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# Thermodynamic Partitioning Model for Hydrophobic Binding of Polypeptides by GroEL

## I. GroEL Recognizes the Signal Sequences of β-lactamase Precursor

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From equilibrium measurements with urea we found a three-state thermodynamic and kinetic folding behavior for the precursor and mature form of *Escherichia coli* β-lactamase TEM2. The thermodynamic intermediate H of *Escherichia coli* β-lactamase and its precursor had no enzymatic activity, and a quenched tryptophan fluorescence intensity, but a native-like wavelength of maximum intensity. State H of mature β-lactamase was 8·7 kcal mol$^{-1}$ less stable than the native state N and about 4·2 kcal mol$^{-1}$ more stable than the unfolded state U, extrapolated to absence of urea. In contrast, state H of precursor β-lactamase was even more stable than N by about 0·5 kcal mol$^{-1}$ and about 6·9 kcal mol$^{-1}$ more stable than U. Native pre-β-lactamase could be stabilized by lowering the pH value from 7·0 to 5·5, probably by protonating a histidine residue leading to an improved solubility of the signal sequence.

Synthetic peptides, containing 23 or 38 N-terminal amino acid residues of pre-β-lactamase, were unable to compete with pre-β-lactamase for binding to GroEL. However, GroEL prevented the inactivation of mature β-lactamase by p38, consistent with competition between GroEL and mature β-lactamase for binding to p38. The equilibrium constant for dissociation $K_D$ of the complex between GroEL and p23, a peptide containing exclusively the signal sequence of pre-β-lactamase, was measured with the BIAcore™ instrument to be in the range $10^{-7}$ to $10^{-8}$ M. Our results are consistent with co-operative binding of GroEL to the mature part and to the signal sequence of pre-β-lactamase. We suggest a thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL.

*Keywords:* protein folding; molecular chaperone; molten globule; peptide binding; BIAcore™

### 1. Introduction

Protein folding *in vivo* is facilitated by the recently discovered molecular chaperone proteins (Gething & Sambrook, 1992; Ellis, 1993; Jaenicke, 1993). These proteins are involved in the transport, folding and assembly of other proteins, but are not components of the final structure of the substrate proteins (Ellis, 1987). The main action of molecular chaperones seems to be to prevent irreversible aggregation reactions of proteins, which are not in their native state. The precise mechanism, by which a protein is recognized as non-native, and of the folding reactions on the chaperones, have remained largely obscure.

One of the best studied molecular chaperones is Cpn60, whose *Escherichia coli* variant is termed GroEL. It is a cylinder-shaped 14-mer of 60 kDa subunits, which are arranged in two stacked rings with 7-fold rotational symmetry (Hendrix, 1979; Hohn *et al.*, 1979; Hemmingsen *et al.*, 1988). The molecule shows a high plasticity and, upon loss of its symmetry, can adapt to a crystal lattice (Zahn *et al.*, 1993). There are many structurally unrelated *in vitro* systems with which the general action of GroEL has been investigated, for example ribulose-1,5-biphosphate carboxylase (Goloubinoff *et al.*, 1989; Viitanen *et al.*, 1990; van der Vies *et al.*, 1992), β-lactamase

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precursor (Laminet *et al.*, 1990; Zahn & Plückthun, 1992), citrate synthase (Buchner *et al.*, 1991; Zhi *et al.*, 1992), lactate dehydrogenase (Badcoe *et al.*, 1991), rhodanese (Martin *et al.*, 1991; Mendoza *et al.*, 1991, 1992), dihydrofolate reductase (Martin *et al.*, 1991; Viitanen *et al.*, 1991), or barnase (Gray & Fersht, 1993; Gray *et al.*, 1993). GroEL binds to one or two substrate proteins, most probably in its central cavity (Langer *et al.*, 1992; Braig *et al.*, 1993). Upon binding to GroEL the exchange of all protected amide protons of cyclophilin is accelerated at least $10^4$-fold, indicating that GroEL binds to a globally destabilized substrate protein, and no secondary structure is conserved in the complex (Zahn *et al.*, 1994). The transient breaking of all elements of secondary structure allows a selection for the most stable interactions within the substrate proteins and thus a pathway to the native state independent of the final topology of the substrate protein (Zahn *et al.*, 1994).

In the presence of ATP (Viitanen *et al.*, 1990; Gray & Fersht, 1991; Bochkareva *et al.*, 1992; Jackson *et al.*, 1993), the substrate protein is released in a state that has largely lost the ability to aggregate (Buchner *et al.*, 1991; Zahn & Plückthun, 1992). As a cofactor for release of some substrate proteins (Goloubinoff *et al.*, 1989; Lissin *et al.*, 1990; Buchner *et al.*, 1991; Martin *et al.*, 1991; Mendoza *et al.*, 1991) GroEL needs GroES, which is a ring shaped heptamer of 10 kDa subunits (Chandrasekhar *et al.*, 1986). Both GroEL and GroES are essential *in vivo* (Fayet *et al.*, 1989).

This paper is the first of two papers on the interaction between the molecular chaperone GroEL and the mature form of *Escherichia coli* β-lactamase TEM2. We believe this to be a particularly attractive model system since the differences in interaction between the precursor form (pre-β-lactamase) and the mature form of β-lactamase with GroEL may allow the delineation of the decisive features of substrate recognition by GroEL. An enzymatically inactive state (X) of pre-β-lactamase has been previously shown to bind to GroEL when added to the chaperone in the urea denatured (U) or in the native (N) form (Laminet *et al.*, 1990). While the binding of the denatured state appears to be instantaneous, the binding of the native protein is extremely slow, suggesting that it needs to unfold first. One can thus set up a reversible system, described by an overall equilibrium describing folding and binding, where we formally refer to each independent binding site as $EL_B$, so that the following scheme is independent of there being one or more of them:

$$EL_BX \rightleftharpoons EL_B + N. \qquad \text{Scheme (I)}$$

After release of pre-β-lactamase from GroEL in the presence of ATP and GroES, there was no difference in the kinetics and equilibrium of pre-β-lactamase folding compared with the reaction from urea, but a decrease in the tendency to aggregate (Zahn & Plückthun, 1992). This is consistent with the binding of GroEL to a non-native state of pre-β-lactamase and a partial folding of the substrate protein in the complex before the final productive ATP-mediated release occurs.

Here, we compare the urea equilibrium and kinetic folding of pre-β-lactamase and mature β-lactamase, and their binding to GroEL at constant temperature (25°C). We show that the reason for the higher affinity of GroEL for pre-β-lactamase, in comparison with mature β-lactamase, lies in the relatively low thermodynamic stability of pre-β-lactamase and a direct recognition of the hydrophobic signal sequence by GroEL. In the accompanying paper (Zahn & Plückthun, 1994) we show that GroEL does bind with high affinity to thermally unfolded mature β-lactamase. The reason for the recognition of thermally unfolded enzyme by GroEL, as opposed to urea-unfolded protein at low temperatures, is both the destabilization of the native form of mature β-lactamase at high temperature and the increase in hydrophobic interaction at higher temperatures. From our results we suggest a thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL.

## 2. Materials and Methods

### (a) *Expression and purification of proteins*

Pre-β-lactamase and mature β-lactamase were purified as described previously (Laminet & Plückthun, 1989). GroEL and GroES were purified from French press lysates of cells harboring the multi-copy plasmid pOF39 (Fayet *et al.*, 1989), as described previously (Viitanen *et al.*, 1990). The main steps in this procedure were DEAE-Sephacel chromatography (Pharmacia), followed by gel filtration on a Sephacryl S-300 column (Pharmacia). The protein concentration was determined by quantitative amino acid analysis, and the concentration for GroEL is always given for the 14-subunit oligomeric form, while the concentration of GroES is given for the 7-subunit oligomeric form.

### (b) *Urea folding experiments*

Urea folding of pre-β-lactamase and mature β-lactamase was carried out at 25°C in a folding buffer containing 100 mM potassium phosphate (pH 7·0), 100 mM urea, 100 mM ammonium sulfate, 0·01% Tween, 10 mM DTT as described previously (Laminet & Plückthun, 1989). Thus the reduced form of pre-β-lactamase and mature β-lactamase is compared in all cases. In one case (see Figure 1a, ■) the solution was buffered with 100 mM Mes (pH 5·5) (with the same additions as above). In the equilibrium experiments, the folding was started either from the urea denatured state (incubation for at least 1 h at 25°C in 8 M urea, 10 mM potassium phosphate (pH 7), 10 mM DTT before refolding) or from native β-lactamase. In the kinetic experiments, the folding reaction was started by diluting the reduced and urea denatured enzyme 1:200 into folding buffer. The folding state of pre-β-lactamase and mature β-lactamase was followed by determining enzymatic activities or spectroscopic properties (see below).

### (c) *Assay of β-lactamase activity*

The enzymatic activity of pre-β-lactamase and mature β-lactamase was assayed spectrophotometrically at 486 nm with the chromogenic substrate nitrocefin at pH 7·0 as described previously (Laminet & Plückthun, 1989). For the assay, 2 to 20 μl of the folding reaction mix were diluted into

1 ml assay solution containing nitrocefin. β-lactamase activity was determined by following the linear increase in absorption for 60 s. The assay temperature was 25°C for pre-β-lactamase and 10°C for mature β-lactamase, at which temperatures further folding during the activity assay (60 s) is slow and insignificant.

(d) *Fluorescence spectroscopy*

Experiments were carried out with a Shimadzu RF-5000 fluorescence spectrometer. The excitation wavelength was 280 nm and the spectral bandwidth was 1·5 nm for excitation and 10 nm for emission. The emission spectra were recorded with a scan speed of 6·7 nm/s and an instrument response of 0·5 s. For the kinetic experiments the solutions (2 ml) were stirred during measurement.

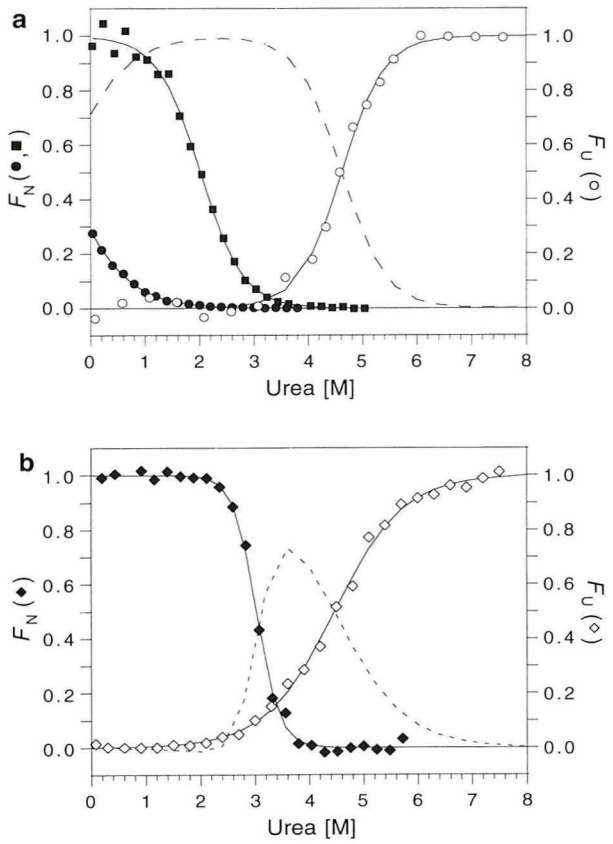


**Figure 1.** Three-state equilibrium folding of precursor and mature β-lactamase. a, Urea equilibrium curves of pre-β-lactamase at 25°C in folding buffer. Folding was started from the 8 M urea state. The enzymatic activity at pH 7·0 (●) or pH 5·5 (■) and the fluorescence maximum at pH 7·0 (○) was measured after 18 h. Data points were fitted to eqn (1) to calculate the fraction of native ($F_N$) and the fraction of unfolded ($F_U$) pre-β-lactamase with eqns (2) and (3), respectively. The enzymatic activity curve at pH 7·0 was scaled as shown, by assuming the same intrinsic specific activity as at pH 5·5 and attributing the lower specific activity observed at 0 M urea at pH 7·0 than at pH 5·5 to a smaller $F_N$. This is justified since the specific activity of mature β-lactamase is the same at pH 5·5 and pH 7·0. The broken line (- - -) indicates the fraction of intermediate pre-β-lactamase ($F_H$) at pH 7·0, calculated from eqn (4). b, Same as in a, but with mature β-lactamase. Enzymatic activity (◆) and fluorescence maximum (◇) were measured at pH 7·0. The broken line (---) indicates $F_H$ of mature β-lactamase.

(e) *Peptide syntheses*

Peptides were synthesized by solid-phase synthesis methodology using a Tentagel (TG) resin (Rapp) on a Milligen 9050 peptide synthesizer or an Ultrasyn C (UsC) resin (Pharmacia) on a Biolynx 4175 peptide synthesizer (LKB) with fluorenylmethoxycarbonyl (FMOC†) chemistry (Atherton & Sheppard, 1987), using benzotriazolyl-*N*-oxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP) or pentafluorophenylester coupling, respectively. The peptides were cleaved from the resin and deprotected with 5% trifluoroacetic acid (TFA) in dichloromethane for 1·5 h at room temperature (RT) followed by treatment with 95% TFA for 1 h at RT (for the TG resin) or with 95% TFA for 1 h at RT (for the UsC resin). The crude peptides were purified by Sephadex G-25 gel filtration (Pharmacia) and by reversed-phase HPLC. Gradients of 0.1% TFA in water (solvent A) against 0·08% TFA in acetonitrile (solvent B) were used. Peptide p23 (see Figure 3) was purified on a HireSil-10 $C_{18}$ column (250 x 20 mm, Chemdata) with a linear gradient from 70%A, 30%B to 60%A, 40%B at a flow rate of 8 ml/min. The longer peptide, p38, was purified on a Nucleosil 300-5 $C_4$ column (250 x 4·6 mm, Macherey-Nagel) with a linear gradient from 75%A, 25%B to 50%A, 50%B at a flow rate of 1 ml/min. Peptide m15 was purified on a Nucleosil 100-5 $C_{18}$ PPN column (250 x 10 mm, Macherey-Nagel), with isocratic elution of 76%A, 24%B at a flow rate of 2 ml/min. The purified peptides were homogeneous as determined by analytical reversed-phase HPLC. The composition and sequence were confirmed by amino acid analysis, gas phase sequencing and fast atom bombardment mass spectroscopy. The lyophilized peptides were dissolved in 8 M urea before addition to the folding buffer.

(f) *Evaluation of folding and binding experiments*

Data points were fitted to appropriate curves with the Macintosh program KaleidaGraph. In equilibrium experiments of pre-β-lactamase and mature β-lactamase, the enzymatic activity or the fluorescence maximum ($y$) were measured as a function of the denaturant concentration [D]. From these data, the free energy change $\Delta G$ in the absence of denaturant $\Delta G(H_2O)$ and the dependence of $\Delta G$ on the denaturant concentration $m$ was determined from a six-parameter fit ($y_f$, $y_u$, $m_f$, $m_u$, $\Delta G(H_2O)$, $m$) using eqn (1), as described by Pace (1990):

$$y = \frac{(y_f + m_f[D]) + (y_u + m_u[D])\, e^{-\{\Delta G(H_2O) - m[D]\}/RT}}{1 + e^{-\{\Delta G(H_2O) - m[D]\}/RT}} \quad (1)$$

where $m_f$ and $y_f$, and $m_u$ and $y_u$ are the slope and the intercept of the pre- and post-transition baselines of the denaturation curve, respectively; $T$ is the absolute temperature; $R$ is the gas constant. The fraction of native β-lactamase $F_N$ was determined from enzymatic activity, while the fraction of completely unfolded β-lactamase $F_U$ was obtained from fluorescence maximum measurements,

† Abbreviations used: FMOC, fluorenylmethoxycarbonyl; PyBOP, benzotriazolyl-*N*-oxytripyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; RT, room temperature; EDC, *N*-ethyl-*N*'-[(3-di-methylamino)propyl)-carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; SPR, surface plasmon resonance; IFC, integrated fluidic cartridge; RBP, ribose-binding protein; MBP, maltose-binding protein.

using eqns (2) and (3). The fraction of intermediate β-lactamase $F_H$ was calculated from eqn (4):

$$F_N = \frac{y - (y_u + m_u[D])}{(y_f + m_f[D]) - (y_u + m_u[D])} \quad (2)$$

$$F_U = \frac{(y_f + m_f[D]) - y}{(y_f + m_f[D]) - (y_u + m_u[D])} \quad (3)$$

$$F_H = 1 - F_N - F_U. \quad (4)$$

The free energy change, $\Delta G$, is assumed to be a linear function of the denaturant concentration, [D], as described by eqn [5]:

$$\Delta G = \Delta G(H_2O) - m[D]. \quad (5)$$

The parameter $D_{1/2}$ is defined as the [D] at the midpoint of the unfolding transition and can be calculated from eqn (5) by setting $\Delta G = 0$.

In the kinetic folding experiments, the enzymatic activity and the fluorescence intensity ($y$) were measured as a function of time $t$. From these data, the overall folding rate constant $k_f$ was determined from a three-parameter fit ($y_\infty$, $y_0$, $k_f$) using eqn (6), according to first-order kinetics:

$$y = y_\infty + (y_0 - y_\infty)\, e^{-k_f \cdot t}, \quad (6)$$

where $y_0$ and $y_\infty$ are the starting value and the end value of the observable used to follow the kinetics, respectively.

Apparently biphasic folding kinetics were fitted with a four-parameter fit ($y_0^I$, $k_f^I$, $y_0^{II}$, $y_f^{II}$) using eqn (7):

$$y = y_\infty + (y_0^I - y_\infty)\, e^{-k_f^I t} + (y_0^{II} - y_\infty)\, e^{-k_f^{II} t}, \quad (7)$$

where $y_0^I$ and $k_f^I$, and $y_0^{II}$ and $k_f^{II}$ are the starting value and the rate constant of the fast and slow phase of the folding kinetics, respectively.

In the titration experiments of pre-β-lactamase or mature β-lactamase with peptide, the concentration of remaining free β-lactamase [N] was measured by enzymatic activity as a function of total peptide concentration [P]. Under the assumption that every β-lactamase-molecule S has a defined number $B$ of "binding pockets" $S_B$, which bind independently to the peptide P, the apparent equilibrium constant for dissociation $K_D'$ was determined from a one-parameter fit using eqn (9), which was derived from the mass law (eqn (8)):

$$K_D' = \frac{([S_B] - [PX_B])([P] - [PX_B])}{[PX_B]} \quad (8)$$

$$[N] = [S] - \frac{B[S] + K_D' + [P] - \sqrt{-4B[S][P] + (-B[S] - K_D' - [P])^2}}{2B}, \quad (9)$$

where [S] is the total concentration of β-lactamase; [N] is the concentration of free, enzymatically active β-lactamase; $[PX_B]$ is the concentration of peptide-bound binding pockets of enzymatically inactive β-lactamase $X_B$; $[S_B]$ is the total concentration of β-lactamase-binding pockets ($=B[S]$); [P] is the total concentration of peptide; $K_D'$ is the apparent dissociation constant, which contains both the dissociation constant of the complex and the folding equilibrium of β-lactamase.

### (g) *BIAcore™: immobilization of peptide*

The immobilization of peptide p23 on the dextran matrix of the sensor ship (CM 5) was performed at a flow of 5 μl/min in HBS (10 mM Hepes (pH 7·4), 0·15 M NaCl, 3·4 mM EDTA, 0·05% Tween 20). The carboxylated dextran matrix was activated with 35 μl of a mixture of *N*-ethyl-*N*'-((3-di-methylamino)propyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). Peptide (50 μl) was injected at a concentration of 2 mg/ml in 40% dimethylsulfoxide (DMSO), 200 mM borate (pH 8·9). Unreacted groups were blocked by subsequent injection of 35 μl 1 M ethanolamine-HCl (pH 8·5).

### (h) *BIAcore™ binding kinetics*

GroEL at concentrations ranging from 10 to 1500 nM in folding buffer was injected onto the sensor surface, where p23 had been immobilized; 21 to 42 μl were used for each injection. The experiments were performed at 25°C with a flow rate of 3 μl/min. The dissociation of the complex was followed for 6 to 8 min, after which the sensor surface was regenerated by injecting 24 to 45 μl of 10 mM MgATP and 0·2 μM GroES in folding buffer.

Data points for association and dissociation of immobilized ligand (peptide) and analyte (GroEL) were evaluated with the BIAlogue™ Kinetics Evaluation Software. The model (Karlsson *et al.*, 1991; Fägerstam *et al.*, 1992) used for analyzing kinetic data assumes that interactions are pseudo-first order reactions with the measured binding rate:

$$\frac{dR}{dt} = k_a B[EL](R_{max} - R) - k_d R, \quad (10)$$

where $dR/dt$ is the rate of formation of surface complexes between immobilized peptide and GroEL in free solution ($R$ = response); $k_a$ is the association rate constant; $B$ is the number of equivalent peptide binding sites per GroEL oligomer; [EL] is the total concentration of GroEL; $R_{max}$ is the maximal response, which should correspond to saturation of all accessible peptides with GroEL; $R$ is the response at time $t$, which should correspond to the number of surface complexes at time $t$; and $k_d$ is the dissociation rate constant. Based on eqn (11):

$$\frac{dR}{dt} = k_a B[EL]R_{max} - (k_a B[EL] + k_d)R, \quad (11)$$

plots of $dR/dt$ *versus* $R$ should be linear, with negative slope $k_s$:

$$k_s = k_a B[EL] + k_d. \quad (12)$$

Data with different GroEL concentrations (from several injections) have therefore been combined and, after plotting $k_s$ *versus* [EL] values, $k_a B$ and $k_d$ can be obtained as the slope and the intercept on the abscissa of this plot, respectively. The dissociation rate constant $k_d$ can be determined more exactly from the dissociation phase (when only buffer is pumped over the sensor chip and [EL] is zero). The derivative of the response curve then reflects the dissociation curve. Integration of eqn (10) with respect to time gives:

$$\ln \frac{R_{t_1}}{R_{t_n}} = k_d(t_n - t_1), \quad (13)$$

where $R_{t_n}$ is the response at time $t_n$ and $R_{t_1}$ is the response at an arbitrary starting time.

## 3. Results

### (a) *Thermodynamics and kinetics of* E. coli *β-lactamase folding*

To compare the thermodynamics and kinetics of pre-β-lactamase and mature β-lactamase folding,

**Table 1**

*Urea equilibrium folding of precursor and mature β-lactamase*

| Start† | pH | β-lactamase (μM) | GroEL‡ (μM) | $\Delta G$ ($H_2O$) (kcal $mol^{-1}$) | $m$§ (kcal $mol^{-1}$ $M^{-1}$) | $D_{1/2}$‖ (M) |
|---|---|---|---|---|---|---|
| A. *N-H transition of pre-β-lactamase* | | | | | | |
| U | 7·0 | 0·13 | — | − 0·5 ± 0·1¶ | 1·1 ± 0·1 | − 0·5 |
| U | 5·5 | 0·13 | — | 3·0 ± 0·4 | 1·5 ± 0·2 | 2·0 |
| B. *H-U transition of pre-β-lactamase* | | | | | | |
| U | 7·0 | 0·26 | — | 6·9 ± 0·9 | 1·5 ± 0·2 | 4·6 |
| C. *N-H transition of mature β-lactamase* | | | | | | |
| U | 7·0 | 0·05 | — | 8·7 ± 0·6 | 2·9 ± 0·2 | 3·0 |
| N | 7·0 | 0·05 | — | 7·4 ± 0·5 | 2·5 ± 0·2 | 3·0 |
| N | 7·0 | 0·05 | 0·1 | 6·4 ± 0·5 | 2·2 ± 0·2 | 3.0 |
| D. *H-U transition of matue β-lactamase* | | | | | | |
| U | 7·0 | 0·26 | — | 4·2 ± 0·4 | 0·9 ± 0·1 | 4·5 |

Conditions as in Materials and Methods (b to d, f). Data are analyzed according to eqns (1) to (5). Standard errors are given.

†Folding was started from protein denatured in 8 M urea (U) or from the native state (N).

‡Concentration of 14-mer.

§Dependence of $\Delta G$ on denaturant concentration.

‖Denaturant concentration at the midpoint of the folding transition.

¶The intercept of the pre-transition baseline ($y_f$) was determined at pH 5.5.

urea equilibrium and kinetic folding experiments were carried out. In the equilibrium experiments, the enzymatic activity and fluorescence maximum of reduced β-lactamase was determined as a function of urea concentration at 25°C and pH 7·0. Folding was started from the denatured state in 8 M urea. For pre-β-lactamase (Figure 1a) and mature β-lactamase (Figure 1b), there were two transitions: one detectable by enzymatic activity and the other detectable by a change in fluorescence maximum, which had a shift in emission wavelength from 342 nm to 352 nm after excitation at 280 nm. Thus, both for pre-β-lactamase and mature β-lactamase we propose the following folding scheme involving three species:

$$U \rightleftharpoons H \rightleftharpoons N, \qquad \text{Scheme (II)}$$

where N is native β-lactamase, H is enzymatically inactive β-lactamase with native-like fluorescence maximum (H stands for intermediate at neutral pH), U is totally unfolded β-lactamase. The native-like fluorescence maximum of H indicates that the tryptophan residues of this state are in a similar non-polar, buried environment as in the native state, N. The quenched fluorescence of H, which was intermediate between N and U (data not shown), might be due to increased mobility of side chains leading to dynamic quenching or reduced energy transfer from tyrosine to tryptophan. Fluorescence quenching with a native-like fluorescence maximum has also been observed for the molten globule state of *Bacillus cereus* β-lactamase 569, detected under acidic (A state) and basic (B state) conditions (Goto & Fink, 1989; Calciano *et al.*, 1993). This β-lactamase contains the same buried tryptophan residues at positions 210 and 229 as *E. coli* β-lactamase TEM2.

There was only a small difference in the midpoint of the H–U transition between pre-β-lactamase (Figure 1a) and mature β-lactamase (Figure 1b). Both proteins had their transition midpoints at about 4·5 M urea (Table 1), while the transition of pre-β-lactamase appears to be more co-operative leading to a $\Delta G_U^H(H_2O)$ of 6·9 kcal $mol^{-1}$ instead of 4·1 kcal $mol^{-1}$. These data are consistent with a similar H–U transition for both enzymes.

When comparing equilibrium curves determined from enzymatic activity at 25°C and pH 7·0, however, there was a much more stable state N for mature β-lactamase (Figure 1b) than for pre-β-lactamase (Figure 1a). While state N of mature β-lactamase was 8·7 kcal $mol^{-1}$ more stable than state H, state N of pre-β-lactamase was 0·5 kcal $mol^{-1}$ less stable than state H (Table 1). Because the enzymatic activity of pre-β-lactamase, in contrast to mature β-lactamase, was found to be strongly pH-dependent between pH 5 and pH 7 (Zahn & Plückthun, 1992), we also measured urea equilibrium folding at pH 5·5. At this pH-value, there was a considerable increase in stability of native pre-β-lactamase, relative to state H (Figure 1a and Table 1) evidenced by a higher absolute enzymatic activity and indicated by a midpoint of transition much closer to the mature form. Under the assumption that at pH 5·5 all pre-β-lactamase molecules were in the native state in the absence of urea, the fraction of native pre-β-lactamase ($F_N$) at pH 7·0 was calculated to be 0·3 at zero denaturant (Figure 1a). A possible reason for the higher stability of native pre-β-lactamase at pH 5·5 may be the protonation of His − 19 (see Figure 3), which leads to better solubility of the hydrophobic signal sequence at lower pH (data not shown) and, as a consequence, to a weaker interaction between the signal sequence and the mature portion of the protein. It thus appears that the signal sequence interacts with state H in a pH dependent manner, thus in effect destabilizing N.

An intermediate state I, with similar properties regarding the enzymatic activity and fluorescence

behavior of state H, was observed during kinetic folding experiments with pre-β-lactamase and mature β-lactamase. When diluting both proteins from 8 M urea to 0·1 M urea, the folding from U to I, as determined by following the change in fluorescence maximum, occurred within the time required for a spectral scan and thus must have a half-life of seconds or smaller. In contrast, the folding from U to N could be followed by measuring β-lactamase activity or fluorescence intensity (Table 2), indicating that the folding from I to N is the rate-determining step:

$$U \longrightarrow I \xrightarrow{k_f} N. \qquad \text{Scheme (III)}$$

The folding kinetics of both mature and precursor enzyme is consistent with a first order reaction (Laminet & Plückthun, 1989) with the folding rate constant of the rate-determining step $k_f$ being higher for mature β-lactamase than for pre-β-lactamase (Table 2). Therefore the signal sequence seems to stabilize state I relative to the transitions state for the folding from I to N. At pH 5·0, where native pre-β-lactamase is more stable than at pH 7·0 (Zahn & Plückthun, 1992), the folding of the precursor protein was biphasic with the faster folding phase still being slower than the monophasic folding of the mature enzyme at pH 7·0 (Table 2). Thus, the low stability of native pre-β-lactamase at pH 7·0 cannot simply be explained by the slower folding rate, rather the signal sequence seems to increase the unfolding rate of the native protein.

At high urea concentrations, there was also a biphasic folding behavior for the mature enzyme (Table 2). One possible reason for multi-phasic folding kinetics is *cis-trans*-isomerization of proline residues (Schmid *et al.*, 1993). However, there are no data yet for or against a kinetically relevant isomerization of the *cis*-proline in position 82 (Herzberg, 1991; Jelsch *et al.*, 1993) during the folding of β-lactamase.

### (b) *Folding of mature β-lactamase in the presence of GroEL*

There is a strong binding of GroEL to pre-β-lactamase at 25°C and between pH 5 and pH 7 (Laminet *et al.*, 1990; Zahn & Plückthun, 1992), which is accompanied by a loss of enzymatic activity (scheme (I)). In contrast, there was no interaction at all between GroEL and the urea denatured or native form of mature β-lactamase under identical conditions, even at 70 molar equivalents of GroEL relative to β-lactamase (data not shown). This confirms and extends the previous experiments at stoichiometric GroEL concentrations (Laminet *et al.*, 1990; Zahn & Plückthun, 1992). There are three potential reasons for the lack of interaction between mature β-lactamase and GroEL: (1) the high stability of the N state against the N–H transition of mature β-lactamase, (2) the fast folding of mature β-lactamase from I to N, or (3) a significant affinity of the signal sequence to GroEL.

To differentiate the reasons for the difference in behavior of pre-β-lactamase and mature β-lactamase concerning their binding to GroEL, urea equilibrium and kinetic folding experiments of mature β-lactamase were carried out in the presence or absence of GroEL. Equilibrium measurements were started from the native form of mature β-lactamase or by refolding the protein from 8 M urea, and both were found to be identical (Figure 2a). Thus, folding is reversible, and a thermodynamic equilibrium can be measured. The same equilibrium curves were obtained with or without two equivalents of GroEL (Figure 2a). GroEL is stable up to at least 2·5 M urea

**Table 2**

*Folding kinetics of precursor and mature β - lactamase*

| pH | $T$ (°C) | β -lactamase (μM) | GroEL† (μM) | GroES‡ (μM) | MgATP (mM) | Urea§ (M) | $k_f$ ($10^{-4}$ $s^{-1}$) |
|---|---|---|---|---|---|---|---|
| A. *Pre-β-lactamase followed by enzymatic activity* | | | | | | | |
| 7·0 | 25 | 0·13 | — | — | — | 0·14 | 6·0 ± 0·6 |
| 7·0 | 25 | 0·26 | — | — | 5 | 0·18 | 4·7 ± 0·3 |
| 7·0 | 25 | 0·26 | 0·26 | — | 5 | 0·18 | 3·1 ± 0·5 |
| 7·0 | 25 | 0·26 | 0·26 | 1·03 | 5 | 0·18 | 4·7 ± 0·7 |
| 5·0 | 25 | 0·13 | — | — | — | 0·14 | 18·6 ± 0·1‖ |
| B. *Mature β-lactamase followed by enzymatic activity* | | | | | | | |
| 7·0 | 25 | 0·13 | — | — | — | 2·50 | 9·0 ± 0·4‖ |
| 7·0 | 25 | 0·13 | 0·07 | — | — | 2·50 | 8·5 ± 0·4‖ |
| 7·0 | 25 | 0·13 | — | — | — | 3·00 | 6·9 ± 0·3‖ |
| 7·0 | 25 | 0·13 | 0·13 | — | — | 3·00 | 7·7 ± 0·4‖ |
| 7·0 | 10 | 0·07 | — | — | — | 0·56 | 6·0 ± 0·5 |
| 7·0 | 10 | 0·07 | 0·26 | — | — | 0·56 | 5·1 ± 0·9 |
| C. *Mature β-lactamase followed by fluorescence intensity* | | | | | | | |
| 7·0 | 25 | 0·13 | — | — | — | 0·14 | 59·5 ± 1·2 |
| 7·0 | 25 | 0·10 | — | — | — | 2·00 | 18·1 ± 0·7 |
| 7·0 | 10 | 0·26 | — | — | — | 0·18 | 8·6 ± 0·2 |

Conditions as in Materials and Methods (b to d, f ). Data analyzed according to eqns (6) and (7). Standard errors are given.

†Concentration of 14-mer.

‡Concentration of 7-mer.

§Final urea concentration in folding assay.

‖Biphasic folding behavior. Given is the rate constant $k_f^1$ of the fast folding phase.

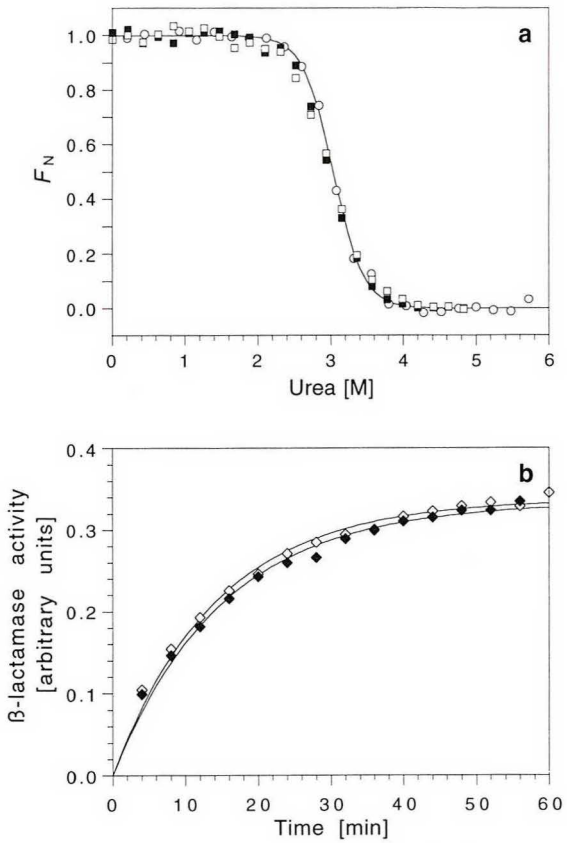


**Figure 2.** Folding of mature β-lactamase in the presence of GroEL. a, Urea equilibrium curves of mature β-lactamase measured by enzymatic activity after 18 h incubation of mature β-lactamase (0·05 μM) in folding buffer (pH 7·0). Folding equilibration was started either from 8 M urea (○) or from the native state in 0 M urea (squares) and in the absence (□) or presence (■) of GroEL (0·1 μM). Data points were fitted to eqn (1) to calculate $F_N$ with eqn (2). b, Folding kinetics of mature β-lactamase (0·65 μM) was followed by measuring enzymatic activity in folding buffer containing 2·5 M urea and in the presence (◆) or absence (◇) of GroEL (0·13 μM). Data were fitted to eqn (7).

at room temperature (data not shown), and there was no significant decrease of $F_N$ in the presence of GroEL at lower urea concentrations. This indicates that there is no interaction between GroEL and mature β-lactamase, either at 2·5 M urea, where H is more populated (although urea would probably weaken the interaction with GroEL), or at very low urea concentrations. In contrast, there is a strong interaction between GroEL and pre-β-lactamase at pH 5·5 and 0·1 M urea (Zahn & Plückthun, 1992), where the N–H transition of pre-β-lactamase has about the same free energy $\Delta G_H^N$ ($\approx$3 kcal $M^{-1}$) as the mature protein at pH 7·0 and 2 M urea (Table 1). The precursor also interacts with GroEL in the presence of 2·5 M urea (data not shown). These experiments suggest that the different thermodynamic stabilities of pre-β-lactamase and mature β-lactamase, caused by the signal sequence, are insufficient to explain the difference in interaction with GroEL, further suggesting that state H of mature β-lactamase is not recognized by GroEL.

When the folding rate of mature β-lactamase (as detectable by measuring enzymatic activity) was slowed either by lowering the temperature (10°C instead of 25°C; Zahn & Plückthun, 1992) or by a high final urea concentration (2·5 M urea) to a folding rate comparable with that of pre-β-lactamase at 25°C, no retardation of the folding reaction in the presence of GroEL could be detected (Figure 2b and Table 2). Thus, there is no evidence for an interaction of state I of mature β-lactamase with GroEL. However, it cannot be distinguished by these experiments whether state U cannot interact with GroEL either for thermodynamic reasons or by a very fast kinetic partitioning to I.

### (c) *Non-competition of peptides with GroEL-bound β-lactamase precursor*

To investigate the direct contribution of the N-terminal signal sequence of pre-β-lactamase for binding to GroEL, we synthesized three peptides (Figure 3). The first peptide (p23) corresponds to the N-terminal 23 amino acid residue signal sequence of pre-β-lactamase, containing mostly hydrophobic amino acid residues and only two residues charged at pH 7·0. For synthetic reasons, there are two changes from the original signal sequence of pre-β-lactamase: Met – 23 was changed to norleucine and Cys –6 was changed to serine. A β-lactamase mutant with this change is known to be fully functional (Laminet *et al.*, 1991). The second peptide (m15) encompasses 15 amino acid residues constituting the N-terminal amphiphatic α-helix of mature β-lactamase with a high ratio of charged to hydrophobic amino acid residues. The third peptide (p38) corresponds to the 38 N-terminal amino acid residues of pre-β-lactamase, containing both the hydrophobic signal sequence at the N-terminus followed by the amphipathic α-helix of mature β-lactamase.

We tested the ability of these peptides to compete with pre-β-lactamase for binding to GroEL. Experiments were carried out with peptide alone or the preincubated mixture of GroEL and peptide, to which pre-β-lactamase denatured with 8 M urea was added. After two hours, during which folding of pre-β-lactamase occurred, enzymatic activity was measured. There was a strong interaction between the signal sequence containing peptides p23 and p38, and pre-β-lactamase, leading to an enzymatically inactive state of the protein (Figure 4a, b). The apparent equilibrium constant for dissociation $K'_D$ was in the range $10^{-7}$ to $10^{-8}$ M, calculated under the assumption that there is a dynamic equilibrium between pre-β-lactamase and the peptides. $K'_D$ is an apparent equilibrium constant, because it contains both the dissociation constant of the complex between β-lactamase and peptide, and the equilibrium constant $K_N^X$ for the folding of β-lactamase from state X to state N. Neither peptide p23 nor p38 could compete with the whole pre-enzyme for GroEL-binding (Figure 4a,b) indicating that pre-β-lactamase

is not simply bound by its signal sequence to the chaperone. The strong interaction between the peptides p23 and p38, and pre-β-lactamase did not allow the use of higher peptide concentrations in the titration experiments. Peptide m15 interacted neither with pre-β-lactamase nor with GroEL, even at 269 equivalents of m15 relative to pre-β-lactamase (Figure 4c).

### (d) *Competition of GroEL with mature β-lactamase for binding to peptide p38*

At high peptide concentrations and in the presence of 2·5 M urea (to destabilize the native protein) there was an inactivation of mature β-lactamase by the peptides containing the signal sequence. We used p38 in the following experiments, because of its higher solubility in comparison with p23. Increasing amounts of p38 were added to either mature β-lactamase alone, or the mixture of β-lactamase and GroEL. The presence of GroEL shifted the titration-curve to higher peptide concentrations (Figure 5a), consistent with the idea that GroEL prevents the inactivation of mature β-lactamase by itself binding to p38 and thereby lowering its free concentration. The titration-experiment at constant concentrations of β-lactamase and p38 with varying GroEL concentrations immediately showed that there is a competition between GroEL and mature β-lactamase for binding to p38 (Figure 5b).

### (e) *Kinetic analysis of the interaction between GroEL and peptide p23 with BIAcore™*

To directly study the interaction between the signal sequence and GroEL, we used the BIAcore™ instrument (Pharmacia Biosensor AB), a biosensor-based analytical system, which allows the kinetic characterization of interactions between biomolecules in real time in a flow system (Jönsson *et al.*, 1991; Malmqvist, 1993). The biosensor uses surface plasmon resonance (SPR) to monitor the binding of biomolecules on the sensor surface (Figure 6).

The detector of BIAcore™ works with polarized light from a light-emitting diode, which is reflected by a gold layer on the sensor chip surface and detected in a diode array. SPR describes a resonance phenomenon between incoming photons and the free electrons in the metal film. Resonance occurs at a specific angle $\theta$ of incident light, at which energy is transferred to the electrons of the gold film causing a decrease of light intensities. The resonance angle $\theta$ changes with changes in the refractive index in the vicinity of the surface, due to binding of molecules to a ligand immobilized on the sensor chip. The immobilization of the ligand takes place in a 100 nm thick dextran matrix composed of 2 to 3% flexible dextran, which is covalently bound to the surface of the gold film and which in turn is in contact with the flow channel of a miniaturized integrated fluidic cartridge (IFC) to deliver reagents (i.e. GroEL) to the sensor chip.

Peptide p23 was immobilized to the sensor chip by coupling the N-terminal amino group of p23 (solubilized in 40% DMSO, 200 mM borate (pH 8·9) to the dextran matrix. The injection of buffer (same as in the folding experiments) into the IFC, containing nanomolar to micromolar concentrations of GroEL, led to an increase of SPR response over time (Figure 7a). This change in refractive index was not observed in either the absence of GroEL, or when no peptide was immobilized on the sensor chip, indicating that the increase in response was a result of the interaction between immobilized peptide and GroEL. However, we obtained the same binding characteristics, when the carboxyl groups of the dextran matrix were not activated during the immobilization procedure. Thus the attachment of p23 to the matrix, and the orientation of the molecules once bound, seems to be dictated more by the hydrophobic properties of p23 than the coupling chemistry used. Further evidence for this came from the continuously decreasing baseline, which indicated the dissociation of the non-covalently bound p23 even when the surface was activated for covalent coupling.

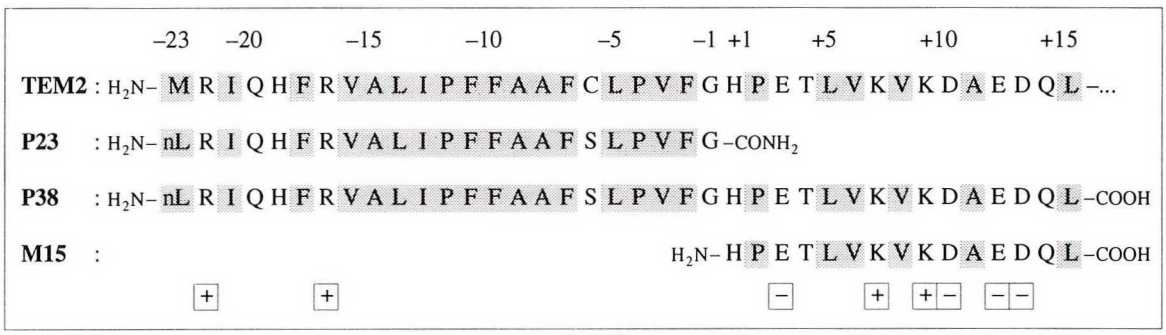


**Figure 3.** N-terminal sequence of precursor β-lactamase and peptides synthesized. The sequence differs in several places from the *E. coli* TEM2 β-lactamase sequence found on many plasmids. The substitutions A –1G and S –22R were introduced for restriction site reasons (Kadonaga *et al.*, 1984) into the protein used in these studies, which is known to be fully functional. C –6S was introduced into the synthetic peptide for synthetic reasons, but it is also known to be fully functional (Laminet *et al.*, 1991). The N-terminal methionine (M) was converted to norleucine (nL) also for synthetic reasons; shaded boxes, hydrophobic amino acids; charged amino acid residues (at pH 7·0) are as indicated.

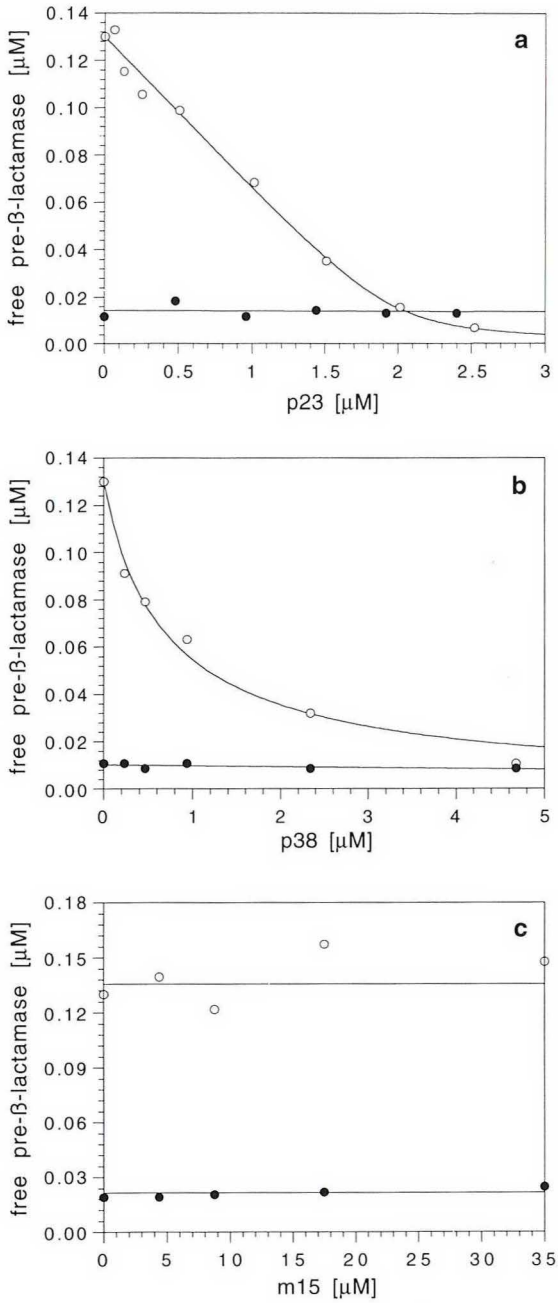


**Figure 4.** Interaction between precursor β-lactamase and peptides a, p23; b, p38; and c, m15 in the absence (○) and presence (●) of GroEL. Pre-β-lactamase (0·13, μM), denatured in 8 M urea was folded in folding buffer containing the indicated total peptide concentrations and either no GroEL (open symbols) or 0·065 μM GroEL (filled symbols). After 2 h, the concentration of free pre-β-lactamase was determined by measuring enzymatic activity. Data points were fitted to eqn (9).

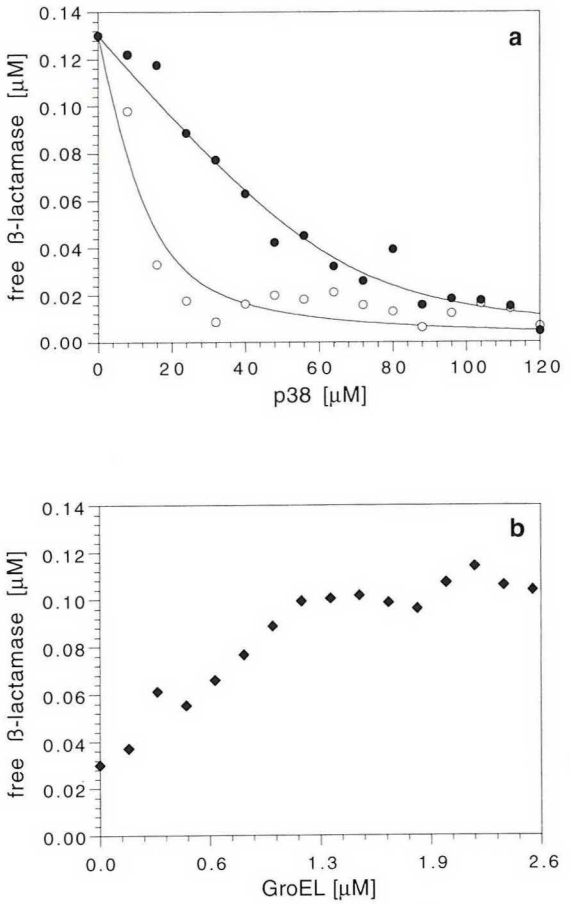


**Figure 5.** Competition between mature β-lactamase and GroEL for binding to peptide p38. a, Titration of mature β-lactamase (0·13 μM) with p38 in the presence (●) or absence (○) of GroEL (1·3 μM). Data points were fitted to eqn (9). b, Titration of mature β-lactamase (0.13 μM) and p38 (26 μM) with GroEL (◆). The folding buffer in a and b contained 2·5 M urea and the enzymatic activity was measured 18 h after addition of β-lactamase to the mixture of GroEL and peptide.

Because this decrease in baseline was very slow, it could be neglected for the kinetic analysis (see below). When, after the association phase, only buffer was injected into the flow cell, there was a continuous decrease in response as a result of a dissociation of GroEL and peptide. The injection of folding buffer containing MgATP and GroES caused the complete removal of GroEL bound to p23, indicating that this peptide behaves like a typical substrate protein, and responds to the factors mediating release of specifically bound substrates.

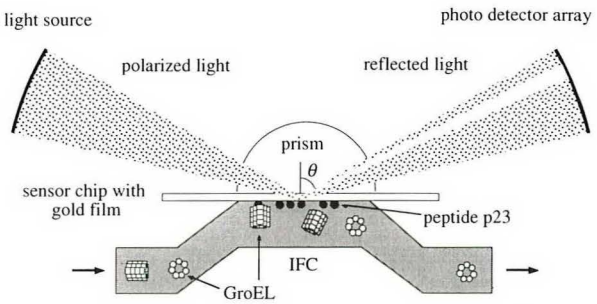


**Figure 6.** The arrangement of the surface plasmon resonance (SPR) detector, the sensor chip and the integrated micro-fluidic cartridge (IFC) in the BIAcore™ system (description in the text), as used for the binding of GroEL to the immobilized peptide p23.

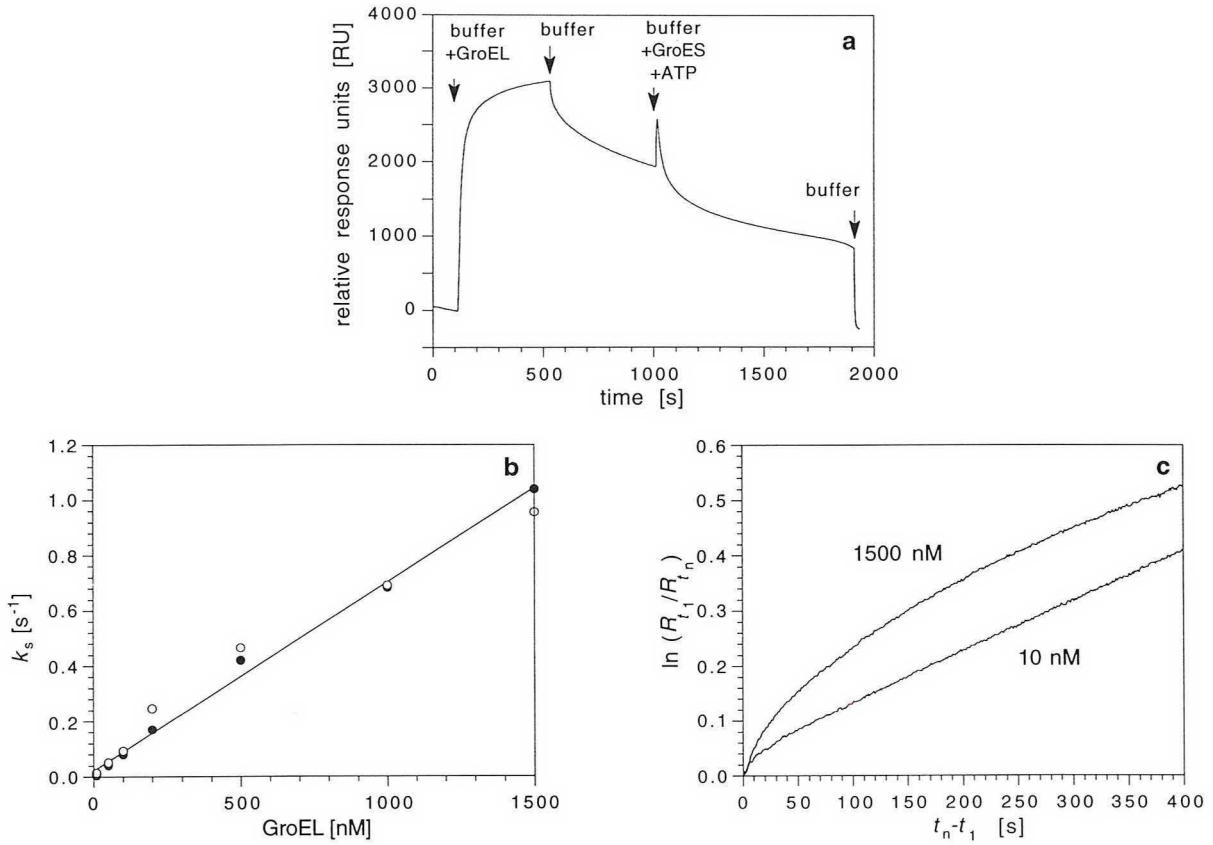


**Figure 7.** Binding of GroEL to peptide p23 measured with BIAcore™. a, Example of a sensorgram with p23 immobilized as a ligand to the sensor surface and GroEL used as the analyte. The refractive index of the sensor surface is given as relative response in resonance units (RU). In the association phase, GroEL was injected at a concentration of 1000 nM through the flow cell of the IFC. In the dissociation phase, only buffer was injected. The sensor chip was regenerated with buffer containing 0·2 μM GroES and 10 mM MgATP. Note that the addition of GroES and MgATP to the folding buffer causes first a jump to a higher response value because of a difference in the bulk refractive index between the buffer solution and the regeneration solution. b, Plot of $k_s$ *versus* the total concentration of GroEL. $k_s$ was determined from the derivative of the association phase $dR/dt$ plotted as a function of the response $R$ (see eqns (10) to (12)). Immobilization of p23 was carried out with (○) or without (●) EDC and NHS mediated activation of the dextran matrix. c, Logarithmic plot of the dissociation phase, where $R_{t_n}$ and $R_{t1}$ are the response at time $t_n$ and at the starting time $t_1$, respectively (see eqn (13)). The GroEL concentration, used for the preceding association phase, was 10 nM (lower curve) or 1500 nM (upper curve).

When repeating this cycle of association, dissociation and regeneration with different GroEL concentrations in the association phase, there was a linear increase in slope ($k_s$) of the $dR/dt$ *versus* $R$ plot with increasing GroEL concentrations (Figure 7b), from which an association rate constant $k_a$ of the order of $10^5$ M$^{-1}$ s$^{-1}$ could be calculated, the exact value of $k_a$ is dependent on the number of peptide binding sites $B$ per GroEL oligomer (Table 3). The logarithmic plot of the dissociation phase showed a biphasic behavior with a short, fast phase at the beginning and a slower second phase (Figure 7c), which could be a result of rebinding of GroEL to p23. If rebinding occurs, it is expected to be more pronounced when low concentrations of GroEL are injected, since this will leave more binding sites unoccupied on the immobilized p23. Thus the five-fold faster dissociation phase with a dissociation rate constant $k_d$

**Table 3**
*BIAcore™: kinetics of GroEL-p23 interaction*

| $B$ † | $k_a$ ($10^5$ M$^{-1}$ s$^{-1}$) | $k_d$ (s)‡ ($10^{-3}$ s$^{-1}$) | $k_d$ (f)‡ ($10^{-3}$ s$^{-1}$) | $K_D$§ ($10^{-8}$ M) |
|---|---|---|---|---|
| 1 | 6·83 ± 0·22 | 1·03 ± 0·01 | 4·63 ± 0·17 | 0·68 |
| 14 | 0·49 ± 0·16 | 1·03 ± 0·01 | 4·63 ± 0·17 | 9·49 |

Conditions as in Material and Methods (g, h). Data analyzed according to eqns (10) to (13). Standard errors are given.

†Number of peptide-binding pockets per GroEL 14-mer.

‡Dissociation rate constant $k_d$ from slow (s) and fast (f) phase of dissociation curve.

§Equilibrium constant for dissociation calculated from $k_a$ and $k_d$ (f).

of $1 \times 10^{-3}$ s$^{-1}$ may be the more relevant number, leading to a $K_D$ of $10^{-7}$ to $10^{-8}$ M, dependent on the number of peptide binding sites $B$ present per GroEL oligomer, which is not known at present (Table 3).

## 4. Discussion

### (a) *Three-state folding of precursor and mature β-lactamase*

The thermodynamic folding behavior of the mature form of *E. coli* β-lactamase TEM2 (Figure 1b) can be described by a three-state equilibrium (scheme (II)). A three-state folding has also been observed for *Staphylococcus aureus* β-lactamase PC1 during equilibrium measurements in guanidinium chloride and urea (Robson & Pain, 1976*a*,*b*; Mitchinson & Pain, 1985). The physical properties of the intermediate state of the *S. aureus* β-lactamase, which is called state H by Pain and co-workers, have been shown to be intermediate in compactness between N and U with a native-like secondary structure but the tyrosine residues exposed to the solvent. This state H fulfils the criteria of a thermodynamic stable molten globule state (Christensen & Pain, 1991). Because of the similar folding behavior and the similarity in sequence and in folding topology (Herzberg, 1991; Jelsch *et al.*, 1993), the intermediate states of *S. aureus* β-lactamase and of *E. coli* β-lactamase seem to have a similar conformation. This state H has apparently no biological activity but a considerable amount of native structure.

The results of the kinetic experiments (Table 2) agree also with the observations of Pain and co-workers, who have demonstrated that a transient intermediate accumulated during folding of *S. aureus* β-lactamase (Ptitsyn *et al.*, 1990; Christensen & Pain, 1991). This state I has been shown to be similar to the equilibrium state H, but was more compact than the latter and has the properties of a kinetic molten globule state. As with the *E. coli* enzyme, the folding from I to N was rate-limiting (scheme (III)). Thus, there is a three-state kinetic and thermodynamic folding of mature β-lactamase of *E. coli* similar to the homologous *S. aureus* enzyme. Ptitsyn *et al.* (1990) suggested that both the H-state and the I-state are on the kinetic folding pathway of β-lactamase:

$$\mathrm{U} \longrightarrow \mathrm{H} \longrightarrow \mathrm{I} \overset{k_f}{\longrightarrow} \mathrm{N} \quad \text{Scheme (IV)}$$

At intermediate urea concentrations there is a biphasic folding behavior for both the *E. coli* and the *S. aureus* β-lactamase (Table 2; Robson & Pain, 1976*b*). Although both enzymes have a *cis*-proline at position 82 (Herzberg, 1991; Jelsch *et al.*, 1993), there is no information yet about a kinetically relevant *cis-trans*-isomerization during folding, which is often the reason for a biphasic folding kinetics (Schmid *et al.*, 1993).

The precursor β-lactamase of *E. coli* has been purified to homogeneity (Laminet & Plückthun, 1989). It could be shown that pre-β-lactamase, as the mature form, has a thermodynamically stable state H (Figure 1a), and a kinetically stable state I (Table 2). Thus the signal sequence has qualitatively no influence on the folding behavior of the β-lactamase. Quantitatively, the signal sequence causes a considerable destabilization of the native protein N relative to state H (Table 1). The physical reason for the destabilization of N is most probably a hydrophobic interaction between the signal sequence and the mature part of the protein, because an additional protonation of His −19 at pH 5·5 results in a better solubility of the signal sequence as well as in a higher stability of N relative to H (Figure 1a). The thermodynamic effect appears to be restricted to the N–H equilibrium, because the signal sequence has only a small influence on the stability of state H relative to the totally unfolded state U (Figure 1).

With regard to the thermodynamic and kinetic properties, pre-β-lactamase differs from the precursor forms of the ribose-binding protein (RBP) and the maltose-binding protein (MBP), whose folding can both be described by a two-state model. While the pre-form of RBP is as stable as the mature protein (Teschke *et al.*, 1991), the precursor form of MBP has a lower free energy for the N–U transition than the mature MBP (Park *et al.*, 1988; Liu *et al.*, 1988). In contrast to pre-β-lactamase, the main reason for the lower stability of N relative to U is not an increased unfolding rate, but a decrease in the folding rate.

### (b) *Non-recognition of urea unfolded mature β-lactamase by GroEL*

Neither when the native form of mature β-lactamase was destabilized by urea to a $\Delta G_H^N(H_2O)$ of 3 kcal mol$^{-1}$, nor when it was not (Laminet *et al.*, 1990; Zahn & Plückthun, 1992), was there kinetic or thermodynamic evidence for an interaction between GroEL and mature β-lactamase under the conditions measured (25 °C and pH 7·0), which would be detectable by enzymatic activity (Figure 2). However, GroEL is able to prevent aggregation of pre-β-lactamase (which occurs in the absence of detergent in the folding buffer; Zahn & Plückthun, 1992) when folding is carried out in the presence of GroEL in up to at least 2·5 M urea (data not shown), indicating that GroEL is stable and does have binding activity at least up to these urea concentrations. This is consistent with the observed binding activity between GroEL and rhodanese in 2·5 M urea (Horovitz *et al.*, 1993). *Vice versa*, when native pre-β-lactamase was stabilized by lower pH to a $\Delta G_H^N(H_2O)$ of 3 kcal mol$^{-1}$, there was still formation of a complex between GroEL and pre-β-lactamase. Thus the lack of binding of mature β-lactamase to GroEL cannot be sufficiently explained simply by the higher stability of the N state with respect to the N–H transition. In addition, there was no evidence of any recognition of the kinetic molten globule state I of mature β-lactamase by GroEL.

### (c) *Recognition of the signal sequence by GroEL*

From the thermodynamic and kinetic folding experiments it is reasonable to propose a significant

contribution of the signal sequence to the binding energy of the GroEL-pre-β-lactamase complex. We tested this by competition and BIAcore™ experiments with synthetic peptides. The competition experiments (Figure 4) show that the signal sequence containing peptides p23 and p38 are unable to compete with pre-β-lactamase for GroEL-binding. However, a similar experiment carried out with mature β-lactamase in 2·5 M urea (Figure 5) demonstrates that p38 binds to GroEL with an estimated $K_D$ in the micromolar range. With BIAcore™ we measured a $K_D$ of the GroEL-p23 complex in the range $10^{-7}$ to $10^{-8}$ M (Figure 7). This low $K_D$ value of the complex demonstrates a significant contribution of the signal sequence to the binding of pre-β-lactamase to the chaperone.

Because of the lack in competition of p23 and p38 with GroEL-bound pre-β-lactamase, there must be additional GroEL-binding sites within the mature portion of pre-β-lactamase, leading to a co-operative binding of the substrate protein to GroEL. This is consistent with GroEL-bound pre-β-lactamase having no enzymatic activity. Since one GroEL-molecule can bind two molecules of β-lactamase (Zahn & Plückthun, 1992), pre-β-lactamase might be bound at up to seven GroEL interaction sites, if every subunit makes a contribution to several hypothetical recognition motifs of the unfolded substrate protein. This co-operative binding of a protein may not be inhibitable, at reasonable concentrations, by peptides which bind to only one such interaction site.

### (d) *Hydrophobic interaction*

Hydrophobic interactions are generally assumed to be one of the main forces in protein folding and stability (Kauzmann, 1954; Dill, 1990). Since most hydrophobic residues are found in the interior of a protein (the protein core), whereas the hydrophilic residues are exposed to the solvent, it seems attractive to suppose that GroEL recognizes an unfolded protein by its exposed hydrophobic residues. Supporting this idea is the finding of Mendoza *et al.* (1991), who suggested by means of bis-anilinonaphthalene-disulfonic acid (bisANS) fluorescence studies that GroEL contains an exposed hydrophobic surface, which is reduced in size in the presence of ATP. Furthermore, the chaperonin can substitute for detergents in preventing aggregation reactions during folding (Laminet *et al.*, 1990). Hydrophobic binding of the substrate has also been suggested from the lack of salt effects on binding (Mendoza *et al.*, 1992).

The experiments carried out in the present study directly show that there is a strong interaction between GroEL and the signal sequence of β-lactamase. One common characteristic of all signal sequences is a stretch of 7 to 15 primarily hydrophobic amino acid residues. In β-lactamase, there are 14 non-polar amino acid residues between residues −16 and −2 (Figure 3). In contrast to the signal sequence containing peptides p23 and p38, there was no measurable binding of GroEL to peptide m15, containing the N-terminal amphiphatic α-helix of mature β-lactamase. This is consistent with the results of Gierasch and co-workers (Landry & Gierasch, 1991; Landry *et al.*, 1992), who measured the interaction between GroEL and the 13 amino acid peptide corresponding to the N-terminal amphiphatic α-helix of rhodanese. From NMR experiments, they estimated a $K_D$ of about $10^{-3}$ to $10^{-5}$ M. According to these results, the pro-sequence of barnase, which consists mainly of polar amino acid residues, causes only a two-fold decrease in the rate constant for the transient binding of barnase to GroEL (Gray *et al.*, 1993). Thus, the much stronger binding of GroEL to the signal sequence suggests a hydrophobic GroEL binding pocket.

Possibly the two positive charges at positions −22 and −17 of the signal sequence also influence the GroEL-binding. This would be consistent with the postulated positively charged GroEL-binding site of granulocyte ribonuclease, containing four arginines out of 18 amino acid residues (Rosenberg *et al.*, 1993). Of the amino acid residues, 50% are apolar, and, as for the signal sequence, there are no negatively charged residues in the sequence. Thus, GroEL may favor polypeptide chains with a high compactness of hydrophobic amino acid residues, and a high ratio of positive to negatively charged side chains. However, the non-specific recognition of solvent exposed hydrophobic residues may be a prerequisite for the observed substrate promiscuity (Viitanen *et al.*, 1992) of this chaperone.

### (e) *Thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL*

It has been shown *in vitro* and *in vivo* that there is no measurable affinity between pre-β-lactamase and the molecular chaperone SecB (Laminet *et al.*, 1991). SecB is a highly charged, soluble and tetrameric *E. coli* protein with a molecular mass of 16·4 kDa per subunit (for a review see Hardy & Randall, 1993). This chaperone maintains some precursor proteins in a transport competent form. Hardy and Randall could show for MBP that both the urea denatured precursor and the mature form of MBP were transiently bound to SecB during refolding (Randall *et al.*, 1990) indicating that any recognition of the signal sequence is not crucial in this case. Furthermore, there was no interaction between SecB and the native form of precursor or mature MBP. They explained their results with a kinetic partitioning model of selective binding of non-native protein by SecB (Hardy & Randall, 1991).

From our results, we suggest that the reason for the lack in interaction between SecB and pre-β-lactamase is the fast folding of pre-β-lactamase from U to I, with I not being recognized by SecB. Alternatively, we cannot exclude that there are linear or three-dimensional motifs in folding intermediates which are recognized by SecB and are absent in pre-β-lactamase. In contrast, GroEL binds to a non-native state of pre-β-lactamase independent of the state in

which the substrate is first presented (Laminet *et al.*, 1990), indicating that an equilibrium is established. This points to a different mechanism of binding for the two molecular chaperones, SecB and GroEL.

From the results of the present investigation we suggest a thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL. Whether a protein is able to interact with GroEL depends on the thermodynamic stability and the hydrophobicity of the substrate protein. There is a thermodynamic partitioning between the stability of a native protein, and the stability of the complex between GroEL and the bound protein. The main criterion for binding may be the solvent exposure of hydrophobic amino acid residues, which are recognized by GroEL and thus screened against irreversible aggregation reactions. The complex between GroEL and substrate is most stable, when a maximal number of hydrophobic residues of the substrate are in contact with a maximal number of GroEL binding sites. As a consequence, the conformation of the substrate protein in the complex with GroEL is suggested to depend on (1) the parameters determining its thermodynamic stability, (2) the distribution of hydrophobic amino acid residues in the primary structure and (3) the molecular weight, the longer the polypeptide chain, the more binding sites for GroEL are available. Depending on the strength of these interactions, association may be possible under all conditions (such as pre-β-lactamase) or only at high temperature (see accompanying paper, Zahn & Plückthun, 1994). Dissociation, once a complex has been formed, may again be spontaneous (by lowering the temperature), require ATP to decrease the hydrophobic surface of GroEL or require both ATP and GroES.

These substrate properties which are recognized by GroEL may explain the unusual molecular design of GroEL. The ring-like arrangement of GroEL-subunits allows a co-operative and concomitant high affinity binding of 7 interaction sites to the unfolded substrate protein. The observed high plasticity of the GroEL-molecule (Zahn *et al.*, 1993) may support the co-operative polypeptide-binding by adjustment of the GroEL subunits or domains to the unfolded polypeptide chain. The cylindrical arrangement of GroEL allows the exposure of hydrophobic binding sites within the central cavity, without the risk of an aggregation of native GroEL itself.

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