shifted 1−2 nm and the quantum yields are lower (Figure 7C,D).

In addition to the changes in fluorescence emission, the EcDHFR mutants show an increased sensitivity to proteinase K when bound to GroEL, as does MuDHFR (data not shown). In contrast, the wild-type EcDHFR shows little or no change in proteinase K sensitivity in the presence of GroEL. Together, these data demonstrate that a partially unfolded conformation of the EcDHFR-$i_9$36 and EcDHFR-$i_7$136 proteins binds to GroEL, as has been shown previously for MuDHFR (Viitanen et al., 1991).

Stoichiometry of Binding for MuDHFR and EcDHFR-$i_7$136. It is difficult to quantitate the binding stoichiometry based on changes in DHFR enzymatic activity as a result of binding to GroEL. Interpretation of the activity data is complicated by the competition between GroEL and DHFR substrates (NADPH and H_2F) for binding to DHFR. The GroEL·DHFR complex is inactive, and the DHFR·H_2F complex is not a substrate for GroEL (Viitanen et al., 1991). As a result, the activity does not reach zero, even at high concentrations of GroEL (Figure 3). Rather, the activity profiles demonstrate a lag phase followed by an acceleration in activity. Interpretation of the fluorescence changes shown in Figure 7 is not subject to the same ambiguities as the activity data (Figure 3) since the system contains only DHFR and GroEL. The binding stoichiometries for MuDHFR and EcDHFR-$i_7$136 were determined by quantitating the fluorescence changes which occur upon binding to GroEL. The GroEL used in these studies contained no contaminating tryptophan-containing peptides, so the background correction was minor, even at high concentrations of GroEL relative to DHFR.

At 338 nm, the fluorescence emission of MuDHFR was increased by approximately 25% when bound to GroEL (Figure 7B). In contrast, the fluorescence emission of EcDHFR-$i_7$136 was quenched by approximately 25% when bound to GroEL (Figure 7D). Because the fluorescence emission at 338 nm of EcDHFR-$i_9$36 was quenched only about 5% when bound to GroEL, it was not possible to determine accurately the binding stoichiometry for this mutant from the fluorescence data.

Both MuDHFR and EcDHFR-$i_7$136 were titrated with GroEL in a "reverse" titration experiment (Lohman & Bujalowski, 1991), and the change in fluorescence emission was monitored at 338 nm (Figure 8). For MuDHFR at 1 μM, the fluorescence emission saturates at a GroEL concentration of 0.5 μM, indicating that 2 mol of MuDHFR bind to 1 mol of GroEL tetradecamer. For EcDHFR-$i_7$136 at 1 μM, the quenching of fluorescence emission at 338 nm saturates at a GroEL concentration of approximately 0.2−0.25 μM, indicating that 4 or 5 mol of EcDHFR-$i_7$136 bind to 1 mol of GroEL tetradecamer. The equilibrium dissociation constants were estimated for each protein by fitting the data shown in Figure 8 to a simple titration equation (A + B ↔ AB). For MuDHFR and EcDHFR-$i_7$136 the K_d is

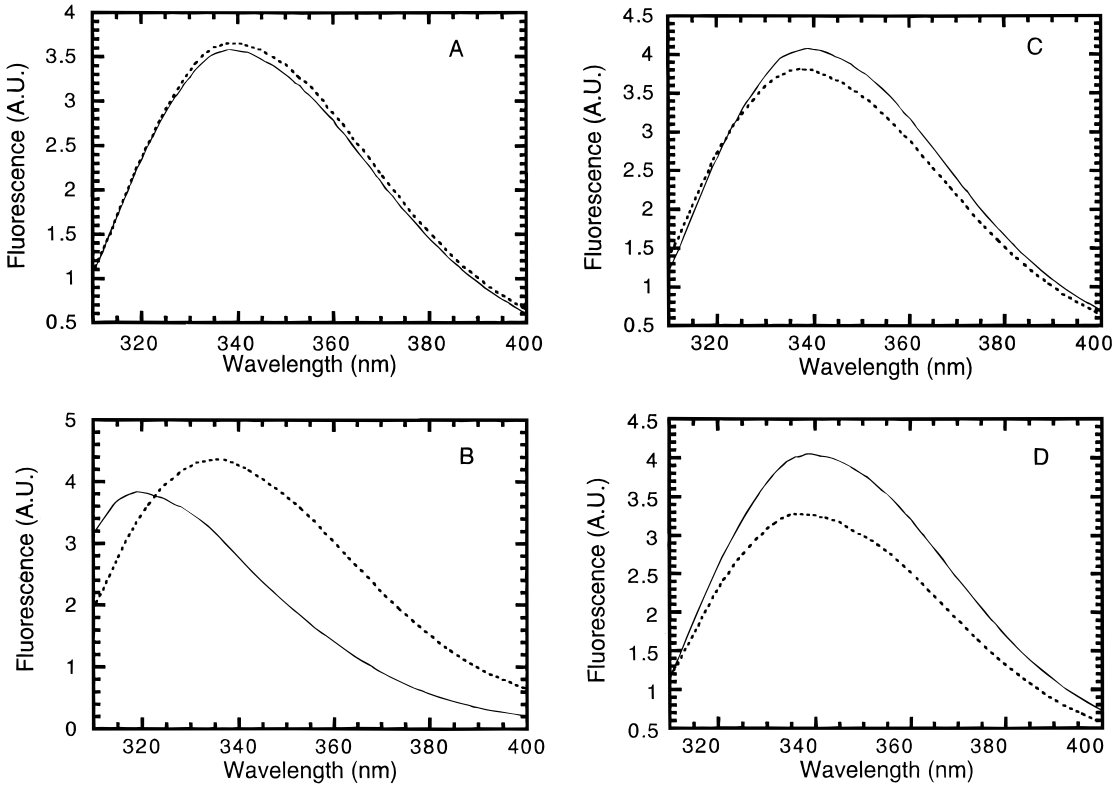

FIGURE 7: Fluorescence spectra of DHFR in the absence and presence of GroEL. Wild-type EcDHFR (A), MuDHFR (B), EcDHFR-$i_9$36 (C), and EcDHFR-$i_7$136 (D) were incubated with GroEL as described in Materials and Methods. For panels A−D, the solid line represents the fluorescence emission of DHFR in the absence of GroEL, and the dashed line represents the fluorescence emission of DHFR in the presence of GroEL. The concentration of DHFR was 0.3 μM, and that of GroEL was 1.2 μM.

estimated to be less than 85 nM. Fluorescence titration experiments over a range of DHFR concentrations indicate that binding is stoichiometric between DHFR concentrations of 100 nM and 1 μM (data not shown) and show that under these solution conditions (50 mM Bis-tris, pH 7.2, 100 mM KCl, 1 mM DTT, 22 °C) an equilibrium dissociation constant of 85 nM defines the upper limit for each system.

DISCUSSION

We have shown that dihydrofolate reductase from *E. coli* does not interact with GroEL, but the structurally homologous DHFR from mouse does. This is comparable to studies of malate dehydrogenase (Staniforth et al., 1994) and aspartate aminotransferase (Mattingly et al., 1995; Widmann & Christen, 1995) which show that mitochondrial and cytosolic isoenzymes bind differentially to GroEL. These and other studies [see review by Lorimer (1996)] indicate that GroEL does not interact universally with all proteins.

The regions of MuDHFR responsible for the interaction with GroEL are suggested by inserting two loops, separately, from MuDHFR into the corresponding regions of EcDHFR. Both EcDHFR mutants demonstrated an interaction with GroEL regardless of whether the interaction was initiated from the folded or unfolded state of DHFR. The binding of the EcDHFR mutants was specific since the presence of MgATP caused the release of the proteins from GroEL. A mutant of MuDHFR resulting from the replacement of residues 99−108 (loop 2) with a four amino acid sequence remained competent to bind to GroEL. These data show that, of the three surface loops in MuDHFR which differ from the homologous regions in EcDHFR, two may be involved in the interaction of MuDHFR with GroEL.

Scheme 1

$$U \rightleftharpoons I \rightleftharpoons N \longrightarrow N^*$$
$$\downarrow\uparrow$$
$$GroEL{:}I$$

However, preliminary studies (Clark and Frieden, unpublished data) suggest that the mutations in EcDHFR have resulted in structural changes that may not be confined to local regions. Thus, it is not yet clear whether GroEL interacts directly with the amino acids in these loops or whether the mutations in *E. coli* DHFR have resulted in structural changes which allow GroEL to interact with other regions of the protein. A mutation in EcDHFR similar to that of EcDHFR-$i_9$36 has been shown to affect the structure and enzymatic properties of the protein (Posner et al., 1996).

Intrinsic tryptophan fluorescence emission and proteolysis studies demonstrated that a nonnative conformation of EcDHFR-$i_9$36 and EcDHFR-$i_7$136 was bound to GroEL. This agrees with the model proposed by Viitanen et al. (1991), a modified form of which is shown in Scheme 1. In this scheme, U, I, and N refer to the unfolded, intermediate, and native conformations of DHFR, respectively. GroEL:I refers to the complex of the GroEL tetradecamer with the intermediate conformation of DHFR. N* refers to the native conformation of DHFR with either NADPH or H_2F, or both, bound. From the data presented here, it is not possible to distinguish this mechanism from one in which the native state of DHFR binds to GroEL followed by an isomerization to a nonnative conformation.

A comparison of the activities shown in Figures 3−6 demonstrates that, in the absence of GroEL, the folding reaction for MuDHFR and for the EcDHFRs is very efficient.

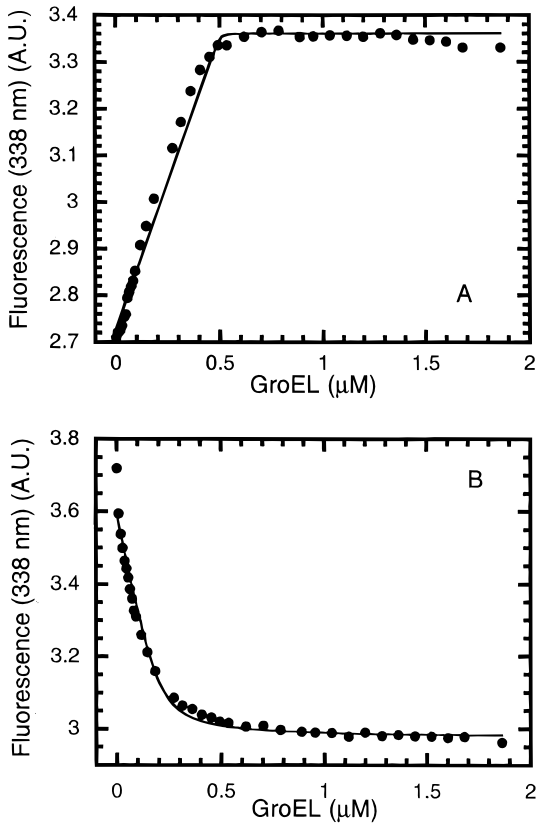

FIGURE 8: Titration of MuDHFR and EcDHFR-i$_7$136 with GroEL. GroEL was added to either MuDHFR (A) or EcDHFR-i$_7$136 (B) to give the final concentrations shown. The concentration of DHFR in each case was 1 μM. The fluorescence emission at 338 nm was monitored and corrected for background fluorescence and for change in volume. The solid lines represent a fit to the data using a simple titration equation (A + B ↔ AB) assuming a stoichiometry of 2 mol of MuDHFR per mol of GroEL tetradecamer (A) or 5 mol of EcDHFR-i$_7$136 per mol of GroEL tetradecamer (B).

For MuDHFR this has been shown previously in folding studies (Viitanen et al., 1991) and in translocation studies using isolated mitochondria (Vestweber & Schatz, 1988; Manning-Krieg et al., 1991).

It is not yet clear why EcDHFR-i$_9$36 and EcDHFR-i$_7$136 show a weaker interaction with GroEL during refolding than does MuDHFR. Assuming there are no other structures in these proteins responsible for binding, it is possible that the rate constant for the I → N transition shown in Scheme 1, a first-order process, effectively competes with complex formation, a multiordered process, at the protein concentrations used here. If so, the I → N transition for MuDHFR is either slower than for EcDHFR-i$_9$36 and EcDHFR-i$_7$136 or the I + GroEL → GroEL:I transition is much faster for MuDHFR than for the EcDHFR mutants. Little is known at present about the folding pathway of MuDHFR or the conformational stabilities of the EcDHFR mutants. The answers to this problem require a detailed kinetic and thermodynamic analysis of this system.

Viitanen et al. (1991) suggested that for MuDHFR the stoichiometry of binding to GroEL may be greater than 1:1, and Hartl and co-workers have shown that 1−2 mol of chicken DHFR bind 1 mol of GroEL (Martin et al., 1991). We show in a fluorescence titration experiment that 2 mol of MuDHFR bind to 1 mol of GroEL tetradecamer. The binding stoichiometry of 4−5 mol of EcDHFR-i$_7$136 to 1 mol of GroEL tetradecamer is more unusual but is not without precedent. Under certain solution conditions, mutants of barley chymotrypsin inhibitor 2 (Itzhaki et al., 1995) and barnase (Corrales & Fersht, 1995) form multiply-bound states. It is not clear why the binding stoichiometries are different between MuDHFR and EcDHFR-i$_7$136, but it is clear that GroEL must contain multiple binding sites. This would allow the chaperone potentially to bind several proteins at once, with the binding stoichiometry determined primarily by steric constraints. It is likely that both loops in MuDHFR bind concomitantly and therefore could bind to different sites in one GroEL toroid. Similarly, Hartl and co-workers have suggested that GroEL binds to two interdomain α-helices in rhodanese and thereby prevents aggregation during refolding (Hlodan et al., 1995). It is not clear from these studies that the substrate binding sites in GroEL are all structurally equivalent or that the substrate proteins all bind to one toroid of the double toroid structure.

There is increasing evidence for the role of hydrophobic interactions in the binding of a substrate protein to GroEL (Hayer et al., 1994; Landry & Gierasch, 1991; Zahn et al., 1994; Zahn and Pluckthun, 1994). Lin et al. (1995) have reported a positive change in enthalpy and a negative change in heat capacity upon binding of either subtilisin BPN′ PJ9, an unfolded variant of subtilisn BPN′, or α-casein to GroEL, suggesting the burial of hydrophobic residues upon binding and thus an entropic driving force for the binding reaction. Most of the sequences that have been shown to interact with GroEL contain several hydrophobic amino acid residues (Gray et al., 1993; Hlodan et al., 1995; Landry & Gierasch, 1991; Landry et al., 1993; Rosenberg et al., 1993; Zahn et al., 1994). In proteins for which binding sites have been determined, there does not appear to be a canonical sequence for binding to GroEL. Nor can one, at present, correlate the degree of hydrophobicity with an equilibrium dissociation constant because there are very few systems for which both the K_d and the amino acid residues responsible for binding have been determined. These data are available only for murine DHFR (this study) and pre-β-lactamase (Zahn et al., 1994). Proteins for which equilibrium dissociation constants have been reported include lactate dehydrogenase (Badcoe et al., 1991), subtilisin BPN′ PJ9 and α-casein (Lin et al., 1995), and α-lactalbumin (Hayer et al., 1994). To our knowledge, the amino acid residues or structures responsible for the binding of these proteins to GroEL have not been determined. It is not clear, therefore, what effect there would be on the binding affinity due to changes in the degree of hydrophobicity.

The binding of EcDHFR-i$_7$136, which contains a loop of MuDHFR close to the C-terminal end, is consistent with the role of hydrophobic residues in the binding to GroEL since five of the seven residues in this loop are hydrophobic. However, based solely on this argument, it is not clear why EcDHFR-i$_9$36, which contains a loop from MuDHFR close to the N-terminal end, binds to GroEL since only one of the nine residues is hydrophobic. It is possible that part of this sequence (V-E-G-K) mimics part of the "mobile loop" of GroES (V-E-T-K). Alternatively, the presence of the single, hydrophobic, valine residue may be sufficient to facilitate binding. This predicts that the binding affinity of EcDHFR-i$_9$36 to GroEL is lower than for either MuDHFR or EcDHFR-i$_7$136. The activity studies shown here suggest that this may be the case, but further binding studies are required. The conclusions based on these data agree with the suggestion

(Lilie & Buchner, 1995) that the surface properties, especially the hydrophobic properties, which are presented to GroEL by the substrate protein may contribute more to binding than a specific sequence of amino acids.

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