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Identification of the Binding Surface on β -Lactamase for GroEL by Limited Proteolysis and MALDI-Mass Spectrometry[†]

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ABSTRACT: *Escherichia coli* β -lactamase, alone or as a complex with GroEL at 48 °C, was partially digested with trypsin, endoproteinase Glu-C, or thermolysin. Peptides were analyzed by matrix-assisted laser desorption and ionization mass spectrometry and aligned with the known sequence. From the protease cleavage sites which become protected upon binding and those which become newly accessible, a model of the complex is proposed in which the carboxy-terminal helix has melted, two loops form the binding interface and the large β -sheet become partially uncovered by the slight dislocation of other structural elements. This explains how hydrophobic surface on the substrate protein can become accessible while scarcely disrupting the hydrogen bond network of the native structure. An analysis of the GroEL-bound peptides bound after digestion of the β -lactamase showed no obvious sequence motifs, indicating that binding is provided by hydrophobic patches in the three-dimensional structure.

The chaperonin GroEL and its co-chaperonin GroES protect newly synthesized polypeptides from aggregation and assist their folding in the bacterial cytosol in an ATP-dependent reaction (1, 2). GroEL is a homo-oligomeric complex composed of two heptameric rings of 57 kDa subunits, stacked back-to-back. The X-ray crystal structure of free GroEL (3) and in complex with its co-chaperonin GroES (4) reveals that each subunit consists of three distinct domains: equatorial, intermediate, and apical. The equatorial domain, connected to the apical domain by a hingelike intermediate domain, contains the ATP binding site and provides most of the contacts between the subunits within one heptameric ring and all contacts between the two rings. The flexible apical domain forms the opening of the cylinder and exposes hydrophobic residues to the inside of the toroid. This is the location where the substrate proteins are bound, and after the apical domain moves to expose these residues toward the top of the ring, also GroES is bound (4–9).

A central question in the mechanism of the chaperonin regards the structural properties of a polypeptide chain bound to GroEL. Many experiments performed with a variety of substrate proteins and under different conditions of complex formation have led to very different proposals about the nature of the bound states. Thus, it was suggested that nativelike conformations (10–12) and molten globule states (13–15) can be bound to the chaperonin. Furthermore, it was suggested that small proteins can undergo at least transient global unfolding while bound to GroEL (16–18). Until now, the only common feature of all substrate proteins

of GroEL seems to be the presence of hydrophobic patches (19, 20), although the importance of electrostatic interactions has also been discussed (21–25).

In the present work we used TEM-1 β -lactamase as a model substrate in order to define the parts of the protein that are recognized by GroEL and are required to form the complex. Its X-ray structure has been solved at 1.8 Å resolution (26), and its interaction with GroEL has already been extensively characterized (10, 19, 27–29). It was previously shown that a stable reversible complex between native, disulfide-containing β -lactamase and GroEL could only be detected starting from native enzyme at 48 °C (19) and that β -lactamase retains a nativelike conformation upon binding under these conditions (10). At room temperature no complex could be detected when chemically unfolded β -lactamase was allowed to refold in the presence of GroEL (30). However, when the single disulfide bond of β -lactamase was removed by two Cys→Ala mutations, the mutated form was able to bind to GroEL when folding in the presence of GroEL and shown to have no residual exchange-resistant protons. Thus, even for the same protein two different sets of conformations (fully unfolded and nativelike) can be bound, depending on the conditions used to form the complex (29).

In this study, the structural features of wt¹ β -lactamase recognized by GroEL at high temperature have been investigated using a combination of protease digestion, matrix-assisted laser desorption and ionization mass spectrometry (MALDI-MS), and sequence matching (31, 32).

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¹ Abbreviations: (Cys→Ala) β -lactamase, Cys→Ala double mutant β -lactamase in which the disulfide bond has been removed; GdmCl, guanidinium hydrochloride; ESI-MS, electrospray ionization mass spectrometry; MALDI-MS, matrix-assisted laser desorption and ionization mass spectrometry; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse phase-high performance liquid chromatography; wt, wild-type.

This method was already successfully used for mapping antigen epitopes (33, 34), DNA-binding proteins (35), and protein-protein interactions (36, 37). The peptide distributions of the digested β -lactamase, identified by their masses and the known cleavage properties of the proteases by matching to the amino acid sequence of β -lactamase, were used to delineate the accessibility of polypeptide bonds and relate it to the chain flexibility of the enzyme in free form and in the complex with the chaperonin. Consequently, regions of β -lactamase involved in binding with GroEL are identified by comparing the digestion profile of the GroEL-bound β -lactamase with that of the free β -lactamase. This method should not be disturbed by the occurrence of secondary digestion sites (peptide bonds that are originally protected from proteolysis but that become proteolytic targets only after an initial cut elsewhere), as these cuts will also be observed in the free β -lactamase. Regions of the enzyme that are directly involved in the interaction with the apical domains of GroEL, buried within the channel of the chaperonin oligomer or part of a rigid structure, will show protection against protease digestion. In contrast, regions of β -lactamase that are not directly involved in the interaction with the apical domains of GroEL, solvent exposed, and flexible will be less protected and therefore subjected to protease digestion. Furthermore, some regions of β -lactamase might undergo conformational changes only upon binding to GroEL and consequently be accessible to proteolysis only in the bound state. We found both regions of protection against proteolysis upon binding and regions of enhanced proteolysis, and we propose a model of the sequence of events upon partial thermal unfolding and binding to GroEL.

MATERIALS AND METHODS

Reagents

Chemicals used were either analytical or HPLC grade. Sequencing grade trypsin (bovine pancreas) and endoproteinase Glu-C (*Staphylococcus aureus* strain V8) were obtained from Sigma Chemical Co. (St. Louis, MO); thermolysin (from *Bacillus thermoproteolyticus*) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Soybean trypsin inhibitor and the protease inhibitor Pefabloc SC were from Boehringer Mannheim GmbH, and EDTA was from Sigma Chemical Company.

Protein Preparations

Wild-type and (Cys \rightarrow Ala) β -lactamase were produced and purified by methods described elsewhere (10). The chaperonin GroEL was purified as described in Gervasoni and Plückthun (28), except for the use of an additional Reactive Red 120 agarose (type 3000-CL) column (Sigma) to remove the tryptophan-containing contaminating peptides as described elsewhere (38). The purity of the GroEL samples was controlled by Trp-fluorescence and MALDI-MS. Protein concentrations were measured using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) and are always given for the oligomeric state. All measurements in this study were carried out in one of the following two buffers: buffer A consists of 50 mM MOPS, 50 mM KCl, 5 mM MgCl₂, pH 7.2; buffer B is 50 mM ammonium

carbonate, pH 7.5. For experiments with buffer B, the GroEL samples were stored in buffer A and were always freshly dialyzed against buffer B before use, since storage in this buffer for several days at 4 °C leads to GroEL aggregation.

Enzymatic Activity Assay

The β -lactamase enzyme assay was carried out at 25 °C as described previously (39).

Limited Protease Digestion

Limited Protease Digestion Monitored by Enzymatic Activity. To form the complex between thermally destabilized β -lactamase and GroEL in buffer A, the enzyme was incubated in the presence of three molar equivalents of GroEL for 15 min at 42 °C, when the disulfide-lacking mutant (Cys \rightarrow Ala) β -lactamase was used, and at 48 °C, when the wt β -lactamase was used. The final concentration of β -lactamase was 7.8 μ M in both cases. Complex formation was assayed by the disappearance of the β -lactamase enzymatic activity. Three molar equivalents of GroEL with respect to the enzyme were used to ensure quantitative complex formation. The reference samples were treated identically but in the absence of GroEL. Digestion was started by addition of the protease. Various final concentrations of trypsin, endoproteinase Glu-C, or thermolysin, between 0.05 and 0.8 μ M, were tested. The protease concentrations were chosen such that the digestion of the free and of the GroEL-bound β -lactamase was maximized while the digestion of the chaperonin was minimized. Aliquots were taken at different time points within a 30-min period, and inhibitors were added to stop the reaction. Soybean trypsin inhibitor was used at a final concentration of 50 μ M, endoproteinase Glu-C inhibitor Pefabloc SC at a final concentration of 0.8 mM, and the thermolysin inhibitor EDTA at a final concentration of 0.3 mM. To achieve complete complex dissociation and quantitative refolding of undigested β -lactamase the protein samples were first incubated for 1 h at 4 °C in the presence of 7.8 μ M fresh GroEL, since some of the GroEL has also been digested, and 1 mM ATP (incubation of β -lactamase with GroEL at 42 or 48 °C has no measurable effect on the extent of GroEL proteolysis). This ATP step allows a quantitative dissociation of β -lactamase from GroEL and quantitative refolding. Finally, the enzymatic activity of refolded β -lactamase was measured. Two reference samples, with and without GroEL, were treated as described above, but no protease was added, and used as the 100% activity value.

Limited Protease Digestion Monitored by Tricine-SDS-PAGE and Western Blotting. Complex formation and digestion with trypsin, endoproteinase Glu-C, and thermolysin were performed as described above. Reference samples were treated identically, but without the addition of protease. After proteolysis of β -lactamase has been stopped by the addition of the inhibitors (see above), the samples were immediately taken up and boiled in Lämmli buffer and separated by Tricine-SDS-PAGE according to Schägger and von Jagow (40). After electrophoresis, the peptides were either stained with Coomassie Brilliant Blue or transferred onto nitrocellulose membranes in a buffer containing 200 mM glycine, 25 mM Tris, 20% ethanol (v/v) using a semi-

dry blotting apparatus for 30 min at 1.4 mA/cm². The nitrocellulose membranes were blocked with 5% milk powder (w/v) in PBS (phosphate-buffered saline: 100 mM sodium phosphate, 150 mM NaCl, pH 7.2) and then incubated with anti- β -lactamase rabbit serum (27), diluted 1:500 in PBS, during 1 h at 25 °C. Membranes were washed with PBST (PBS containing 0.05% Tween-20 (v/v)) and incubated with anti-rabbit IgG peroxidase conjugate (Sigma), diluted 1:5000, for 30 min. The blots were finally washed with PBST and developed with BM blue POD-substrate (Boehringer Mannheim) according to the manufacturer's instructions.

Limited Protease Digestion Monitored by MALDI-MS. Complex formation was performed as described above, and either buffer A or buffer B was used in the experiment. We used here a constant final concentration of 0.8 μ M for each protease (trypsin, endoproteinase Glu-C, or thermolysin) and adjusted the digestion times in order to minimize the digestion of the chaperonin, but simultaneously allowing substantial β -lactamase proteolysis. These conditions were established by monitoring the proteolysis by Western blotting and β -lactamase enzymatic activity assay (see above). The proteolytic reaction was stopped after 1 min by addition of 10% TFA. The samples were then immediately frozen at -70 °C prior to mass spectrometric analysis where GroEL and wt β -lactamase were analyzed together. SDS-PAGE of β -lactamase in the presence of the proteases showed that under these conditions the proteolysis was completely stopped. When the proteolytic reaction was stopped by the addition of the protease inhibitors instead of TFA, the MALDI mass spectra showed peaks that could be matched neither to β -lactamase nor to GroEL. Therefore, to obtain interpretable mass spectra, protease inhibitors were omitted and the reactions were stopped with TFA.

Assignment of Protease Cleavage Sites

The positions of the protease cleavage sites in the β -lactamase amino acid sequence were identified by considering the molecular masses of the polypeptide fragments detected by MALDI-MS and the specificity of the proteases used. The search of the corresponding fragments in the amino acid sequence of β -lactamase was carried out using the program PAWN (<http://www.mann.embl-heidelberg.de>). The molecular masses of all peptides measured matched the theoretical ones, obtained from the β -lactamase amino acid sequence, within an accuracy of 0.15% or better.

Mass Spectrometry

Mass spectrometric analyses were carried out on a Voyager Elite mass spectrometer (PerSeptive Biosystems, Framingham, MA) using a 25 kV accelerating voltage. The mass spectra were acquired by adding the individual spectra from 32 laser shots. For protein analysis the samples were run in linear mode and for peptide analysis in reflectron mode. The peptide solutions were diluted 1:1 (v/v) with the matrix solution, α -cyano-4-hydroxycinnamic acid (Aldrich, Gillingham, England), 10 mg/mL in 50% CH₃CN in 0.1% TFA, and allowed to air-dry on the sample target before analysis. Adrenocorticotrophic hormone (2465.2 Da, Sigma) and horse heart myoglobin (16952 Da, Sigma) were used as external standards.

RESULTS

To obtain information about accessible protease sites in the GroEL-bound state of β -lactamase, partial enzymatic digestions were performed with three proteases, trypsin (which hydrolyzes peptide bonds at the carboxyl side of Arg and Lys), endoproteinase Glu-C (which in ammonium carbonate preferentially hydrolyzes peptide bonds at the carboxyl side of Glu), and thermolysin (an unspecific protease which hydrolyzes peptide bonds involving the amino group of hydrophobic amino acid residues). The proteolysis conditions were first optimized by following the disappearance of the β -lactamase enzymatic activity and by monitoring the digestion products with tricine-SDS-PAGE (40) which resolves the small digestion products, and by Western blot analysis using polyclonal antibodies against β -lactamase. (Cys \rightarrow Ala) β -lactamase was diluted from GdmCl into a native buffer, which in the absence of GroEL leads to quantitative refolding, but which leads to complex formation in the presence of GroEL. The kinetics of digestion, followed by measuring the disappearance of the enzymatic activity of β -lactamase as a function of the digestion time, are shown in Figure 1a. The refolded, native (Cys \rightarrow Ala) β -lactamase at 25 °C is completely resistant against proteolysis under the conditions used. In contrast, when the refolding of the (Cys \rightarrow Ala) β -lactamase is prevented by GroEL, the GroEL-bound form shows low protection against trypsin treatment. When the GroEL-(Cys \rightarrow Ala) β -lactamase complex was formed starting from thermally destabilized (Cys \rightarrow Ala) β -lactamase at 42 °C (Figure 1b) or from thermally destabilized wt β -lactamase at 48 °C (Figure 1c), the sensitivity against trypsin digestion is again increased by complex formation, compared to free β -lactamase. However, the differences are not as pronounced as when GroEL binds the protein during folding at 25 °C. Similar results were also obtained using endoproteinase Glu-C or thermolysin (data not shown).

The enhanced sensitivity to proteolytic digestion for the GroEL-bound substrate proteins is well documented in the literature (13, 41–44) and is usually interpreted as non-native substrate proteins binding to GroEL. Prior proton exchange experiments indicated a nativelike state being bound for thermally destabilized β -lactamase, and a very unprotected state for (Cys \rightarrow Ala) β -lactamase being trapped in the folding reaction started from a GdmCl unfolded state. In all cases, the GroEL-bound form is more labile against proteolysis (see below), but the relative differences are smaller at high temperatures, when the complexes are formed from thermally destabilized molecules.

The measurement of the disappearance of the enzymatic activity, however, only gives information about the amount of native protein still present. After different digestion periods, aliquots were therefore also analyzed by PAGE and Western blotting. Figure 2a,b shows the digestion pattern of wt β -lactamase alone and in complex with GroEL, respectively. Interestingly, not only the kinetics of β -lactamase enzymatic activity disappearance are different between the free and the GroEL-bound β -lactamase, with full length β -lactamase disappearing faster in the GroEL-bound state (Figure 1), but also the peptide pattern generated by proteolysis is different. Furthermore, the Western blots show that the free enzyme is more extensively proteolyzed than the GroEL-bound protein, where there is an accumulation

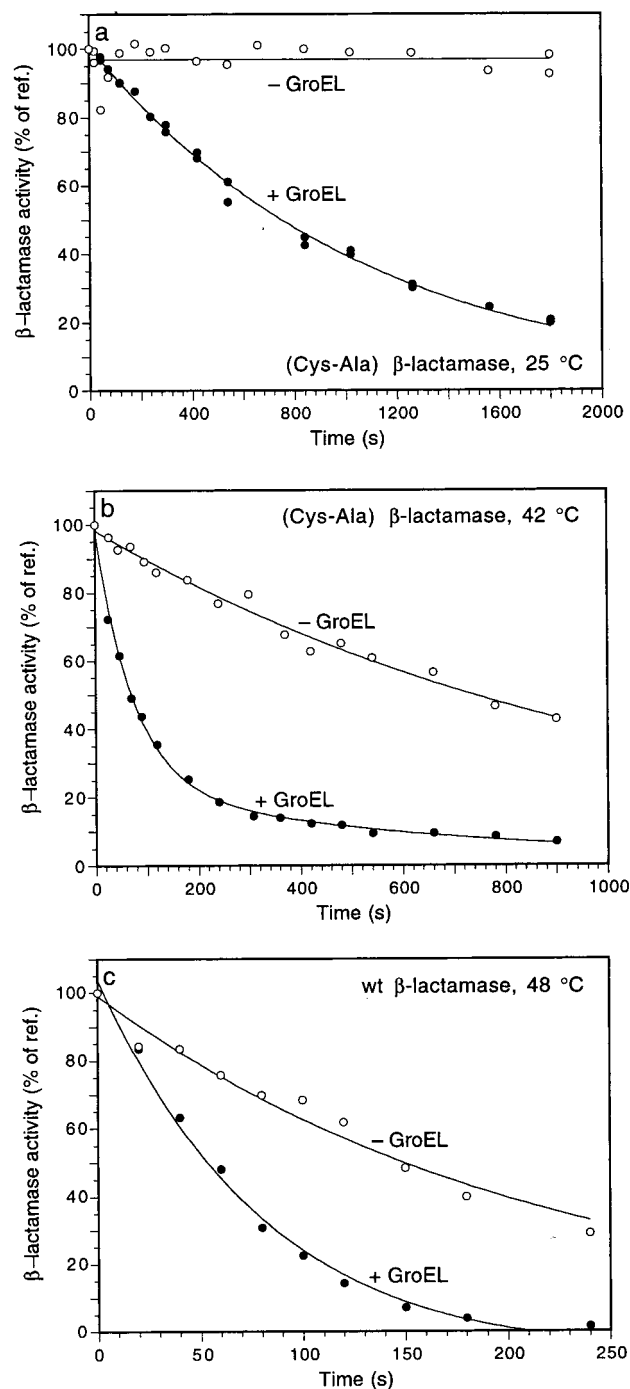


FIGURE 1: The proteolytic digestion of β -lactamase in the presence and absence of GroEL, followed by enzymatic activity. (a) Digestion of (Cys \rightarrow Ala) β -lactamase with trypsin at 25 °C was started 15 min after dilution of the enzyme from the denaturant into buffer A in the absence (○) and in the presence of 10-fold molar excess of GroEL (●). At time 0, trypsin was added to the protein samples, aliquots were withdrawn after different periods of time and treated as described in Materials and Methods to refold remaining enzyme before β -lactamase enzymatic activity was measured. (b) Digestion of (Cys \rightarrow Ala) β -lactamase with trypsin was started 20 min after incubation of native enzyme at 42 °C in the absence (○) and in the presence of three molar equivalents of GroEL (●). (c) Digestion of wt β -lactamase with trypsin was started 20 min after incubation of native enzyme at 48 °C in the absence (○) and in the presence of three molar equivalents of GroEL (●). The final concentration of wt and (Cys \rightarrow Ala) β -lactamase was 7.8 μ M and that of trypsin was 0.17 μ M.

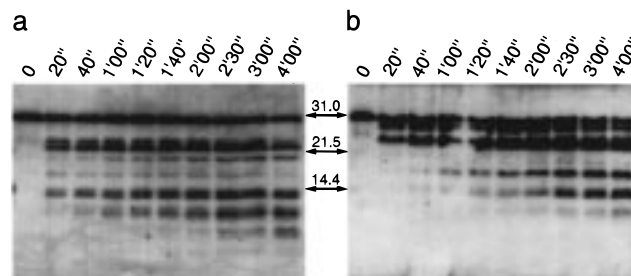


FIGURE 2: Western blot showing peptide fragments of wt β -lactamase derived from partial digestion with trypsin (final concentration was 0.17 μ M) at 48 °C in the absence (a) and in the presence (b) of three molar equivalents of GroEL over wt β -lactamase. The final concentration of β -lactamase was 7.8 μ M. At time 0 almost only full-length wt β -lactamase is seen (molecular mass, 28906.7 Da).

of high molecular mass fragments which appear to be protected against further proteolysis. Once the free β -lactamase is partially proteolyzed, new recognition sites that were previously nonaccessible to the proteases become exposed and the protein can be further digested (Figure 2a). In contrast, the GroEL-bound β -lactamase is sterically protected by the interaction with the GroEL apical domains from a more extensive digestion, thus producing a limited number of peptide fragments (Figure 2b). These differences in the accessible protease sites can be used to obtain information on the structural features of the free and the GroEL-bound β -lactamase.

For analyzing the peptides of wt β -lactamase, obtained from limited protease digestion, by MALDI-MS, a higher purity of the GroEL sample was required. Thus, GroEL purified as described previously (28) was additionally subjected to Reactive Red 120 Agarose chromatography, by which the contaminating peptides, which could make the interpretation of the MALDI mass spectra more difficult, can be completely removed (38).

Limited protease digestion of the free and of the GroEL-bound β -lactamase was first attempted in buffer A, but because of the high salt concentration of this buffer only poor quality MALDI mass spectra were obtained. Ammonium carbonate buffer (buffer B) was therefore used for all of the MALDI-MS measurements. Complex formation between wt β -lactamase and freshly dialyzed GroEL at 48 °C in buffer B (see Materials and Methods) and its reversible dissociation after cooling were monitored and confirmed by β -lactamase enzymatic assay (data not shown). To quantitatively bind wt β -lactamase to GroEL, three molar equivalents of the chaperonin compared to the enzyme were used; this corresponds to a molecular mass ratio GroEL/ β -lactamase of about 80. Nevertheless, the peptides of wt β -lactamase obtained by limited proteolysis could be identified and assigned even in the presence of this large excess of the chaperonin. While some peptides could be identified as coming from the digestion of some GroEL, the surface of the apical domains of the chaperonin that directly interact with β -lactamase and which therefore should show an increased protection against proteolysis, cannot be mapped. It is not possible to distinguish which peptides come from the large amount of uncomplexed GroEL present in the sample and which come from complexed GroEL.

Figure 3 shows the MALDI mass spectra obtained by enzymatic digestion for 1 min at 48 °C of the free (a, c) and

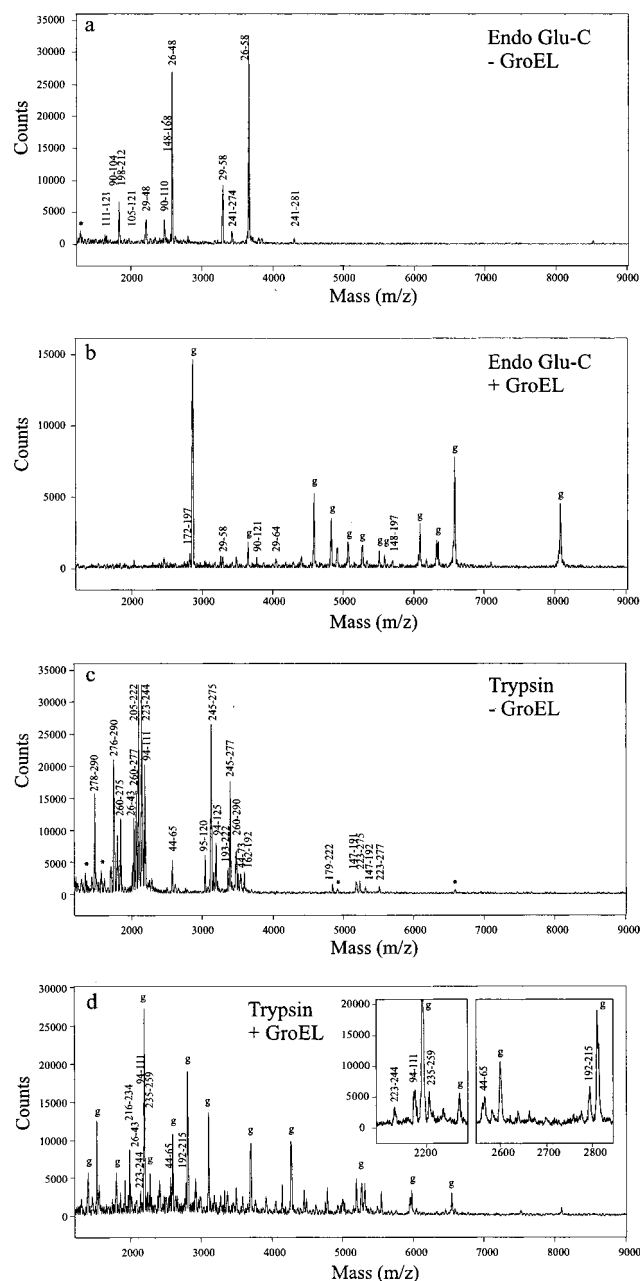


FIGURE 3: MALDI mass spectra of peptide fragments produced by partial enzymatic digestion of wt β -lactamase alone (a, c) and in complex with GroEL (b, d). (a, b) Peptide fragments produced by endoproteinase Glu-C enzymatic digestion. (c, d) Results of an identical experiment using trypsin. The peaks labeled with g refer to digested GroEL, and those labeled with an asterisk refer to unidentified impurities. Ion signals are labeled with residue numbers defining the peptide. The numbering is according to Ambler et al. (45) with the mature protein (263 amino acids) numbered from 26 to 290, with two gaps of 1 amino acid after Gly-238 and after Pro-252.

of the GroEL-bound wt β -lactamase (b, d) with endoproteinase Glu-C and trypsin. Some peptides observed in Figure 3c,d were unequivocally assigned to GroEL, a sign that some GroEL has also been digested. While the percentage of digested GroEL is very small, due to the large amount of the chaperonin present (see above) the digestion of GroEL becomes observable. Peptide bonds of the substrate protein that are originally protected from proteolysis by the interaction with GroEL and become proteolytic targets only after GroEL digestion could be detected and assigned as not

important for the interaction of β -lactamase with GroEL, as these cuts would occur in the control without GroEL as well. This will only decrease the "resolution" in mapping the surface of wt β -lactamase which is involved in the binding to GroEL. To limit this possible "smoothing" effect we used conditions in which the substrate protein was substantially digested but at the same time the proteolysis of GroEL was minimized (see Materials and Methods). Due to the broad specificity of thermolysin it was not possible, in contrast to the other two proteases, to unequivocally match the molecular mass of the peptides obtained within the β -lactamase amino acid sequence.

The three-dimensional structure of TEM-1 β -lactamase (ref 26; PDB code 1bt1) can be described as consisting of two domains which are closely packed together (Figure 4a). The N-terminal or β -domain is made of a five-stranded β -sheet (S1-S5) which is shielded from the solvent on one side by three helices (the amino- and carboxy-terminal α -helices, H1 and H11, respectively, and the short 3_{10} helix H10). The interface between this β -sheet and the three helices is a large hydrophobic core. The second or α -domain is made up of eight helices (H2-H9) and two short two-stranded antiparallel sheets (SB and SC). In this domain Cys-77 and Cys-123 [numbering according to Ambler et al. (45)] form the single disulfide bridge in the wt enzyme. These cysteines have been mutated in the Cys \rightarrow Ala mutant. The substrate binding site is located between these two domains that are connected to each other by two hinge regions, preventing very large conformational changes (26).

Figure 4b,c,d summarizes the results obtained from the analysis of the MALDI mass spectra of Figure 3, and represents the peptide backbone of TEM-1 β -lactamase (26), in which the amino acid residues whose carboxyl groups are proteolytic targets have their side chains shown in a ball-and-stick representation. In Figure 4b, all cleavage sites are shown, occurring either only in the free enzyme, only in the bound form, or in both. A clear correlation exists between sites that are targets for the proteases and sites that are exposed and mobile (Figure 4b, Figure 5). The cleavage sites are randomly distributed on the protein surface and occur mostly on longer stretches of not defined secondary structures. The few cleavage sites that do occur within regular secondary structures can be explained by the high experimental temperature (48 °C) used to perform the limited protease digestion (see below). Figure 4c shows those residues that are accessible to proteolysis only for the free wt β -lactamase, but not when the enzyme is bound to GroEL (labeled F in Figure 5). Similarly, Figure 4d shows the only three residues (labeled B in Figure 5; Glu-171 on the loop connecting the α -helix H7 to H8, Lys-234 at the interface between the two domains, and Lys-215 on the loop connecting α -helix H9 to the 3_{10} -helix H10) that become proteolytic targets only when the enzyme is bound to GroEL, but which are not accessible in the free form—indicating a local but substantial conformational change of wt β -lactamase upon binding to GroEL. These three particular digestion sites cannot be alone responsible for the high molecular mass peptides observed in Figure 2b, since simultaneously to these three sites also other residues are proteolytic targets in the GroEL-bound wt β -lactamase, as shown in Figure 4b.

When Figure 4c is compared with Figure 4d, a region of wt β -lactamase becomes apparent that is protected from

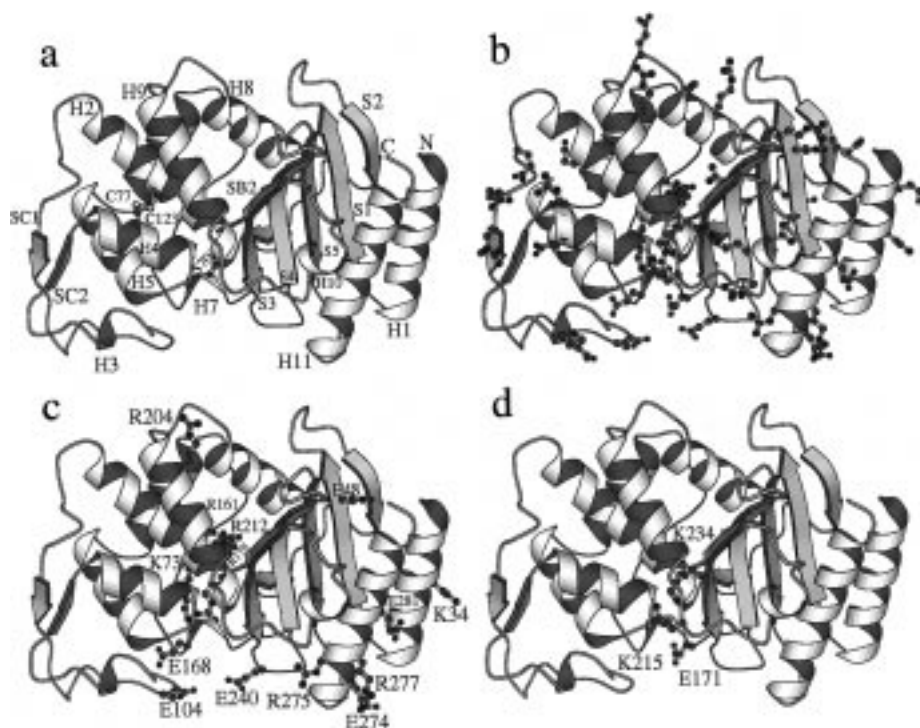


FIGURE 4: (a) Ribbon diagrams of the structure of TEM-1 β -lactamase in which the secondary structure elements are labeled according to Jelsch et al. (26). H denotes helices, S denotes sheets, and SB and SC denote the two small two-stranded antiparallel sheets. The single disulfide bridge between Cys-77 and Cys-123 and the catalytic Ser-70 are shown. (b, c, d) The amino acid residues, whose carboxylamide group are targets for proteolysis, have their side chains shown in a ball-and-stick representation. (b) All the proteolytic targets of the wt β -lactamase either in complex or in free form are shown. Note that this plot contains more sites than (c) and (d) together, since also those sites common to both experiments are shown. (c) Only those amino acid residues that are proteolytic targets in the free wt β -lactamase, but not in the GroEL-bound form, are shown. (d) Only those amino acid residues that are proteolytic targets in the GroEL-bound wt β -lactamase, but not in the free form, are shown. The numbering is according to Ambler et al. (45) with the mature protein numbered from 26 to 290. The figures were prepared with the program MOLSCRIPT (59).

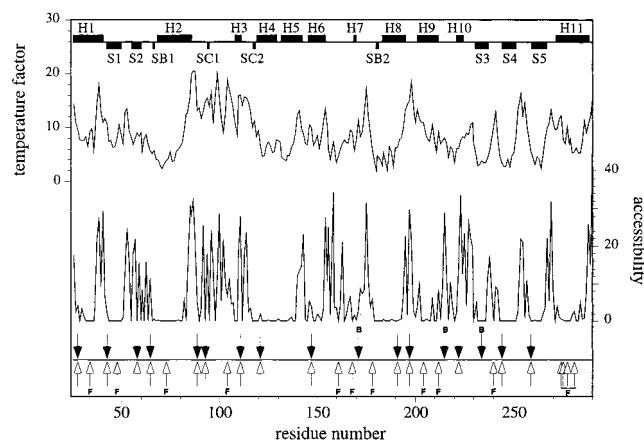


FIGURE 5: Plot of the main-chain accessibility and of the temperature factor (B value) vs the polypeptide chain of the wt β -lactamase. Bars at the top of the figure indicate segments of secondary structure (α -helices and β -strands). The line at the bottom of the figure represents the 263 amino acid residues of β -lactamase [numbering according to Ambler et al. (45) with the mature protein numbered from 26 to 290]. Open arrows indicate sites of limited proteolysis of the free wt β -lactamase, and filled arrows indicate sites of limited proteolysis of wt β -lactamase in complex with GroEL. F, unique cleavage sites of the free wt β -lactamase. B, unique cleavage sites of the GroEL-bound wt β -lactamase.

proteolysis in the complex. This region includes the carboxy-terminal α -helix H11, the loops connecting SC1 to H3 and S3 to S4, and finally a portion of the interface between the two domains. In contrast, the loop connecting the α -helix H9 to the 3_{10} -helix H10 is proteolyzed at Lys-234, only once

wt β -lactamase is bound to GroEL (see above). The α -domain shows the same proteolytic pattern for both free and GroEL-bound wt β -lactamase, arguing against complex formation by strong interaction of this region of β -lactamase with the apical domain of the chaperonin. The slightly faster disappearance of the enzymatic activity during protease treatment of the GroEL-bound wt β -lactamase at 48 °C compared to the free form (Figure 1c) may be explained in the two following ways: The proteolytic reaction at the sites common to both free and GroEL-bound wt β -lactamase is faster in the bound state because the chaperonin stabilizes the non-native form; in this case the GroEL-bound wt β -lactamase, although not native, retains a large amount of native secondary structure as determined by H/D exchange experiments (10). Alternatively, the catalytically important Lys-234 (46), which shows no accessibility and has a low-temperature factor in the native structure (Figure 5), becomes sensitive to protease digestion only upon binding to GroEL (Figure 4d). All these results are summarized in Figure 5, where the accessibility and the mobility (given by the temperature factors or B values) of the main chain of the TEM-1 β -lactamase are both given, obtained from its X-ray structure determined at 1.8 Å resolution (26). As expected, the highest mobility occurs in loops that connect regions of regular secondary structures, and preferential cleavage of the free wt β -lactamase is observed at these sites and at other sites that are exposed.

Even though there is clear evidence for the role of hydrophobic interactions for the binding of a substrate protein

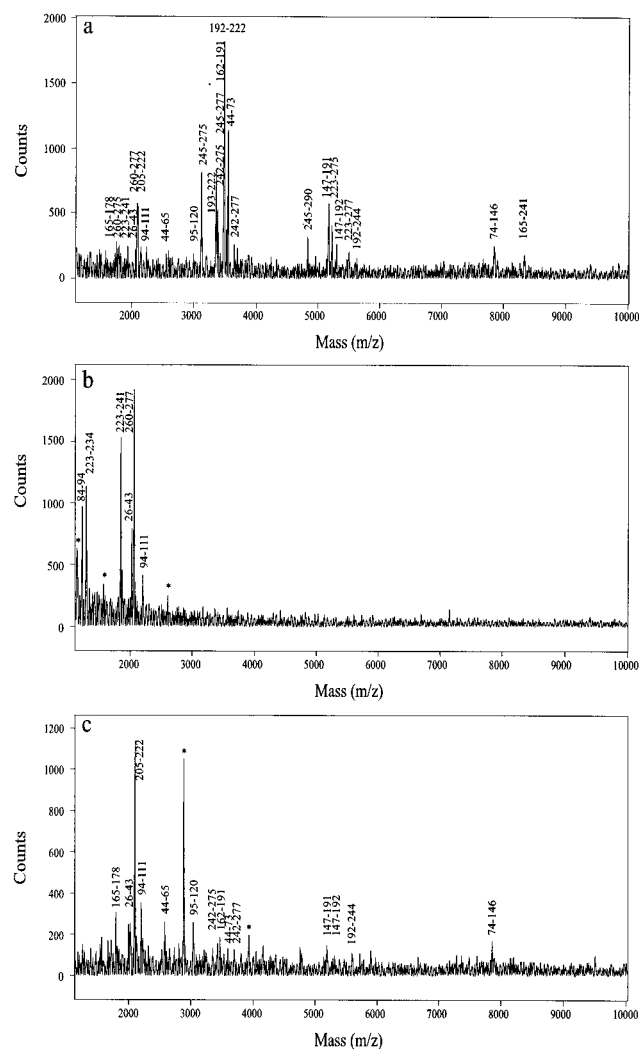


FIGURE 6: (a) MALDI mass spectrum of peptide fragments produced by partial enzymatic digestion of 15 μ M wt β -lactamase with 0.17 μ M trypsin for 6 min at 48 $^{\circ}$ C. The reaction was stopped by addition of trypsin inhibitor at 24 μ M final concentration. (b) MALDI mass spectrum of peptide fragments of wt β -lactamase produced as in (a) that do not bind to GroEL. (c) MALDI mass spectrum of peptide fragments of wt β -lactamase produced as in (a) that do bind to GroEL. Ion signals are labeled with residue numbers. The peaks labeled with an asterisk refer to unidentified impurities. The residue-numbering is according to Ambler et al. (45) with the mature protein numbered from 26 to 290.

to GroEL (7, 19, 20, 25, 43), it is still not clear if a specific hydrophobic amino acid sequence or only the hydrophobic surface properties of a substrate protein are necessary for binding to GroEL. To answer this question the peptide pool, obtained after limited protease digestion of wt β -lactamase with trypsin, was incubated with an excess of GroEL. This step should allow the binding of all the peptides with sufficient affinity to GroEL. The chaperonin was then loaded on a G3000SWXL TSK-gel filtration column to separate the GroEL-bound peptides from those that do not bind with high-enough affinity. The GroEL-containing fractions eluted from the gel filtration, corresponding to the GroEL-bound polypeptide fractions, and all the fractions corresponding to a molecular mass smaller than 29000 Da, containing the free peptides, were pooled. Finally, the peptides in each of the three sets (total, bound, and free) were resolved by MALDI-MS (Figure 6). Not all the peptides detected in the total set

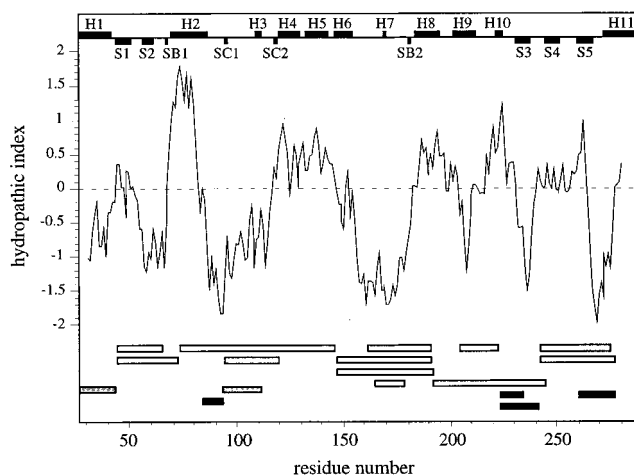


FIGURE 7: Plot of the main-chain hydrophobicity vs the polypeptide chain of the wt β -lactamase. A window of 13 residues was used with the hydrophobic scale of Kyte and Doolittle (50). The bar at the top of the figure indicates segments of secondary structure (α -helices and β -strands). White bars at the bottom represent peptides that bind to GroEL; gray bars, peptides that bind GroEL but with low affinity; black bars, peptides that do not bind to GroEL. The residue-numbering is according to Ambler et al. (45) with the mature protein numbered from 26 to 290.

(Figure 6a) were also detected in the MALDI mass spectra of either the free peptide pool (Figure 6b) or of the GroEL-bound peptides (Figure 6c). Therefore, during the preparation of the samples (gel filtration column) some peptides may have been lost.

From the mass of the peptides and the known specificity of trypsin it was possible to unequivocally identify the amino acid sequence of all the fragments. Only peptides with a minimal length of about 13–14 amino acids residues were bound with sufficiently high affinity to be isolated in the complex with GroEL, an observation reported already previously (22, 30, 43, 47–49). Interestingly, the peptide 26–43 [numbering according to Ambler et al. (45)], which corresponds to the complete amino-terminal amphipathic α -helix, and the peptide 94–111, were detected both in the free and in the bound pool, suggesting a low binding constant to GroEL for these two peptides. Competition experiments performed with a slightly shorter version of the amino-terminal peptide (residues 26–40) had failed to detect any interaction with GroEL (30). Taken together, these results suggest only a minor role of the amino-terminal α -helix in complex formation with GroEL. Figure 7 displays the location of the peptides observed in Figure 6b,c on the β -lactamase sequence and the peptide hydrophobicity, using a window of 13 residues with the hydrophobicity scale of Kyte and Doolittle (50). A strong correlation exists between peptides that do not bind to GroEL or only with low affinity and a negative hydrophobic index. The six highly charged peptides (displayed in gray and black in Figure 7) are too short to contain enough hydrophobic residues to form a stable complex with GroEL. The longer peptides that were found to tightly bind to GroEL (in white in Figure 7) show no correlation with the hydrophobic index. This is not unexpected, because these peptides, with a molecular mass between 2000 and 8000 Da, could assume stable amphipathic structures and thus expose enough hydrophobic patches to be recognized by the chaperonin. However, it is possible that some peptides which are classified as nonbinders to

GroEL from this experiments, might contribute to binding in the intact β -lactamase, being part of a larger assembly.

DISCUSSION

In this study, limited proteolysis analyzed with MALDI-MS was used to investigate the structural features of GroEL-bound wt β -lactamase. This method allows a fast identification of those sites of wt β -lactamase which are accessible to the proteases in the free form and in complex with GroEL, and consequently, the identification of regions involved in the interaction with the chaperonin. The masses and the sequences of the peptides generated by limited proteolysis are directly obtained by a combination of MALDI-MS and sequence matching. In contrast to previous reports (43, 51), the time-consuming peptide purification and N-terminal sequencing are no longer necessary.

We have previously shown that β -lactamase binds GroEL in two clearly different sets of conformations, depending on the conditions used to form the complex (10, 19, 29). Wt β -Lactamase or (Cys \rightarrow Ala) β -lactamase bind to GroEL with a nativelylike conformation—which cannot be unfolded further by the chaperonin—when the complex is formed starting from thermally destabilized enzyme, while (Cys \rightarrow Ala) β -lactamase binds with a conformation lacking any stable secondary structures when the complex is formed starting from GdmCl-unfolded enzyme. The obligatory prerequisite to identify the surface of the enzyme that directly interacts with the GroEL apical domains is that there is a difference in accessibility to proteases between the bound state of β -lactamase and the free state. It is expected that when β -lactamase binds to GroEL in an unfolded conformation, the enzyme will not show significant protection against proteolysis, which would not be very informative. We therefore analyzed only the GroEL-wt β -lactamase complex that was formed under the conditions where the enzyme binds to GroEL with a nativelylike conformation, although it is likely that the substrate protein is bound to GroEL in a dynamic equilibrium involving several conformations.

With this strategy we were able to identify regions of wt β -lactamase that are responsible for the binding to GroEL: in particular the region comprising the carboxy-terminal α -helix H11 and the region located at one face of the β -lactamase structure (at the bottom in Figure 4c), delineated by the residues Glu-104, Arg-161, Glu-240. Complex formation has the interesting consequence that three new sites (Glu-171, Lys-215, Lys-234) become available only in the GroEL-bound state which are located close to the interface (Figure 4d), suggesting that they become uncovered by removing the SC1-H3 loop and the S3-S4 loop and perhaps slightly dislocating the two domains.

These results can be correlated to a high-temperature molecular dynamics unfolding simulation of PC1- β -lactamase from *S. aureus* (52). PC1- β -lactamase, whose structure has very similar topology to the RTEM β -lactamase used in the present study (26, 53), shows a different stability for α -helices and β -sheets against thermal unfolding in the simulation, with the melting of helical motifs preceding that of sheets. Yet, it is important to note that molecular dynamics simulations of unfolding, performed at high temperature (e.g., 600 K), may actually change the unfolding pathway rather than simply accelerate the same process (54).

At the end of the simulation (200 ps at 600 K), the five-stranded antiparallel β -sheet is still fairly stable, compared to the carboxy-terminal α -helix that has almost completely melted (52). Taking the simulations and the experimental results together, we can now propose a structural model of complex formation of β -lactamase with GroEL. At high temperatures, complex formation is possible only after the melting and binding of the carboxy-terminal α -helix H11 to GroEL which becomes protected from proteases as a consequence. These conformational changes will cause part of the large hydrophobic surface of the five β -strands, facing the carboxy-terminal helix, and part of the interface between the two domains to be exposed and recognized by GroEL. Consequently, the peptide bonds that are proteolytic targets in the free β -lactamase become inaccessible and are therefore protected against protease digestion upon binding. Three new sites become exposed upon binding, presumably by being uncovered, when neighboring loops are moved away upon binding to GroEL.

Previous studies performed by Fontana and co-workers (55) have clearly shown that limited proteolysis takes place both at exposed sites and at sites displaying high chain flexibility. Limited proteolysis performed at high temperature (in the present study a temperature of 48 °C was used) can therefore give structural information on the melting process of the proteins analyzed. In the case of the free wt β -lactamase, the numerous cleavage sites within the carboxy-terminal α -helix H11 may indicate from where the melting of the enzyme starts. This helix is a target for protease digestion even though it shows both a relatively low-temperature factor and low accessibility in the native structure (Figure 5). The results are consistent with the molecular dynamics unfolding simulations discussed above (52).

Once wt β -lactamase starts to unfold at high temperature, hydrophobic patches become exposed which can give rise to irreversible aggregation (19). In the presence of GroEL, however, such hydrophobic patches are quickly recognized, and presumably bind to the hydrophobic patches in the apical domain which face the central cavity, and a stable reversible complex is formed, thus forestalling aggregation. If enough hydrophobic surface is exposed during thermal destabilization prior to global unfolding, then the substrate protein will bind to GroEL in a conformation that still has native secondary structure elements. Indeed, we could show that thermally destabilized wt β -lactamase at 48 °C binds to GroEL in a nativelylike conformation (10). Under the same conditions, cyclophilin is bound in a conformation in which all protons can exchange (16, 18). Thus, depending on the substrate protein used, the protein engaged in the (mainly hydrophobic) interaction with GroEL may have residual structure of widely different stability and extent. Furthermore, the conformation of a substrate protein bound to GroEL can be very different when the complex is formed starting from a urea, GdmCl, or acid unfolded protein (11, 43, 56), or when the complex is formed at high temperature (29). Depending on the refolding/unfolding conditions used, both kinetic and thermodynamic effects may differently dictate which conformation on the folding/unfolding pathway will be recognized by GroEL.

Hlodan et al. (43) investigated the limited protease digestion of GroEL-bound rhodanese and proposed that both

domains of rhodanese are bound to the chaperonin, but with different affinities: the N-domain might bind with lower affinity and be released from GroEL more rapidly, followed by the more tightly bound C-domain. This sequential release might reduce incorrect folding, and by interacting predominantly with residues that form the domain interface in the native protein, minimize aggregation after release (43). Our results point to a similar, but more detailed structural picture and a mechanism how GroEL may mediate the folding of β -lactamase: due to the unfolding of the carboxy-terminal α -helix, with the consequence of partially exposing the large hydrophobic surface of the five β -strands, wt β -lactamase is recognized by GroEL. The two domains, via the carboxy-terminal α -helix H11 and the loops connecting SC1 to H3 and S3 to S4, can interact with the apical domain of the chaperonin without the necessity of undergoing further global unfolding. The energy of binding will primarily result from the large hydrophobic contribution which results from the accessibility of the large hydrophobic surfaces for binding, such as the five β -strands.

When this work was in preparation, Coyle et al. (57) reported a sequence alignment of nine peptides known to bind to GroEL. No sequence consensus could be identified, but a clear preference for hydrophobic and to some extent also for basic amino acids was demonstrated. As expected, in the present work no recognition binding motif could be obtained from the analysis of all the sequences of the GroEL-bound peptides identified (Figure 6). These results are in agreement with previous studies with a wide variety of substrate-proteins (19–23, 25, 30, 49) which suggest that binding of a substrate to GroEL may not be primarily determined by specific residues or sequence motifs, but by the global surface properties—mainly hydrophobic, but also charged and polar—presented to GroEL.

In conclusion, by using limited proteolysis analyzed by MALDI-MS, we were able to identify those sites of wt β -lactamase responsible for forming a stable complex with GroEL. This method allows a relatively simple and rapid identification of the structural elements where thermal unfolding may begin. A comparison with data obtained from molecular dynamics on unfolding simulations identified the same regions. Taken together, these results suggest a recognition mechanism by which the chaperonin GroEL can prevent β -lactamase aggregation during heat shock. It will now be interesting to investigate whether the folding and unfolding pathway is identical, and whether the identified nativelike structures of the GroEL-bound enzyme are also the conformations assumed by the substrate shortly before release.

After this work was submitted, Torella et al. (58) using a similar approach (limited protease digestion and peptide-detection by RP-HPLC and ESI-MS), described the conformation of mitochondrial aspartate aminotransferase (mAAT) trapped by GroEL. The authors proposed a model in which the compact C-terminal part of mAAT is located in the central cavity of GroEL, and therefore protected from proteolysis, and with the N-terminal part exposed to the surface of the chaperonin. Furthermore, GroEL seems to stabilize the bound mAAT, which may bind with a conformation with considerable amounts of secondary structure and limited conformational flexibility, and no further unfolding or folding of the enzyme by GroEL was detected. In

conclusion, heat-destabilized wt β -lactamase and mAAT seem to bind to GroEL in states with similar characteristics.

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