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Effect of single point mutations in citrate synthase on binding to GroEL

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Abstract Six single point mutants of yeast citrate synthase were analyzed for binding to the molecular chaperone GroEL. In contrast to the wild-type and G276S, all other G276-mutants were able to displace pre-β-lactamase from GroEL. The off-rate constant for pre-β-lactamase must be at least partially rate-limiting, leading to an equilibrium dissociation constant between 10^{-10} M and 10^{-12}M. Direct evidence for binding of citrate synthase was obtained from gel filtration experiments. The results suggest that thermodynamic rather than structural features of the mutants determine the degree of binding to the chaperone.

Key words: Molecular chaperone; Protein folding; Protein stability; GroEL; Pre-β-lactamase; Citrate synthase

1. Introduction

Molecular chaperones are attracting increasing attention in the field of protein folding [1,2] to study this problem under conditions closer to those in the cell. In vitro folding experiments are usually carried out under optimized conditions, i.e. at low temperature and low protein concentration. However, even under these conditions, folding of most proteins is not fully reversible. In particular, large multi-domain proteins tend to aggregate during folding, leading to off-pathway reactions. The existence of molecular chaperones may explain the high efficiency of protein folding under apparently adverse conditions in vivo.

The three-dimensional structure of a high molecular weight chaperone, the *Escherichia coli* chaperonin GroEL [3,4], was solved to 2.8 Å resolution [5]. From previous electron microscopic studies of single particles [6–8] or two-dimensional crystals [9,10] it was already known that GroEL is a cylindrical homo-oligomer composed of two rings, each containing seven 57 kDa subunits. Now, in combination with mutational studies [11] the three-dimensional crystal structure gives a more detailed insight into the domain structure of the GroEL-subunit: the largest equatorial domain provides a nucleotide binding site, and the apical domain, forming the opening of the central cavity, contains the binding sites for the substrate protein and the co-chaperonin GroES [12,13].

The chaperonin GroEL binds to non-native proteins [14,15] by hydrophobic interactions [11,16–19], thus preventing irreversible aggregation reactions [20,21]. By proton exchange experiments detected by nuclear magnetic resonance (NMR) it has been shown that in the complex with GroEL the complete secondary and tertiary structure of cyclophilin is globally destabilized [22]. This is in agreement with the results from mass spectrometry experiments, which show that the GroEL-populated state of α-lactalbumin is similarly or more unfolded than its uncomplexed molten globule state [23]. Recently, amide proton exchange experiments detected by NMR have also been carried out in the presence of catalytic amounts of GroEL [24]. These experiments show that GroEL does indeed bind to a fully unfolded state of a protein, and thus has the potential to correct misfolding in proteins by annealing. There is some evidence that at least part of the refolding of the substrate protein takes place in the complex with GroEL, and that the general folding pathway, i.e. the rate limiting step, is not changed by the chaperonin [21,25–28].

Mutagenesis studies on substrate proteins of GroEL have been carried out with *Bacillus amyloliquefaciens* RNAse (barnase) [27,28] and chymotrypsin inhibitor 2 (CI2) [19]. Both of these rather small substrate proteins bind only transiently to the chaperone, and they differ with respect to the effect of substrate mutations on chaperone binding. In the smaller CI2 (64 amino acids), single point mutations affect the refolding rate in the presence and absence of GroEL differently. These experiments show that introducing a more hydrophobic or positively charged amino acid favors binding to the chaperone, and a more negatively charged amino acid disfavors binding. In the larger protein barnase (110 amino acids), the refolding rates of various mutants in the presence of GroEL parallel those in solution. This indicates that the mutation does not significantly influence the binding energy between barnase and the chaperone, and that the folding pathway of barnase is conserved in the complex.

In this study we investigate the binding of GroEL to *Saccharomyces cerevisiae*-citrate synthase (442 amino acids) and to various mutants containing single amino acid substitutions at Gly^{276}. Eukaryotic citrate synthase consists of two identical 50 kDa subunits, containing almost exclusively α-helical secondary structure [29]. Gly^{276} is part of the hinge region of citrate synthase [29,30] allowing an induced fit mechanism: the binding of oxaloacetate induces a conformational change from an 'open' to a 'closed' conformation leading to an optimized binding site for acetyl coenzyme A. All the mutants investigated have native-like secondary structure, as deduced from circular dichroism experiments, but their native states are less stable than that of the wild-type enzyme [30]. G276K, G276L and

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Abbreviations: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; CS, citrate synthase; Bla, pre-β-lactamase.

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G276P are not able to bind oxaloacetate (Table 1) because of a change in their tertiary structure, as indicated by fluorescence spectroscopy [30]. G276D has a tertiary structure similar to the wild-type, but in contrast to G276S and G276A, it does not bind its substrates. G276A is the only enzymatically active mutant and differs from the wild-type only in having a smaller k_{cat}.

Here, we show by competitive binding experiments with pre-β-lactamase that GroEL has different apparent affinities for the wild-type compared to some of the single point mutants of citrate synthase, and we explain our results by the lower thermodynamic stability of these mutants. The results are in agreement with the previously suggested thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL [16,17].

2. Materials and methods

2.1. Protein expression and purification

Pre-β-lactamase [15] and citrate synthase, which carries both an N-terminal and a C-terminal his_5-tail [30,31], were produced and purified as described previously. GroEL was overexpressed in the *Escherichia coli*-strain W3110 containing the plasmid pOF39 [32] and purified as described [33]. After this procedure, there were still some polypeptides bound to GroEL, which could not be separated by various chromatography procedures. Thus, in order to dissociate and to precipitate these polypeptides, an 'ATP-heating step' was used on a concentrated GroEL solution (> 3 mg/ml): the solution was heated to 58°C and then 20 mM MgATP (pH 7.0) was added. After 20 min the solution was cooled on ice for 10 min and centrifuged. Any denatured GroEL was removed by loading the supernatant again onto a DEAE-Sephacel column (Pharmacia) [33]. GroEL was now more than 98% pure, and was stored at 4°C. Protein concentrations were determined by quantitative amino acid analysis, and are always given for the oligomeric state.

2.2. Competitive binding assay

Pre-β-lactamase (26 μM), denatured in 8 M urea, was diluted into folding buffer (100 mM potassium phosphate pH 7.0, 100 mM urea, 100 mM ammonium sulphate, 0.01 v/v% Tween 20) containing 19 nM GroEL (final concentration), to a final concentration of 37 nM. After 4 h incubation at 25°C, citrate synthase was added to a final concentration of 187 nM. The β-lactamase activity of this solution was measured by taking aliquots of 20 μl and diluting them 50-fold into the enzymatic activity assay. Control experiments were carried out, omitting either GroEL or citrate synthase. The data were fitted to an equation describing a single exponential with offset using the Macintosh program Kaleidagraph: $E(t) = A_o \cdot (1 - \exp(-k_{com}^{obs} t)) + C$, where $E(t)$ is the enzymatic activity at time t, A_o is the amplitude, k_{com}^{obs} the observed rate constant of β-lactamase activity increase in the competition experiment, and C the offset.

2.3. Enzymatic activity assay

The β-lactamase activity was determined at 25°C as described previously [15,21].

2.4. Gel filtration

An equimolar solution of GroEL and citrate synthase (7 μM) was incubated for 21 h at 25°C in folding buffer (containing no detergent) and then loaded onto a Superdex 200 column (Pharmacia) equilibrated at the same temperature with 100 mM potassium phosphate pH 7.0, 1 mM DTT. The column fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie-staining.

2.5. Unfolding kinetics

The unfolding of citrate synthase (50 nM) was determined by measuring fluorescence intensity (with excitation at 280 nm and emission at 342 nm) as a function of time, in 100 mM Tris-HCl pH 8.0, 27.5 mM imidazole, 138 mM sodium chloride, at 25°C [30]. The data were fitted to an equation describing a single exponential with offset using the Macintosh program Kaleidagraph: $F(t) = A_o \cdot \exp(-k_u^{cs} t) + C$, where $F(t)$ is the fluorescence at time t, A_o is the amplitude, k_u^{cs} the unfolding rate constant, and C the offset.

3. Results

Because of the lack in enzymatic activity for five of the six citrate synthase mutants (Table 1), pre-β-lactamase was used as a reporter for binding to GroEL. Pre-β-lactamase has been shown to bind to GroEL with high affinity and independent of whether it is added in the native or the non-native state [15]. In a competitive binding experiment, pre-β-lactamase, denatured in 8 M urea, was diluted into folding buffer at 25°C containing 0.5 molar equivalents of GroEL. Thus, at the concentrations used, the two binding sites of GroEL (14-mer) for pre-β-lactamase were just saturated with substrate protein [21]. After 4 h, when the system was in equilibrium, the various citrate synthase mutants were added in 5-fold molar excess to pre-β-lactamase, and binding was followed by measuring the β-lactamase activity as a function of time.

Even after 22 h, the addition of wild-type citrate synthase and of G276S did not significantly change the β-lactamase activity of the solution (Fig. 1a). In contrast, the mutants containing aspartic acid, leucine, lysine or proline at position 276 increased the β-lactamase activity to a yield similar to that in the control reaction containing only denatured pre-β-lactamase and no GroEL. This indicates that all of the pre-β-lactamase originally bound has been displaced by these mutants. However, the observed rate constant of refolding of pre-β-lactamase (with a half-time of 3 h) was 10-fold slower in the presence of citrate synthase and GroEL than in their absence (see below). The same kinetics as for G276D, G276L, G276K, G276P but a lower binding equilibrium were observed for the G276A mutant, the only one with enzymatic activity. When the same experiment was repeated in the presence of 10 mM oxaloacetate, which is known to stabilize the native state of citrate synthase [30], identical results were obtained (Fig. 1b).

The formation of a stable complex between GroEL and G276A was directly examined by gel filtration experiments. An equimolar solution of the citrate synthase mutant and GroEL was incubated for 21 h at 25°C, and then loaded onto a gel filtration column equilibrated at the same temperature. The column fractions were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2a, some G276A co-eluted

Table 1
Effect of single point mutations at position 276 of yeast citrate synthase

Citrate synthase	Binding to oxaloacetate[a]	k_{cat} (s^{-1})[b]	$k_u^{cs} \times 10^5$ (s^{-1})[c]	Binding to GroEL[d]
Wild-type	+	69	0.5	−
G276A	+	17	2.3	+
G276S	+	0	3.4	−
G276P	−	0	4.0	++
G276L	−	0	4.3	++
G276K	−	0	6.1	++
G276D	−	0	7.2	++

[a] Binding (+) or non-binding (−) was determined by measuring the displacement of Cibacron Blue by oxaloacetate [30].
[b] Enzymatic activity could only be observed for the wild-type and for G276A [30].
[c] Unfolding rate constants were determined as described in section 2.
[d] Conditions for competitive binding assay are given in section 2; ++, +, and − indicate stronger, similar, or weaker binding of citrate synthase than of pre-β-lactamase, respectively.

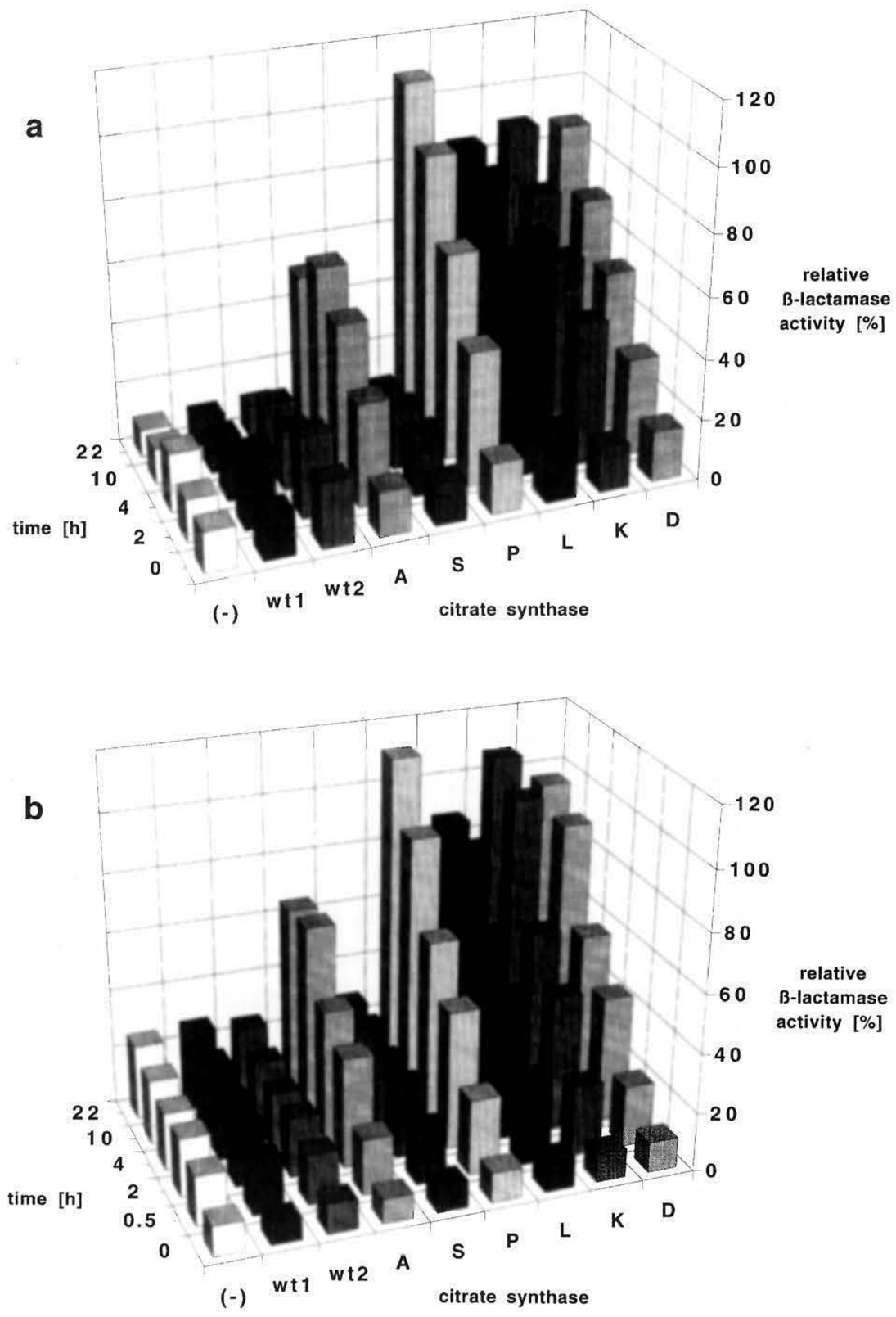

Fig. 1. Competitive displacement of GroEL-bound pre-β-lactamase by citrate synthase. (a) In the absence of oxaloacetate. (b) In the presence of 10 mM oxaloacetate. The relative β-lactamase activity shown was measured at different time points after addition of citrate synthase to the pre-incubated complex of GroEL and pre-β-lactamase. The different mutants of citrate synthase are indicated by the amino acid (single letter code) exchanged at position 276; wt1 and wt2 are two different preparations of the wild-type enzyme. Control experiments were carried out in the absence of citrate synthase (−) or GroEL, which corresponds to 100% activity.

with GroEL, indicating the existence of a stable complex between these two proteins. When the same experiment was carried out with wild-type protein (Fig. 2b), all the citrate synthase eluted together, separately from GroEL, in agreement with the competition experiments.

4. Discussion

The data may be analyzed by the scheme in Fig. 3. In the absence of citrate synthase, there is a formation of a stable complex ($GroEL \cdot Bla_X$) between GroEL and non-native pre-β-lactamase (Bla_X), which is in equilibrium with native pre-β-lactamase (Bla_N), as indicated by the low enzymatic activity. The equilibrium dissociation constant of $GroEL \cdot Bla_X$ has been estimated to be in the nanomolar range or smaller [34]. The concentration of free GroEL, therefore, is less than 1% at equimolar concentrations of substrate protein and GroEL binding sites.

In the absence of GroEL, the various citrate synthase species denature irreversibly, as determined by measuring the time-dependent decrease in fluorescence intensity [30]. The unfolding rate constant (k_u^{cs}) varies for the different G276-mutants and is generally larger for the mutants than for the wild-type (Table 1). In the presence of oxaloacetate, which stabilizes the native state of the wild-type enzyme, G276A and G276S, the unfolding rate is decreased [30].

After addition of G276A, G276D, G276K, G276L or G276P to the pre-incubated complex, $GroEL \cdot Bla_X$, the increase in β-lactamase activity implies that pre-β-lactamase has been displaced by citrate synthase. The observed rate constant for the competitive binding (k_{com}^{obs}) of citrate synthase by GroEL could, in principle, be limited by the rate constant of the unfolding of citrate synthase (k_u^{cs}), the binding of citrate synthase to GroEL (k_{on}^{cs}), the dissociation of $GroEL \cdot Bla_X$ (k_{off}^{bla}), or the refolding of pre-β-lactamase (k_f^{bla}).

The rate constant for increase in β-lactamase activity is, within the experimental error, a constant for these mutants, and is independent of the presence of oxaloacetate (Fig. 4), so that k_u^{cs} can be ruled out as the limiting rate constant. The on-rate for the binding of GroEL to denatured protein has been shown to be diffusion controlled [28], with a second-order rate constant $k_{2nd} = 3.5 \times 10^7\ M^{-1} \cdot s^{-1}$, from which a pseudo-first order rate constant of $1\ s^{-1}$ can be calculated at these GroEL concentrations. This is considerably higher than k_{com}^{obs}, and we can rule

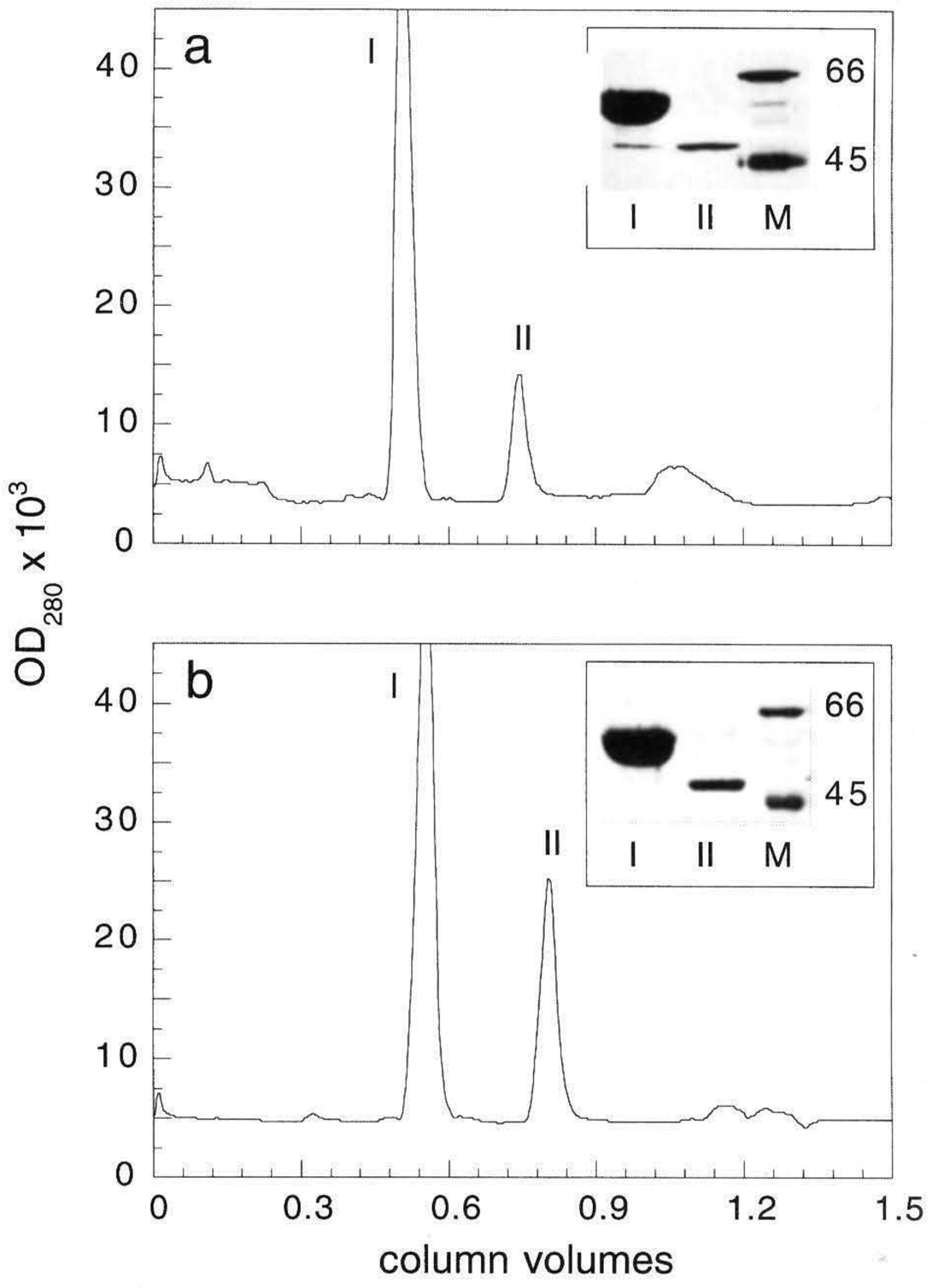

Fig. 2. Gel filtration of citrate synthase in the presence of GroEL. (a) G276A, (b) Wild-type. Inserts indicate the protein composition of peaks I and II after SDS-polyacrylamide gel electrophoresis and Coomassie- staining. The upper and lower bands represent GroEL and citrate synthase, respectively. M indicates two molecular weight markers of 45 and 66 kilodalton.

out the binding of citrate synthase as the rate-determining step. The overall refolding rate constant of pre-β-lactamase k_f^{bla}, determined in the transition region for folding, equals to $6 \times 10^{-4} \cdot s^{-1}$ [16], which is about 10-fold larger than k_{com}^{obs}. Thus, the off-rate constant must be at least partially rate-determining for the competitive displacement of pre-β-lactamase from GroEL by citrate synthase. In the presence of G276D, G276K, G276L or G276P, the binding equilibrium is on the side of the free pre-β-lactamase (Table 1). In order to estimate the dissociation equilibrium constant K_{diss}^{bla}, we have to know k_{off}^{bla} and k_{on}^{bla}. The rate constant k_{com}^{obs} is a function of k_{off}^{bla}, k_{on}^{bla} and k_f^{bla} (Fig. 3). Assuming a steady state for $[GroEL]_{free}$ and $[Bla_X]$ as a first approximation, we can calculate the rate for the increase of β-lactamase activity

$$\frac{dN}{dt} = \frac{k_f^{bla} \cdot k_{off}^{bla}}{k_{on}^{bla} \cdot [GroEL]_{free} + k_f^{bla}} \cdot [GroEL \cdot Bla_X]$$

$$= k_{com}^{obs} \cdot [GroEL \cdot Bla_X].$$

k_{off}^{bla} will be solely rate-determining (and thus equal to k_{com}^{obs}) if $k_{on}^{bla} \cdot [GroEL]_{free} << k_f^{bla}$. This is the case if $[GroEL]_{free} \leq 0.001 \cdot [GroEL]_{tot}$ which depends on the efficiency of the binding competition by citrate synthase. We assume a similar, diffusion controlled on-rate $k_{on}^{bla} = 3.5 \times 10^7$ $M^{-1} \cdot s^{-1}$ as determined previously [28]. Together with $k_{off}^{bla} = k_{com}^{obs}$ this leads to $K_{diss}^{bla} \approx 2 \times 10^{-12}$ M.

Since $k_{com}^{obs} \approx 0.1 \cdot k_f^{bla}$, we can deduce that

$$\frac{k_{off}^{bla}}{k_{on}^{bla} \cdot [GroEL]_{free} + k_f^{bla}} \approx 0.1.$$

Even for the most unfavorable competition by citrate synthase, $[GroEL]_{free} \leq 0.1 \cdot [GroEL]_{tot}$ at the beginning of the reaction, and k_{off}^{bla} could then be as high as $7 \times 10^{-3} \cdot s^{-1}$, leading to $K_{diss}^{bla} \approx 2 \times 10^{-10}$ M. Thus, we conclude that the dissociation constant of pre-β-lactamase lies between 10^{-10} M and 10^{-12} M. G276A is a worse competitor leaving more free GroEL for pre-β-lactamase binding.

The lack of competition of wild-type citrate synthase with pre-β-lactamase for binding to GroEL can be easily explained by its small k_u^{cs} in comparison to the mutants (Table 1, Fig. 4), so that the equilibrium between $GroEL \cdot Bla_X$ and $GroEL \cdot CS_X$ favors the former complex. The lack of competition of G276S

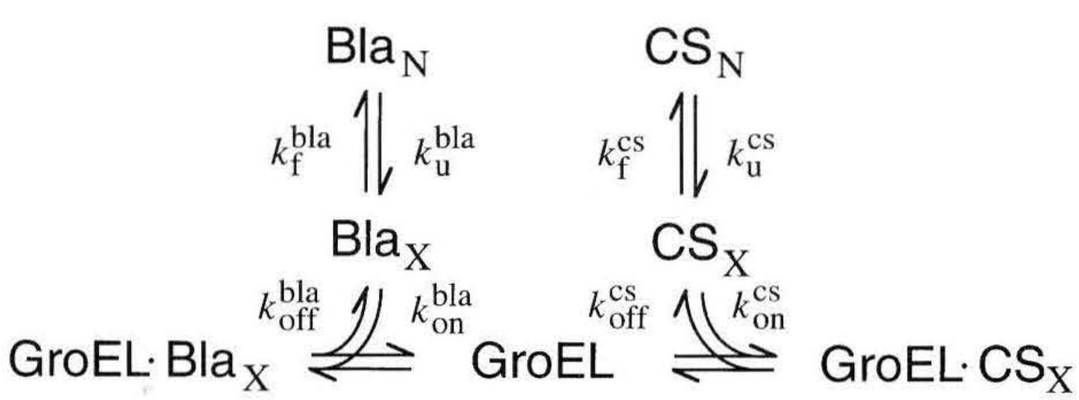

Fig. 3. Minimal scheme for competitive binding of pre-β-lactamase and citrate synthase to GroEL. Bla_N and Bla_X, and CS_N and CS_X are native and non-native pre-β-lactamase and citrate synthase, respectively; $GroEL \cdot Bla_X$ is the complex between non-native pre-β-lactamase and GroEL, and $GroEL \cdot CS_X$ is the complex between non-native citrate synthase and GroEL; k_f, k_u, k_{on}, and k_{off} represent the rate constants for the refolding, unfolding, complex-formation and complex-dissociation of pre-β-lactamase or citrate synthase, respectively.

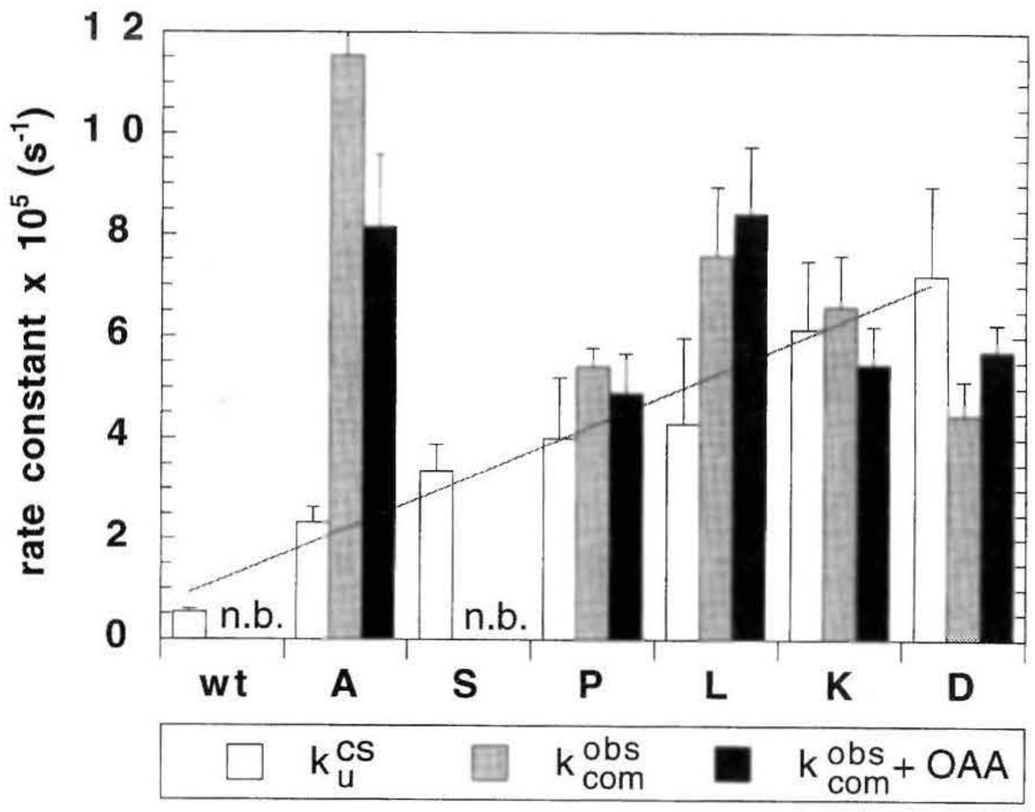

Fig. 4. Rate constants for unfolding and GroEL-binding of citrate synthases. k_u^{cs} is the first-order rate constant for unfolding of citrate synthase, as determined by measuring the decrease in fluorescence intensity [30]. The straight line indicates an increase in unfolding rate constant for the various citrate synthases sorted by k_u^{cs}. k_{com}^{obs} and k_{com}^{obs} + OAA represent the first-order rate constants of the experiments in Fig. 1 (a) and (b), respectively. Standard errors are given. Note, that, for technical reasons, k_u^{cs} and k_{com}^{obs} were determined in different buffer systems (see section 2), so that their absolute values cannot be compared. For wild-type citrate synthase and G276S no binding (n.b.) could be detected.

with pre-β-lactamase indicates that for this citrate synthase, the mutation seems to cause a similar increase in the unfolding rate constant k_u^{cs} and in the refolding rate constant k_f^{cs} so that again the binding equilibrium is on the side of $GroEL \cdot Bla_X$.

It has been shown for pre-β-lactamase that the presence of an N-terminal signal sequence of 23 amino acids can induce formation of a stable complex with GroEL, due to the folding equilibrium of the substrate protein being shifted towards the unfolded state by the signal sequence [16]. From these and other experiments a thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL has been suggested [16,17], saying that the formation of a protein–chaperone complex is dependent on the thermodynamic stability, the length, and the hydrophobicity of the substrate. Here, we show that a single point mutation, which destabilizes the native state of citrate synthase with respect to non-native states, can also allow formation of a stable complex at about equimolar concentrations of substrate and chaperone, which is in agreement with the proposed model. As in barnase [27,28], the single exchange of a side-chain in the substrate protein seems not to alter significantly the net-binding energy of the complex, and this is consistent with the binding of wild-type citrate synthase by GroEL during refolding [20].

A stable complex between mutant proteins and GroEL, which is essential for bacterial growth at all temperatures [32], would cause a considerable problem for the living cell. However, ATP is known to decrease the affinity of GroEL for proteins at micromolar concentrations [33,35–38]. As the intracellular concentration of ATP is in the millimolar range, there are probably no stable complexes formed in vivo between mutant proteins and GroEL. Nevertheless, the steady state concentration of free GroEL may be lowered, and the liganded GroEL-molecules will not be available for other chaperone

dependent cellular processes. Autoregulation mechanisms governing the actual concentration of free chaperone molecules as well as the degradation machinery of the cell recognizing similar stable states may be crucial components in solving this problem.

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