The Intermediate Domain Defines Broad Nucleotide Selectivity for Protein Folding in Chlamyphila pneumoniae GroEL1

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The chaperonin GroEL assists protein folding in the presence of ATP and magnesium through substrate protein capsulation in combination with the cofactor GroES. Recent studies have revealed the details of folding cycles of GroEL from Escherichia coli, yet little is known about the GroEL-assisted protein folding mechanisms in other bacterial species. Using three model enzyme assays, we have found that GroEL1 from Chlamyphila pneumoniae, an obligate human pathogen, has a broader selectivity for nucleotides in the refolding reaction. To elucidate structural factors involved in such nucleotide selectivity, GroEL chimeras were constructed by exchanging apical, intermediate, and equatorial domains between E. coli GroEL and C. pneumoniae GroEL1. In vitro folding assays using chimeras revealed that the intermediate domain is the major contributor to the nucleotide selectivity of C. pneumoniae GroEL1. Additional site-directed mutation experiments led to the identification of Gln400 and Ile403 in the intermediate domain of C. pneumoniae GroEL1 as residues that play a key role in defining the nucleotide selectivity of the protein refolding reaction.

The chaperonin GroEL is a cylindrical homotetradecamer with a molecular mass of 800 kDa (1). Each subunit consists of three functional domains: an apical domain, which has a polypeptide and a co-chaperonin GroES-binding site, and an intermediate domain, which connects the apical domain containing a ligand and GroES-binding sites with the equatorial domain containing a nucleotide-binding pocket. Recent proteomic analysis has shown that ~250 different proteins interact with GroEL in E. coli (2). Of these, only ~85 proteins are obligate GroEL-dependent substrates. The current understanding of the folding pathway for obligate substrates of GroEL is as follows. In the first step, substrate proteins bind to the apical domain of GroEL, which permits ATP binding to the equatorial domain. Subsequently, GroES binds to the substrate-binding site of the apical domain. In the second step, the intermediate domain rises, forming a cis-ATP complex, and then substrate protein is loaded into the central cavity of GroEL. In the third step, ATP hydrolysis induces trans-ring activation followed by ATP and substrate protein binding to the trans-ring. Finally, the cis-ternary complex decays, releasing GroES and ADP from the cis-ring. The substrate protein is then released whether it is folded or not. Consequently, only a small percentage of the molecule is folded in a single round of the ATP- and GroES-mediated cycle (3, 4).

Other GroEL/GroES-assisted folding pathways also exist. One pathway is the trans-ring-mediated pathway for substrate proteins that are too large to be encapsulated (5, 6). Another folding pathway is GroES-independent, in which non-obligate substrates are folded in the presence and absence of GroES, and the yield of the GroES-dependent pathway is generally higher than that of the GroES-independent pathway.

Even though these concepts have been well established in the model microorganism E. coli, it is unknown whether they are generally applicable. Chlamyphila pneumoniae is a Gram-negative bacterium that is an obligate intracellular parasite of eukaryotic cells (7). This bacterium is a human pathogen that causes acute respiratory disease. Most bacterial genomes encode a single copy of the groEL gene that forms an operon with the groES gene, but the Chlamydiae clade has three groEL genes that result from complex events of gene duplication (8). Of these, only the groEL1 gene consists of a GroE operon with a groES gene. Western blot and microarray analyses have shown that all three genes are constitutively expressed, but the expression levels of the groEL2 and groEL3 genes are not influenced by heat shock in Chlamydia trachomatis (9). Therefore, we have expressed the groEL1 and groES genes from C. pneumoniae strain J138 in E. coli and purified the gene products to investigate whether C. pneumoniae GroEL1 (CP GroEL1)2 is able to refold three types of denatured enzymes according to previously characterized mechanisms.

In this study, three types of substrate enzymes were used to characterize CP GroEL1: 5,10-methylentetrathydrofolate reductase (METF), which is an obligate substrate for E. coli

2 The abbreviations used are: CP GroEL1, C. pneumoniae GroEL1; METF, 5,10-methylentetrathydrofolate reductase; LDH, d-lactate dehydrogenase; AGLU, α-glucosidase; EC GroEL, E. coli GroEL; ATP yS, adenosine 5′-O-(thiotriphosphate); AMP-PNP, adenosine 5′-[(β,γ-iminotriphosphate); MOPS, 3-(N-morpholino)propanesulfonic acid.

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GroEL (EC GroEL); d-lactate dehydrogenase (LDH), which partially interacts with GroEL; and α-glucosidase (AGLU), which is too large to be encapsulated in the GroEL/GroES cavity. In characterizing CP GroEL1, we took into consideration the fact that EC GroEL assists refolding of these enzymes in the presence of ATP (2, 10–12). Furthermore, because EC GroEL has been found to enhance refolding of some non-obligate substrates even in the presence of ADP and ATP analogs ATPγS and AMP-PNP (13–16) and CTP and UTP (17), the protein folding assay was also conducted in the presence of various nucleotides (i.e. CTP, UTP, GTP, and their di- or monophosphates) to elucidate the nucleotide selectivity of CP GroEL1 as well as EC GroEL-assisted protein refolding. We report here that CP GroEL1 shows broader nucleotide selectivity for protein refolding compared with EC GroEL and that CP GroEL1 residues that play a critical role in such broad nucleotide selectivity are located in the intermediate domain.

**EXPERIMENTAL PROCEDURES**

**Gene Cloning, Chimera Construction, and Mutagenesis—**The groEL and groES genes from *E. coli* strain K12 and the groEL1 and groES genes from *C. pneumoniae* strain J138 were cloned into the pET22b(+) vector (Novagen). The metf gene fused with His tag sequences at the 3′-end was also cloned into the pET22b(+) vector. Moreover, a series of chimera groEL genes with exchanged apical, intermediate, and equatorial domains of the groEL gene from *E. coli* and the groEL1 gene from *C. pneumoniae* were constructed by an overlap extension method using PCR (18) and subcloned into the pET22b(+) vector. Mutant groEL genes were also constructed by DpnI-mediated site-directed mutagenesis and subcloned into the pET22b(+) vector. All genes were ligated into the Ndel and Xhol sites of the pET22b(+) vector. The primer sets for gene cloning, chimera construction, and mutagenesis using PCR are listed in supplemental Tables S1–S3, respectively. Moreover, schematic representations of primer annealing sites for the preparation of the chimeric genes are shown in supplemental Fig. S3.

**Protein Purification—**The recombinant proteins GroEL GroES, and METF were purified as described previously (19). The molecular masses of EC GroEL, CP GroEL1, and their chimeras were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI micro MX, Waters Corp., Tokyo, Japan).

**METF Refolding Assay—**METF (EC 1.5.1.20) from *E. coli* is a homotetrameric protein with a subunit molecular mass of 33,100 Da. It catalyzes the reduction of 5,10-methylene-tetrahydrololate to 5-methyltetrahydrofolate using the reductant NAD(P)H and coenzyme FAD. Unless stated otherwise, the concentrations of GroEL, GroES, METF, and LDH are expressed as tetradecamer, heptamer, tetramer, and dimer, respectively. METF refolding assay was carried out as described in our previous study (19) with a modification. When testing ADP in the refolding reaction, ATP contamination was eliminated as much as possible by hexokinase/glucose treatment (16).

**LDH Refolding Assay—**LDH (EC 1.1.1.28) from *Staphylococcus* sp. (Amano Enzyme Inc., Aichi, Japan) partially interacts with GroEL and is a homodimeric protein with a subunit molecular mass of 32,000 Da. It catalyzes reversible conversions of pyruvate to lactate and of NADH to NAD⁺. LDH (10 μM) was denatured with 4 M guanidine hydrochloride for 1 h at 25 °C. The unfolded LDH was diluted 100-fold at 25 °C into refolding buffer (50 mM MOPS-KOH, pH 7.0, 10 mM KCl, 10 mM magnesium acetate, and 5 mM β-mercaptoethanol) with or without 0.5 μM GroEL, 1 μM GroES, and 2 mM nucleotide. LDH activity was assayed at 25 °C by monitoring absorbance at 340 nm in LDH assay solution (100 mM Tris-HCl, pH 7.8, 100 μM NADH, and 100 μM sodium pyruvate).

**AGLU Refolding Assay—**AGLU (EC 3.2.1.20) from *Saccharomyces cerevisiae* (Sigma), which is too large to be encapsulated in the GroEL/GroES cavity, is a monomeric protein with a molecular mass of 68,000 Da. It hydrolyzes an α-glucosyl bond present in its substrates. AGLU (10 μM) was denatured with 6 M guanidine hydrochloride for 1 h at 25 °C. The unfolded AGLU was diluted 100-fold at 25 °C into refolding buffer (50 mM MOPS-KOH, pH 7.0, 10 mM KCl, 10 mM magnesium acetate, and 5 mM β-mercaptoethanol) with or without 0.5 μM GroEL, 1 μM GroES, and 2 mM nucleotide. AGLU activity was assayed at 25 °C by monitoring absorbance at 405 nm, resulting from the release of p-nitrophenol in AGLU assay solution (100 mM sodium phosphate buffer, pH 6.8, and 2 mM p-nitrophenyl-α-D-glucopyranoside).

**RESULTS**

**Refolding of Denatured Enzymes by CP GroEL1 and EC GroEL—**First, the time-dependent refolding of the obligate substrate METF, partial substrate LDH, and large substrate AGLU by GroEL proteins was investigated with and without ATP and GroES. Purified METF failed to refold spontaneously in refolding buffer. However, METF was recovered by ~40% within a few minutes in the presence of CP GroEL1 with CP GroES and ATP, but not without CP GroES (Fig. 1a). Simi-
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![Graphs showing effects of various nucleotides on GroEL/GroES-assisted refolding.](image)

In contrast, LDH was recovered by ~60% 7 h after the start of the refolding reaction in the presence of CP GroEL1 with CP GroES and ATP, and even in the presence of CP GroEL1 and ATP. LDH was scarcely recovered in the absence of CP GroEL1 (Fig. 1b). The protein refolding activities of CP GroEL1 and EC GroEL were also observed for AGLU from yeast. AGLU was recovered spontaneously by ~10%, but greater recovery of enzyme activity was observed when CP GroEL1/CP GroES or EC GroEL/EC GroES was added to the reaction in the presence of ATP (Fig. 1c). In the absence of ATP, CP GroEL1/CP GroES allowed spontaneous refolding of the enzyme, whereas EC GroEL/EC GroES completely arrested the refolding reaction. Similar events were also observed when either CP GroEL1 or EC GroEL alone was added to refolding buffer in the absence of ATP (Fig. 1d).

Effects of Various Nucleotides on GroEL-assisted Protein Refolding—Because CP GroEL1 has protein refolding activity, the effects of diverse nucleotides on the protein refolding by the two chaperonins were examined. METF was refolded by CP and EC GroEL/GroES in the presence of nucleotide triphosphates (ATP, CTP, and UTP), excluding GTP (Fig. 2, a and b). Nucleotide monophosphate species (AMP, CMP, GMP, and UMP) and inosine species (ITP, IDP, and IMP) were incapable of enhancing METF, LDH, and AGLU refolding (data not shown). Interestingly, CTP, CDP, UTP, and UDP permitted LDH refolding either by CP GroEL1/CP GroES or by CP GroEL1 alone (Fig. 2c), whereas addition of these nucleotides was ineffective in LDH refolding by EC GroEL irrespective of the presence or absence of EC GroES (Fig. 2d). The nucleotide sensitivity of the refolding reaction for AGLU by CP GroEL1/CP GroES and CP GroEL1 alone resembled that observed for LDH (Fig. 2e). However, both CTP and UTP permitted the EC GroEL/EC GroES-assisted refolding of AGLU (Fig. 2f). In contrast to the observed differences in nucleotide selectivity in the protein refolding reactions, both CP GroEL1 and EC GroEL hydrolyzed CTP and UTP as well as ATP, but not GTP (supplemental Fig. S1, a and b). Also, neither GroEL hydrolyzed ITP (data not shown).

Nucleotide Selectivity for LDH Refolding by GroEL Chimeras—Because a marked difference was observed for nucleotide selectivity in LDH refolding by CP GroEL1 and EC GroEL, we investigated structural features that could explain this result. For this purpose, a series of GroEL chimeras were constructed by exchanging apical, intermediate, and equatorial domains between the two GroEL proteins, and the in vitro LDH refolding assays were carried out in the presence of various nucleotides. GroEL proteins consist of N-terminal side equatorial (Ea), N-terminal side intermediate (Ia), apical (A), C-terminal side intermediate (Ic), and C-terminal side equatorial (Ec) regions (Fig. 3, a and b). In this study, each GroEL chimera is designated by five “C” and “E” letters representing the Ea to Ec regions, where “C” and “E” indicate that the regions are from C. pneumoniae and E. coli, respectively. Schematic structures of the chimeras are shown in Fig. 3, in which the color white indicates “C,” and the color gray indicates “E.” Each GroEL chimera was expressed in E. coli strain BL21(DE3) and purified. All the chimeras were assembled to yield 800-kDa complexes, as revealed by gel filtration and native PAGE analysis.

Exchanging apical domains with each other (CCECC and EECEE) barely affected nucleotide selectivity in GroEL-assisted LDH refolding (Fig. 3, c and f). However, the EECEE chimera-assisted LDH refolding was much lower compared with wild-
type EC GroEL-assisted LDH refolding in the presence of ATP and ADP (Fig. 3f), whereas ATP hydrolysis by the EECEE chimera was greater than that by wild-type EC GroEL (supplemental Fig. S2).

Interestingly, the ECECE chimera, in which the intermediate domain of EC GroEL was replaced with the corresponding region of CP GroEL1, assisted the LDH refolding in the presence of various nucleotides (Fig. 3g). In this case, the LDH refolding yield in the presence of CTP, UTP, and their diphosphates was about half of the corresponding yield achieved by wild-type CP GroEL1. Further addition of a C region to the apical domain reduced the refolding yield of LDH, but retained the broad nucleotide selectivity phenotype (Fig. 3e). These chimeras were capable of METF refolding, ATP hydrolysis, and METF capsulation (supplemental Fig. S2, a–d). In contrast, the CECEC and CEEEC chimeras enhanced LDH refolding in the

![Diagram](https://via.placeholder.com/150)
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![Diagram of GroEL structures](image)

**FIGURE 4.** Nucleotide selectivity for GroEL-assisted LDH refolding in the C-terminal intermediate domain. *a,* amino acid sequences of the Ic regions from *C. pneumoniae* and *E. coli* and the consensus amino acid sequence. The consensus sequence was obtained by multiple alignments of GroEL homologs from 200 bacterial species using the ClustalW program. Yellow boxes indicate different residues compared with the consensus sequence and the sequence from *E. coli,* and red letters indicate specific amino acid residues for *Chlamydia pneumoniae* and *Chlamydia.** The letter X in the consensus sequence indicates various amino acid residues. The 14th strand and the M helix are colored cyan and light green, respectively. b, the structure of the Ic region in GroEL (Protein Data Bank code 1OEL, chain a). This image was created using the RasMol program. The colors are as described for a. Leu<sup>400</sup> and Arg<sup>404</sup> are colored red. Shown is the ratio of LDH refolding in the presence of various nucleotides (2 mM) by EC GroEL mutants (0.5 μM): L400Q (c), R404I (d), L400Q/R404I (e). The refolding ratio is shown as the relative value to that observed for 0.5 μM CP GroEL1 in the presence of 2 mM ATP. − Nuc indicates the refolding yield observed in the absence of nucleotides. The data represent the mean ± S.E. of four independent experiments.

The amino acid sequences of the Ic regions from *C. pneumoniae* and *E. coli* (Fig. 3, i and j). Although the CECEC chimera failed to assemble, the CCCEC chimera assembled to refold LDH. We found that the CCCEC chimera retained weaker nucleotide selectivity in LDH refolding compared with the ECECE chimera in the LDH refolding assay (Fig. 3, i and j). The CCCEC chimera had no METF refolding, ATP hydrolysis, and METF capsulation activities (supplemental Fig. S2, a–d). Moreover, in the presence of ATP, ADP-induced LDH refolding by the CCCEC chimera was completely suppressed (data not shown).

**DISCUSSION**

*GroEL1 from C. pneumoniae Acts as a Chaperonin—* Chlamydiaceae bacteria proliferate as energy-dependent parasites in living cells, taking high energy metabolites such as nucleotide triphosphates and acetyl-CoA from their host cells. The heat shock protein Hsp60 (GroEL) from Chlamydiaceae bacteria has been shown to induce abnormal vascular smooth muscle cell proliferation, thereby possibly contributing to early stages of atherogenesis (20, 21). Such cell proliferation by *C. pneumoniae* probably results from activation of the host cell innate immune system through interactions with Toll-like receptors (22, 23), yet it is unclear whether CP GroEL1 has protein refolding functions similar to EC GroEL. Therefore, for

presence of ADP and CDP, but not ATP (Fig. 3, d and h). These two GroEL chimeras had no METF refolding, ATP hydrolysis, and METF capsulation activities (supplemental Fig. S2, a–d). Moreover, in the presence of ATP, ADP-induced LDH refolding by the CECEC GroEL chimera was completely suppressed (data not shown).

Comparison of the ECECE chimera with wild-type EC GroEL indicated a major role for the *Chlamydia pneumoniae* intermediate domain in providing broad nucleotide selectivity in LDH refolding. Therefore, we further investigated the structural factors of the intermediate domain that could be involved in such a function. Construction of the ECEEE and EEECE chimeras led to the finding that the EEECE chimera has broader nucleotide selectivity. We found that the double mutant could carry out ADP- and CTP-mediated refolding activities (Fig. 4d), whereas the L400Q mutation resulted in enhanced ATP- and CTP-mediated refolding activities (Fig. 4c). Yet the mutant still lacked the refolding function in the presence of UTP. The L400Q/R404I double mutant was then tested to examine whether this structural change leads to a gain in UTP selectivity. We found that the double mutant could carry out the refolding reaction when UTP was added to the buffer (Fig. 4e).
the first time, we have investigated in vitro protein refolding by CP GroEL1/CP GroES. We found that CP GroEL1 has a protein folding function with broader nucleotide selectivity compared with EC GroEL.

CP GroEL1 refolded METF with an activity of ~40% relative to native enzyme activity within a few minutes (Fig. 1a) and hydrolyzed ATP to a similar extent compared with EC GroEL (supplemental Fig. S1). CP GroEL1 utilized only CP GroES for METF folding, whereas EC GroEL utilized both EC GroES and CP GroES (supplemental Fig. S2a). Consistent with this, CP GroEL1-induced ATP hydrolysis was not inhibited by addition of EC GroES (supplemental Fig. S2b), and denatured METF was not capsulated (supplemental Fig. S2, c and d). These results suggest that CP GroEL1 has protein folding functions and that there is stringent selectivity for GroES.

AGLU recovered spontaneously from the denatured state by 10% compared with the activity of the native enzyme, as reported previously (11). In the absence of ATP, EC GroEL completely arrested the denatured AGLU, whereas CP GroEL1 did not, instead permitting the spontaneous refolding of the enzyme. Therefore, CP GroEL1 is thought to have a lower binding capacity for AGLU compared with EC GroEL.

Unique Nucleotide Selectivity of GroEL1 from C. pneumoniae—The chaperonins GroEL and GroES fold newly synthesized and denatured proteins in the presence of ATP in cells. Because they utilize ATP for the protein folding reaction, ATP is thought to have high affinity for GroEL and is present at high concentrations in cells compared with other nucleotides. However, many ATPases can bind not only ATP but also CTP and UTP (24). In addition, EC GroEL even enhances protein refolding of some substrates in the presence of ADP and ATP analogs (17) and CTP but not UTP for ribulose-bisphosphate carboxylase refolding (24). In addition, EC GroEL even enhances protein refolding of some substrates in the presence of the ADP and ATP analogs ATPyS and AMP-PNP (13–15). However, it was shown that stringent substrates are not refolded by EC GroEL in the presence of unhydrolyzed species ADP, ATPyS, and AMP-PNP (16). In the present study, METF, the obligate substrate for EC GroEL (2, 25, 26), was recovered both by EC GroEL/EC GroES and by CP GroEL1/CP GroES in the presence of ATP, CTP, and UTP (Fig. 2, a and b), suggesting that only hydrolyzable nucleotide species can be utilized for the refolding reaction. Consistent with this observation, both CP GroEL1 and EC GroEL hydrolyzed not only ATP but also CTP and UTP, but not GTP (supplemental Fig. S1).

Unlike the case for the METF refolding reaction, a marked difference in nucleotide selectivity was observed between CP GroEL1 and EC GroEL for refolding LDH (Fig. 2, c and d) and AGLU (Fig. 2, e and f). EC GroEL did not refold LDH in the presence of CTP and UTP, whereas CP GroEL1 did. Investigation of the primary sequence showed that all the amino acid residues identified to date as playing important roles in protein folding are completely conserved in CP GroEL1 (100% identity) (27). Therefore, it was thought that unidentified structural features are likely to be involved in the broad nucleotide selectivity of CP GroEL1 for refolding of LDH and AGLU.

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The Intermediate Domain Defines Nucleotide Selectivity for GroEL-assisted Refolding—A broader nucleotide selectivity was observed for CP GroEL1-assisted refolding of LDH and AGLU compared with EC GroEL-assisted refolding of the respective proteins. However, the CP GroEL1-assisted AGLU refolding yield included a spontaneously refolding fraction. Moreover, the nucleotide selectivity of CP GroEL1 for LDH refolding was much broader than that for AGLU refolding (Fig. 2). Therefore, we chose the LDH refolding reaction to identify structural factors contributing to the observed nucleotide selectivity. In these experiments, neither EC GroES nor CP GroES was added to the refolding assay to allow interpretation of the refolding reaction catalyzed by the chimeras and mutants of EC GroEL and CP GroEL1.

Exchanging the apical domain between EC GroEL and CP GroEL1 barely affected the nucleotide selectivity for the LDH refolding reaction (Fig. 3, c and f), excluding the apical domain from factors that make a major contribution to the observed nucleotide selectivity. The EECEE chimera-assisted LDH refolding yield was much lower than that assisted by wild-type EC GroEL, which might be due to enhanced ATP hydrolysis by domain swapping. The most prominent changes in the nucleotide selectivity for the LDH refolding reaction were observed when the intermediate domains of EC GroEL were replaced with the corresponding domains of CP GroEL1 (see the ECEEE chimera-assisted refolding reaction in Fig. 3g). A direct interpretation of this result is that structural factors permitting such broad nucleotide selectivity are likely to be present in this domain, yet replacing the two intermediate domains of CP GroEL1 with those of EC GroEL resulted in a loss of the ATP-assisted refolding reaction as well as the CTP-, CDP-, UTP-, and UDP-assisted refolding reactions (Fig. 3d). Similar results were also obtained for the CEESEC chimera (Fig. 3h). The CCEEC and CEESEC chimeras were incapable of hydrolyzing ATP (supplemental Fig. S2b), which probably underlies the block in the protein refolding reaction cycle.

Recent studies have revealed that EC GroEL undergoes dynamic allosteric transitions by ATP binding and hydrolysis during functional folding cycles (28–30). A high affinity for substrate proteins occurs in the T state, whereas ATP binds in the R state. Following this, the GroEL substrates become ATP-bound substrates (28–30). A high affinity for substrate proteins occurs in the T state, whereas ATP binds in the R state. Following this, the GroEL substrates become ATP-bound substrates (28–30).
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![Diagram showing ribbon structures of GroEL in different states](image)

**FIGURE 5.** Ribbon diagrams of the intermediate domain in various states of EC GroEL. a, T state of the intermediate domain of the crystal structure of GroEL (Protein Data Bank code 1OEL, chain a); b, R state of the intermediate domain of a cryoelectron microscope map of the GroEL D398A mutant-ATP complex (code 2C7E); c, R” state of the intermediate domain of the crystal structure of the GroEL-GroES-ADP complex (code 1AON). Illustrations were obtained using the RasMol program. The 14th strand is colored cyan, whereas the M helix is in green. Leu400 and Arg404 are colored red.

...demonstrates that the Ic region plays a pivotal role in the nucleotide selectivity of the LDH refolding reaction.

A point mutation of either L400Q or R404I in the Ic region of EC GroEL only slightly affected its nucleotide selectivity (Fig. 4, c and d). However, the L400Q/R404I double mutation markedly increased nucleotide sensitivity. Further addition of the A394D mutation to the L400Q/R404I double mutation scarcely influenced the nucleotide selectivity (data not shown), demonstrating that Gln400 and Ile404 contribute cooperatively and mainly to the broad nucleotide selectivity of the CP GroEL1-assisted LDH refolding. This was confirmed by a Q400L/I404R reverse double mutation in CP GroEL1, which reduced its nucleotide selectivity (data not shown).

Leu400 and Arg404 in the T state (Protein Data Bank code 1OEL) (31), R state (code 2C7E) (32), and R” state (code 1AON) (1) of EC GroEL are shown in Fig. 5. Because these two amino acid residues are directed opposite the equatorial domain, they would not be expected to contribute directly to the hydrolysis of nucleotides. A recent study using low frequency normal mode analysis has shown domain movement-triggered ligand binding in EC GroEL, which involves “hot spot” amino acid residues in the elastic network model (33). In that report, both Leu400 and Arg404 were listed as hot spots. Consistent with this, Leu400 markedly moves in the R → R” transition, whereas Arg404 moves in the T → R and R → R” transitions (Fig. 4). Glu172, another hot spot residue in the Ic region, is located in the vicinity of Arg404 in the T state and was thought likely to form a salt bridge with Arg404. However, the E172N/L400Q/R404I triple mutation failed to increase nucleotide selectivity, excluding the formation of a Glu172–Arg404 salt bridge from the factors defining the nucleotide selectivity. Rather, the broad nucleotide selectivity of CP GroEL1 may be the result of other inter-residue communications such as van der Waals contacts that permit the dynamic movement of GroEL in the protein folding reaction.

In conclusion, we have found for the first time that 1) CP GroEL1 can refold the three denatured enzymes METF, LDH, and AGLU and 2) utilize not only ATP but also other diverse nucleotides when refolding LDH and AGLU without GroES. Construction of chimeras in conjunction with amino acid substitutions has led to the identification of two amino acids in the intermediate domain that play an important role in determining the broad nucleotide selectivity of the GroEL-assisted LDH folding reaction. Although the mechanism by which these residues control nucleotide binding remains to be studied in more detail, this study has provided the novel concept that regions other than the nucleotide-binding site possess a hidden ability to control nucleotide-binding enzymes in selecting as well as hydrolyzing nucleotide species.

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