Amino Acid Specificity of the *Escherichia coli* Chaperone GroEL (Heat Shock Protein 60)*

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The chaperones GroEL/hsp60 are present in all prokaryotes and in mitochondria and chloroplasts of eukaryotic cells. They are involved in protein folding, protein targeting to membranes, protein renaturation, and control of protein-protein interactions. They interact with many polypeptides in an ATP-dependent manner and possess a peptide-dependent ATPase activity. The nature of the structural elements of substrate proteins recognized by GroEL/hsp60 is still unknown. In this study, we show that the GroEL chaperone of *Escherichia coli* interacts with single amino acids. The hydrophobic amino acids Ile, Phe, Val, Leu, and Trp present the strongest interaction with GroEL. While most of these hydrophobic amino acids are β-sheet formers, GroEL interacts also with the α-helix formers Glu, Ala, Gln, and His. The multiple interactions of GroEL with the side chains of hydrophobic and polar amino acids, including the strongest α-helix and β-sheet formers would allow this chaperone to act as an amphiphilic organizer of protein folding.

Chaperones constitute a class of polypeptide-binding proteins which are involved in protein folding, protein targeting to membranes, protein renaturation, and control of protein-protein interactions (reviewed in Refs. 1-4). The two main classes of chaperones (hsp70/DnaK and hsp60/GroEL) bind to segments of completely or partially unfolded polypeptides (4) and possess a peptide-dependent ATPase activity (5, 6). The mechanisms of the binding of substrate proteins to chaperones are not precisely known. It has been suggested that chaperones might recognize unfolded polypeptide chains (7), molten globule conformation (8), secondary structures (7), or hydrophobic sequences (6). In this report, we show that GroEL/hsp60 interacts with single amino acids, and we suggest that amino acid side chains of substrate proteins would represent a simple and important structural element recognized by chaperones. In contrast to DnaK (which interacts with hydrophobic amino acids), GroEL interacts not only with hydrophobic amino acids (Ile, Phe, Leu, Val, and Trp), but also with less hydrophobic and with charged amino acids (Ala, Tyr, Thr, Glu, Gln, His, Lys, Arg, and Pro). The common interaction of DnaK and GroEL with hydrophobic amino acids would allow their interaction with hydrophobic residues which are exposed in unfolded or partially denatured proteins (8, 10, 11). The interaction of GroEL/hsp60 with hydrophobic and hydrophilic amino acids including strong α-helix and β-sheet formers could allow this chaperone to behave as an amphiphilic organizer of protein folding (12).

EXPERIMENTAL PROCEDURES

Purification of GroEL—GroEL was purified as described in (13) from the hyperproducing strain of *Escherichia coli* KY 1603 (R40-1) from Dr. T. Yura Laboratory (Institute for Virus Research, Kyoto University) (14). The purified protein was dialyzed against 50 mM Tris-hydrochloride, pH 7.4, 0.06 M sodium phosphate, pH 7.4, 50 mM KCl, and 5 mM 2-mercaptoethanol.

ATPase Assay—1 μl of purified GroEL (0.12 pmol) in 50 mM Tris-hydrochloride, pH 7.4, 50 mM KCl, 0.06 M sodium phosphate, and 5 mM 2-mercaptoethanol was incubated for 1 h at 20 °C with 1 μl of 100 μM [3H]ATP (1.5 Ci/mmol) containing 300 μM MgCl₂ and 1 μl of amino acid as indicated. The reaction was linear as a function of time, and it was terminated by applying 2 μl of sample to polyethylenimine cellulose thin layer chromatography plates that had been spotted with carrier nucleotide as described (6). Negligible amounts of AMP were produced during the reaction. A relative activity of 1 represents 6 nmol/min/mg of protein.

Materials—ATP disodium salt and α-casein were from Sigma. [3H]ATP was obtained from Amersham and was used at 1.5 Ci/mmol. l-Amino acids were used in solutions adjusted to pH 7.4. All the other products were from Sigma and were reagent grade.

RESULTS

Amino Acid Specificity of GroEL—The dependence of the GroEL ATPase on the concentration of three amino acids (Ile, Glu, and Gln) taken as an example, is shown in Fig. 1A. The hydrophobic amino acid isoleucine stimulates GroEL 3-fold (Kᵣ = 0.2 mM). The polar amino acid glutamate stimulates GroEL 30-fold (Kᵣ = 0.7 mM) while glycine does not affect the ATPase activity of GroEL. α-Casein (a protein which possesses certain properties of partially denatured proteins) stimulates the GroELATPase 3-fold with a Kᵣ of 0.5 mM (not shown). Thus, the stimulation factor of the GroELATPase by amino acids is similar to the stimulation factor of GroEL by α-casein or by other peptides (8), and the Kᵣ of the amino acid stimulation is approximately 500-fold higher than the Kᵣ of the stimulation by α-casein (Mᵣ = 120,000), a protein composed of several hundreds of amino acids. Furthermore, the stimulation of the GroEL ATPase by Ile or Glu is not observed when GroEL is already stimulated by α-casein (Fig. 1B), suggesting that the stimulation of GroEL by amino acids involves the peptide-binding sites of GroEL.

The effects of the 20 amino acids on the ATPase activity of GroEL are summarized in Table I. A first set of amino acids stimulates GroEL with a Kᵣ ~0.2 mM and comprises the hydrophobic amino acids Ile, Phe, Leu, and Trp. A second set of amino acids stimulates GroEL with a higher Kᵣ ~1 mM, and comprises the amino acids Val, Met, Ala, Tyr, Pro, Thr, His, Glu, and Gln. A third set of amino acids inhibits GroEL with a Kᵣ ~1 mM (Lys and Arg), and a fourth set does not significantly affect the ATPase activity of GroEL (Gly, Cys, Ser, Asn, and Asp).

The ability of chaperones to interact with single amino acids suggests that the minimal motif for their interaction with poly

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The GroEL ATPase activity was measured as described in Fig. 1. A relative activity of 1 represents the unstimulated activity of GroEL, which amounts to 6 nmol/min/mg of protein. The stimulation (or inhibition) factors of the GroEL ATPase and the $K_r$ (or $K_i$) are the mean values from three independent experiments. The standard deviations of the ATPase activities and of the $K_r$ (or $K_i$) were, respectively, less than 15 and 35%. Data analysis was made by non-linear regression as described (25).

| Amino acid | GroEL ATPase | $K_r$ or $K_i$ |
|------------|--------------|----------------|
| Ile        | 2.7          | 0.2            |
| Phe        | 2.3          | 0.3            |
| Val        | 2.3          | 0.6            |
| Leu        | 1.9          | 0.3            |
| Trp        | 2.2          | 0.3            |
| Met        | 1.4          | 1.1            |
| Ala        | 2.5          | 0.6            |
| Gly        | 1.0          |                |
| Cys        | 1.0          |                |
| Tyr        | 2.1          | 0.7            |
| Phe        | 1.8          | 1.9            |
| Thr        | 2.7          | 0.4            |
| Ser        | 1.3          | 3.2            |
| His        | 2.1          | 0.8            |
| Glu        | 2.8          | 0.7            |
| Asn        | 1.1          |                |
| Gln        | 2.0          | 0.9            |
| Asp        | 1.0          |                |
| Lys        | 0.5          | 1.0            |
| Arg        | 0.5          | 1.8            |

peptides could consist of a single (or a very few) amino acid side chain. The preferential interaction of chaperones with non-native states of proteins would result from the exposure of a specific subset of amino acids (mainly hydrophobic amino acids) by non-native proteins, or from a greater accessibility of the amino acid side chains in unfolded proteins.

Interaction of GroEL with Arginine and Lysine—Since arginine and lysine produce an inhibition of the GroEL ATPase, in contrast to the other amino acids, their effects on GroEL were further investigated. As shown in Fig. 2, when assayed in the presence of a stimulating amino acid (tyrosine or isoleucine), lysine produces a stimulation of the GroEL ATPase instead of inhibiting it. Similar results were obtained with arginine (not shown). Thus, the ability of arginine and lysine to stimulate GroEL in the presence of other amino acids suggests that they are also implicated in the interaction of GroEL with peptides.

Interaction of GroEL with Amino Acid Derivatives—The interaction of GroEL with amino acid derivatives having their α-amino group blocked by acetylation, and/or their α-carboxylic group blocked by esterification was investigated. As shown in Fig. 3, N-acetyl-L-tyrosine ethyl ester (with amino and carboxylic groups blocked) stimulates the GroEL ATPase in a similar manner to tyrosine (the stimulation factor is similar and the $K_r$ is slightly lower). This suggests that GroEL interacts with the side chain of amino acids, as was expected from the different specificities of GroEL for the 20 amino acids (which differ by their side chains). The decreased interaction of N-acetyl-L-tyrosine and tyrosine ethyl ester with GroEL is probably due to the net negative or positive charge carried by these amino acid derivatives. Similar results were obtained with leucine and tryptophan derivatives (not shown).

Amino Acid Hydrophobicity and GroEL Stimulation—The ability of amino acids to stimulate the GroEL ATPase correlates well with their hydrophobicity (19, 40). The most hydrophobic amino acids Ile, Phe, Val, Leu, and Trp give the strongest stimulation of the GroEL ATPase. As previously discussed for DnaK/hsp70, the specific interaction of chaperones with hydrophobic amino acids (which are buried in native globular proteins and exposed in non-native forms (11)) would allow an interaction of these chaperones with nascent polypeptides or with denatured proteins (8, 10). Such a hydrophobic interaction might also be involved in the interaction of chaperones with the hydrophobic sequences of nascent membrane proteins or with

Fig. 1. Stimulation of the GroEL ATPase by amino acids. 1 pl of purified GroEL (0.12 pmol) in 50 mM Tris-hydrochloride, pH 7.4, 50 mM KCl, 0.06 M sodium phosphate, and 5 mM 2-mercaptoethanol was incubated for 1 h at 20 °C with 1 pl of 100 µM [γ-32P]ATP (1.5 Ci/mmol) containing 300 µM MgCl$_2$ and 1 pl of isoleucine (C), glutamate (x), or glycine (●) at the final concentrations indicated in the abscissa (A), or 1 pl of isoleucine (C), 1 pl of glutamate (x), in the presence of α-casein at a final concentration of 1 µg. Each point represents the mean value of three experiments. A relative activity of 1 represents the unstimulated activity of GroEL, which amounts to 6 nmol/min/mg of protein.

Fig. 2. Effects of Lysine on the GroEL ATPase. 1 pl of purified GroEL (0.12 pmol) in 50 mM Tris-hydrochloride, pH 7.4, 50 mM KCl, 0.06 M sodium phosphate, and 5 mM 2-mercaptoethanol was incubated for 1 h at 20 °C with 1 pl of 100 µM [γ-32P]ATP (1.5 Ci/mmol) containing 300 µM MgCl$_2$ and 1 pl of lysine at the indicated final concentrations, in the presence of 1 pl of water (C), 1 pl of 4 mM Tyr (x), and 1 pl of 2 mM Ile (●). Each point represents the mean value of three experiments. A relative activity of 1 represents the unstimulated activity of GroEL, which amounts to 6 nmol/min/mg of protein.

TABLE 1

| Amino acid | GroEL ATPase | $K_r$ or $K_i$ |
|------------|--------------|----------------|
| Ile        | 2.7          | 0.2            |
| Phe        | 2.3          | 0.3            |
| Val        | 2.3          | 0.6            |
| Leu        | 1.9          | 0.3            |
| Trp        | 2.2          | 0.3            |
| Met        | 1.4          | 1.1            |
| Ala        | 2.5          | 0.6            |
| Gly        | 1.0          |                |
| Cys        | 1.0          |                |
| Tyr        | 2.1          | 0.7            |
| Phe        | 1.8          | 1.9            |
| Thr        | 2.7          | 0.4            |
| Ser        | 1.3          | 3.2            |
| His        | 2.1          | 0.8            |
| Glu        | 2.8          | 0.7            |
| Asn        | 1.1          |                |
| Gln        | 2.0          | 0.9            |
| Asp        | 1.0          |                |
| Lys        | 0.5          | 1.0            |
| Arg        | 0.5          | 1.8            |
signal sequences of exported proteins. The interaction of chaperones with hydrophobic amino acids of substrate proteins might involve either consecutive amino acids (signal sequences, hydrophobic sequences of membrane proteins) or non-consecutive amino acids (favoring the formation of collapsed folding intermediates), thus allowing a great flexibility in their interactions with substrate proteins.

As shown in Fig. 4 and in Table I, GroEL interacts also (although with lower affinity) with less hydrophobic, and with polar or charged amino acids. In particular, Thr, Pro, His, Glu, Arg inhibit GroEL by 50%, with a $K_i$ equal to 1 mM. None of these amino acids (except Thr) had any effect on DnaK. The interaction of GroEL/hsp60 with a wider palette of amino acids than DnaK/hsp70 is reminiscent of a more complex function of GroEL (scaffold for protein folding), than DnaK (antifolding activity) (12).

**DISCUSSION**

Our results suggest that amino acid side chains of substrate proteins would be an important motif recognized by chaperones. While GroEL appears to interact with 15 amino acids, the strongest $\beta$-sheet formers are also hydrophobic amino acids (Val, Ile, Tyr, Trp, Phe, and Thr), and by the $\beta$-turn formers at the top of the figure (Gly, Asn, Pro, Ser, and Asp). The conformational preference of amino acids are taken from Ref. 23. The specificity factor of amino acids for GroEL stimulation is defined in the legend to Fig. 4.

and Gln stimulate GroEL 3-fold with a $K_i$ equal to 1 mM, and Lys and Arg inhibit GroEL by 50%, with a $K_i$ equal to 1 mM. None of these amino acids (except Thr) had any effect on DnaK. The interaction of GroEL/hsp60 with a wider palette of amino acids than DnaK/hsp70 is reminiscent of a more complex function of GroEL (scaffold for protein folding), than DnaK (antifolding activity) (12).

**Implication of Amino Acids in Protein Secondary Structures and GroEL Stimulation**—In Fig. 5, the amino acids have been ranked according to their ability to form secondary structures in proteins (15, 16). The $\alpha$-helix formers interact with GroEL and comprise both hydrophobic amino acids (Leu and Met) and less hydrophobic or polar and charged amino acids (Ala, Glu, Gln, His, Lys, and Arg). Glu and Gln stimulate GroEL despite their lack of hydrophobicity, and in contrast to Asp and Asn, which are involved in the formation of $\beta$-turns.

The $\beta$-sheet formers (Val, Ile, Tyr, Trp, Phe, and Thr) strongly stimulate GroEL. However, this result is confusing since the strongest $\beta$-sheet formers are also hydrophobic amino acids (17). Meanwhile, Tyr and Thr which are moderately hydrophobic interact with GroEL, while being important for the formation of amphiphilic $\beta$-sheets.

The $\beta$-turn forming amino acids do not interact significantly with GroEL (Gly, Asn, Ser, and Asp) except proline which constitutes a unique case among amino acids in the formation of protein structure, and possesses the strongest $\alpha$-helix and $\beta$-sheet breaking properties of all amino acids. Thus, GroEL appears to interact with amino acids that are frequently found in $\alpha$-helices and $\beta$-sheets.
number of different amino acid-binding sites is probably less, since similar amino acids are likely to interact with the same binding site (as in the case of proteases). Even if the number of amino acid-binding sites is reduced to about five on each GroEL protomer, this would make 70 amino acid-binding sites on each GroEL 14-mer, and the same number of possible interactions between the chaperone and its substrate protein, since each 14-mer appears to bind only one molecule of protein (8).

The ability of groEL to recognize hydrophobic residues, which are buried inside native proteins (11, 18), and exposed in their non-native forms would allow the specific interaction of the substrate protein with the hydrophobic and hydrophilic sites of GroEL resulting in a displacement of the protein to the surface of the chaperone.

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