

Fatty Acyl Benzamido Antibacterials Based on Inhibition of DnaK-catalyzed Protein Folding^{*[S]}

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We have reported that the hsp70 chaperone DnaK from *Escherichia coli* might assist protein folding by catalyzing the *cis/trans* isomerization of secondary amide peptide bonds in unfolded or partially folded proteins. In this study a series of fatty acylated benzamido inhibitors of the *cis/trans* isomerase activity of DnaK was developed and tested for antibacterial effects in *E. coli* MC4100 cells. *N*^α-[Tetradecanoyl-(4-aminomethylbenzoyl)]-L-asparagine is the most effective antibacterial with a minimal inhibitory concentration of $100 \pm 20 \mu\text{g/ml}$. The compounds were shown to compete with fluorophore-labeled σ^{32} -derived peptide for the peptide binding site of DnaK and to increase the fraction of aggregated proteins in heat-shocked bacteria. Despite its inability to serve as a folding helper *in vivo* a DnaK-inhibitor complex was still able to sequester an unfolded protein *in vitro*. Structure activity relationships revealed a distinct dependence of DnaK-assisted refolding of luciferase on the fatty acyl chain length, whereas the minimal inhibitory concentration was most sensitive to the structural nature of the benzamido core. We conclude that the isomerase activity of DnaK is a major survival factor in the heat shock response of bacteria and that small molecule inhibitors can lead to functional inactivation of DnaK and thus will display antibacterial activity.

The rapid emergence of bacterial strains that are resistant to current antibiotics requires the development of antimicrobial compounds based on novel mechanisms of action. *De novo* protein synthesis and assisted protein folding are processes essential for viability of cells thus representing attractive targets for the design of antibacterials. The hsp70 chaperone DnaK is a folding helper enzyme showing ATPase and secondary amide peptide bond *cis/trans* isomerase (APIase)² activity that assists

together with its co-chaperones the *de novo* protein folding of ~20% of the *Escherichia coli* proteins larger than 30 kDa (1–4). Whereas the deletion of the DnaK gene alone is not deleterious for *E. coli* cells, combined deletion of the ribosome-associated peptidylprolyl-*cis/trans*-isomerase trigger factor and DnaK genes is lethal under normal growth conditions (4, 5). Furthermore, DnaK is essential under various stress conditions like heat, oxidative stress, and nutritional deprivation (6–8). In *E. coli* cells lacking DnaK, heat shock treatment at 42 °C causes strong protein aggregation comprising 150–200 different protein species and ~10% of the amount of pre-existing *E. coli* proteins (9).

During infection pathogens encounter stress conditions generated by the host defense mechanisms to eliminate the infection. They respond by the rapid acceleration in the rate of expression of heat shock and other stress proteins (10). The DnaK/DnaJ chaperone machinery of *Salmonella enterica* has been shown to be involved in bacterial invasion of epithelial cells, and the DnaK/DnaJ-depleted mutant could not survive or proliferate at all within macrophages (11). In the case of the pathogen *Brucella suis* it was shown that DnaK but not DnaJ is required for intracellular multiplication (12). DnaK of *Listeria monocytogenes* is essentially required for survival under high temperatures and acidic conditions, and it is essential for the efficient phagocytosis of the facultative intracellular pathogen with macrophages (13). The DnaK homologues HscA and HscC seem to have more specialized functions in the cell; HscA appears to be involved in the assembly of Fe/S proteins (14, 15), and HscC seems to play a role in the response to special types of stress (16).

It has been already shown that proline-rich cationic oligopeptides such as pyrrolicorin appear to kill responsive bacteria by binding to DnaK (17). Dimeric analogues of pyrrolicorin showed improved features with respect to broad applicability and stability in mammalian sera (18). However, the relationship between the formation of the DnaK/pyrrolicorin and the antibacterial action remained unexplained. From gene deletion studies, the potential of small molecule inhibitors of the APIase activity of DnaK to act as antimicrobial agents can be inferred. However, no small molecule inhibitors or even

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental HPLC, mass spectrometry, and ¹H NMR data for compounds 1–18.

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² The abbreviations used are: APIase, secondary amide peptide bond *cis/trans* isomerase; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MIC, minimal inhibitory concentration; IPTG, isopropyl 1-thio- β -D-galactopyranoside; HATU, *N*-[(dimethylamino)-

1H-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; RCMLA, reduced carboxymethylated lactalbumin.

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non-peptidic binders for the APlase site of DnaK have been reported so far.

The existence of a distinct APlase site in DnaK serving the catalysis of the *cis/trans* isomerization of secondary amide peptide bonds opened up the new possibility of assaying compounds for inhibiting this essential DnaK function. Here we report that the fatty acylated benzamido derivatives such as N^α -[fatty-acyl-(4-aminoalkylbenzoyl)]-L- α -amino acids reversibly block the APlase site of DnaK, inhibit DnaK-mediated refolding of firefly luciferase, increase protein aggregation in the cell, and exhibit antibacterial activity against *E. coli* MC4100 strain. To examine structure-activity relationships, we modified the α -amino acid residue, the benzoic acid linker, as well as the fatty acid part of the compounds (see Fig. 1).

EXPERIMENTAL PROCEDURES

Materials—Monoclonal anti-DnaK antibody was purchased from Stressgen (Victoria, Canada). Kool™ NC-45™ Universal RNA polymerase templates and *E. coli* RNA polymerase core enzyme were purchased from Epicenter (Madison, WI). *E. coli* MC4100 cells (*araD139* Δ (*argF-lac*)205 *flb-5301* *pstF25* *rpsL150* *deoC1* *relA1*) were obtained from DSMZ (Braunschweig, Germany). The *E. coli* MC4100 Δ *dnaK52::cat* *sidB1* (Δ *dnaK*) cells were provided by B. Bukau (BB1553), pET11DnaK was provided by F. U. Hartl, and pPL900-GrpE and pPL871-DnaJ were provided by N. Dixon. DnaK was purified as described by Zylicz *et al.* (19). As an additional purification step DnaK has been treated with alkaline phosphatase and subsequently applied to a gel-filtration column as described by Theyssen *et al.* (20). DnaJ and GrpE were purified according to already published procedures by Zylicz *et al.* (21).

Synthesis—The fatty acylated benzamido derivatives were synthesized by solid-phase peptide synthesis with the synthesizer Syro II (MultiSynTech GmbH, Witten, Germany) using 0.15 mmol of amino acid pre-loaded Wang resins (compounds 1–18) and 0.15 mmol of Rink amide resin for the peptide amide 19. All resins were obtained from NovaBiochem (Läufelfingen, Switzerland). Synthesis was done by Fmoc strategy with Fmoc amino acids as building blocks and PyBOP (NovaBiochem) and *N*-methylmorpholine as coupling reagents in dimethylformamide. Piperidine/*N,N*-dimethylformamide (20%) was the standard cleavage mixture used for Fmoc detachment. The resin was treated twice for 10 min. All couplings were performed using a 4-fold excess of Fmoc amino acid derivative, PyBOP, and *N*-methylmorpholine (eight equivalents) in *N,N*-dimethylformamide. For all amino acid derivatives a 1.5-h double coupling protocol was used. The activation reagent HATU (AB Applied Biosystems, Foster City, CA) was used instead of PyBOP for coupling of the fatty acids in fatty acylated benzamido derivatives 11 and 16.

After detachment of the fatty acylated benzamido derivatives from the resin and site-chain deprotection with trifluoroacetic acid/water (95:5) at room temperature for 12 h, the fatty acylated benzamido derivatives were purified by reversed-phase-HPLC on a Gilson 306 equipment with a column SP 250/10 Nucleosil 100-7 C8 (Macherey-Nagel, Düren, Germany) using a water (0.1% trifluoroacetic acid)/

acetonitrile gradient. The purified fatty acylated benzamido derivatives were than lyophilized.

Conditions for analytical HPLC were: column LiChroCART® (LiChrospher® 100, RP8, 5 μ m) 125 \times 4 mm (Merck, Darmstadt, Germany) using a water (0.1% trifluoroacetic acid)/acetonitrile gradient 5–100% in 30 min; flow rate 1 ml/min; detection at 220 nm. The identity of the fatty acylated benzamido derivatives were verified by electrospray ionization mass spectrometry on a VG-BIO-Q Triple Quadrupole Tandem Electrospray mass spectrometer (Fisons Instruments, San Carlos, CA). All 1 H NMR spectra were obtained at 500.13 MHz on a Bruker DRX500 spectrometer (Bruker Biospin Fällanden, Switzerland). The measurements were carried out in CD₃OD. Two-dimensional total correlation spectroscopy and two-dimensional nuclear Overhauser effect spectroscopy spectra were recorded at 25 °C.

Identification of Compound 1 in *E. coli* Cytosol—*E. coli* MC4100 cells were incubated with 100 μ g/ml 1 for 12 h at 42 °C in Mueller-Hinton broth. The cells were washed extensively with 10 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) ethanol until no compound 1 could be detected in the buffer after washing. Cells were lysed by sonication. After centrifugation (4300 rpm, 15 min, 4 °C) cell lysate was subjected to analytical HPLC (column LiChroCART® (LiChrospher® 100, RP8, 5 μ m) 125 \times 4 mm (Merck, Darmstadt, Germany) using a water (0.1% trifluoroacetic acid)/acetonitrile gradient 5–100% in 30 min; flow rate 1 ml/min; detection at 220 nm). The identity of compound 1 was verified by electrospray ionization mass spectrometry. Pure compound 1 was used as the reference. As a control, *E. coli* MC4100 cells without treatment with compound 1 were subjected to the described procedure.

Luciferase Refolding Assay—Refolding experiments were done essentially as described by Szabo *et al.* (22). Briefly, luciferase from *Photinus pyralis* (Promega) was incubated for 1 h in denaturation buffer (30 mM Tris/HCl, pH 7.4, 5 mM dithiothreitol, 6.0 M guanidinium chloride) at 10 °C at a final concentration of 2.08 μ M. Refolding was initiated by dilution of denatured luciferase into renaturation buffer (10 mM MOPS, pH 7.8, 50 mM KCl, 1 mM ATP, 5 mM MgCl₂, 1 μ M bovine serum albumin, 436 nM DnaK, 160 nM DnaJ, and 436 nM GrpE) to a final concentration of 10.4 nM at 10 °C and 30 °C. Before the refolding reaction was started, compounds 1–18 at different concentrations were incubated in renaturing buffer for 10 min. After 1 h of refolding, luciferase activity was determined with a luminometer (Luminoskan, Ascent) using luciferase assay buffer (20 mM Tricine, pH 7.8, 5 mM MgCl₂, 0.1 mM EDTA, 3.3 mM dithiothreitol, 270 μ M coenzyme A, 500 μ M D-luciferin, 500 μ M ATP). The IC₅₀ values have been calculated for a one site competition model with SPSS Sigmaplot 8.0.

Enzymatic Measurements—The APlase activity of DnaK was determined using RNaseT1 P39A variant as substrate as described in Schiene-Fischer *et al.* at 15 and 30 °C (1). Steady-state ATPase measurements were performed as described in Montgomery *et al.* (23). Compound 1 at 150 μ M has been incubated with 1 μ M DnaK for 10 min at 30 °C. The reaction was started with 330 μ M ATP. The time course was followed for at least 10 min.

Determination of MIC Values—All compounds were dissolved in ethanol/10 mM potassium phosphate, pH 7.8, to 2 mg/ml (final ethanol concentration, 13% (v/v)). After incubation for 12 h at 37 °C insoluble particles were separated by centrifugation at $16,000 \times g$ for 15 min. The final concentration was determined UV-spectrometrically by using the characteristic absorption at 236 nm.

MICs were determined in triplicate in Mueller-Hinton medium by a liquid growth inhibition assay. In a sterile microtiteration plate, 100 μ l of the compound in varying concentrations, or 10 mM potassium phosphate, pH 7.8, 13% (v/v) ethanol as a control, were added to 50 μ l of a mid-logarithmic growth phase culture of *E. coli* MC4100 cells. Plates were incubated for 24 h at 42 °C. Samples were spread on agar plates and incubated overnight at 37 °C. The number of colony forming units was counted, and the MIC value was determined as the concentration of substance that causes a 99.9% inhibition of cell growth (NCCLS criteria (24)).

Erythrocyte Hemolysis—The hemolytic activity of fatty acylated benzamido derivatives was determined by spectrometric analysis as described before (25) using human erythrocytes. Freshly isolated human erythrocytes were washed three times with Tris-buffered saline (10 mM Tris/HCl, pH 7.4, 150 mM NaCl) prior to the assay. The erythrocytes (2.5×10^8 cells/ml) were treated with fatty acylated benzamido derivatives at final concentrations ranging from 3.1 to 50 μ g/ml and incubated under gently shaking at 42 °C for 30 min. The samples were subjected to ice for 2 min and centrifuged at $380 \times g$ for 5 min. The amount of hemoglobin released from the cells was determined by measuring the absorbance of the supernatants at 550 nm after 10-fold dilution with 0.5% NH_4OH . Saponin (100 mg/liter) was used to obtain a positive control hemolysis value (100% hemolysis), and 10 mM potassium phosphate buffer, pH 7.8, was used as a negative control. Inhibitor concentrations causing 50% hemolysis (EC_{50}) were derived from the dose-response curves with SPSS SigmaPlot 8.0.

Heat Shock Experiments—*E. coli* MC4100 Δ dnaK pTrc99A-DnaK cells were grown to mid-exponential growth at 30 °C in Terrific Broth. Cells were incubated for 1 h in the presence of 0, 0.2, 0.5, or 1.0 mM IPTG. Cells were subjected to 57 °C for 210 s in the absence or additional presence of 1.35 mM compound **1** added immediately before heat shock. Immediately afterward heat shock cells were cooled on ice for 2 min. Serial dilutions were plated onto LB plates with selection marker supplemented with the indicated IPTG concentrations. The plates were incubated for 12 h at 30 °C. For analysis of DnaK produced by *E. coli* MC4100 Δ dnaK pTrc99A-DnaK, cells were lysed by sonification after 1-h induction in the presence of varying IPTG concentrations, and samples were analyzed by 12.5% SDS-PAGE and immunoblotting using anti-DnaK antibody.

Aggregation Assay—*E. coli* MC4100 wild type were grown to mid-exponential growth at 30 °C. 0.13 mg/ml or 0.066 mg/ml compound **1** or **2**, respectively, was added, and the cells were cultured further for 15 min. Cells were subjected to heat shock at 45 °C for 30 min and subsequently grown at 30 °C for 3 h. *E. coli* MC4100 Δ dnaK cells in the absence of inhibitors was treated equally and thus used as a control for protein aggregation in the absence of DnaK. Aggregated proteins were isolated

and separated from contaminating membrane proteins as described. Briefly, cells were harvested by 10-min centrifugation at $5000 \times g$ and 4 °C. Pellets were resuspended in buffer A (100 mM Tris/HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 15% (w/v) sucrose, 1 mg/ml lysozyme) and incubated for 30 min on ice. Cell lysis was performed by adding a 7-fold excess of buffer B (100 mM Tris/HCl, pH 7.5, 1 mM EDTA), followed by sonification. After centrifugation at $2,000 \times g$ for 15 min at 4 °C, an insoluble cell fraction was isolated by subsequent centrifugation at $15,000 \times g$ for 20 min at 4 °C. The pellets were frozen, resuspended in 400 μ l of buffer B by sonification, and centrifuged ($15,000 \times g$, 20 min, 4 °C). The washed pellets were again resuspended in 320 μ l of buffer B by sonification, subsequently 80 μ l of 10% (v/v) Nonidet P-40 was added, and aggregated proteins were isolated by centrifugation ($15,000 \times g$, 20 min, 4 °C). This washing procedure was repeated. Nonidet P-40-insoluble pellets were washed with buffer B and resuspended in 50 μ l of buffer B by sonification (26). Fractions corresponding to identical cell numbers were analyzed by 12.5% SDS-PAGE followed by staining with Coomassie Brilliant Blue.

Pull-down Assay of DnaK with Compound 19—MC4100 cells were grown overnight in LB medium at 42 °C. 200 μ l of streptavidin beads was washed with buffer C (20 mM Tris/HCl, pH 7.5, 50 mM KCl, 1 mM MgCl_2) and blocked with 1 mg/ml bovine serum albumin for 1 h at 4 °C. MC4100 cells were lysed by sonification and incubated with 0.7 mM compound **19** for 1 h at room temperature in 1200 μ l of buffer C. Streptavidin beads were added, and the mixture was further incubated for an additional hour at room temperature. Streptavidin beads were pelleted and washed twice with buffer C. DnaK was eluted from beads by adding compound **1** with a final concentration of 1.92 mM. 5 μ l of the different samples was analyzed by 12.5% SDS-PAGE and immunoblotting using anti-DnaK antibody. Controls showed 0.1 μ l of *E. coli* lysate and 0.2 μ g if recombinant DnaK.

RESULTS

Characterization of the Interaction of DnaK with N^α -[Tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine—As exemplified by N^α -[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine (**1**) compounds were synthesized by stepwise solid-phase synthesis using the Fmoc strategy on Wang resin preloaded with isoleucine. After deprotection myristic acid was coupled using PyBOP as reagent. Type **1** anionic compounds consisted of three building blocks, a fatty acid moiety, an aromatic linker, and an α -amino acid residue covering a free carboxylate group (Fig. 1A).

Compound **1** inhibited the APIase activity of DnaK with an IC_{50} value of 2.7 μ M as determined by DnaK-catalyzed RNase T1 P39A refolding assay (Fig. 2A) (1). Similarly, it decreased the yield of native protein from the guanidinium chloride-denatured firefly luciferase in a DnaK/DnaJ/GrpE-assisted refolding assay in a dose-dependent manner with an IC_{50} value of 9.5 μ M (Fig. 2B). According to the refolding pathway, inhibition of the APIase function of DnaK translates into reduction of final folding yield (27). The ATPase activity of DnaK remained unaffected up to a concentration of 150 μ M of **1**. To ascertain the specificity of the refolding assay for APIase function the effect of **2** was examined. Its inhibitory potency was low in both the

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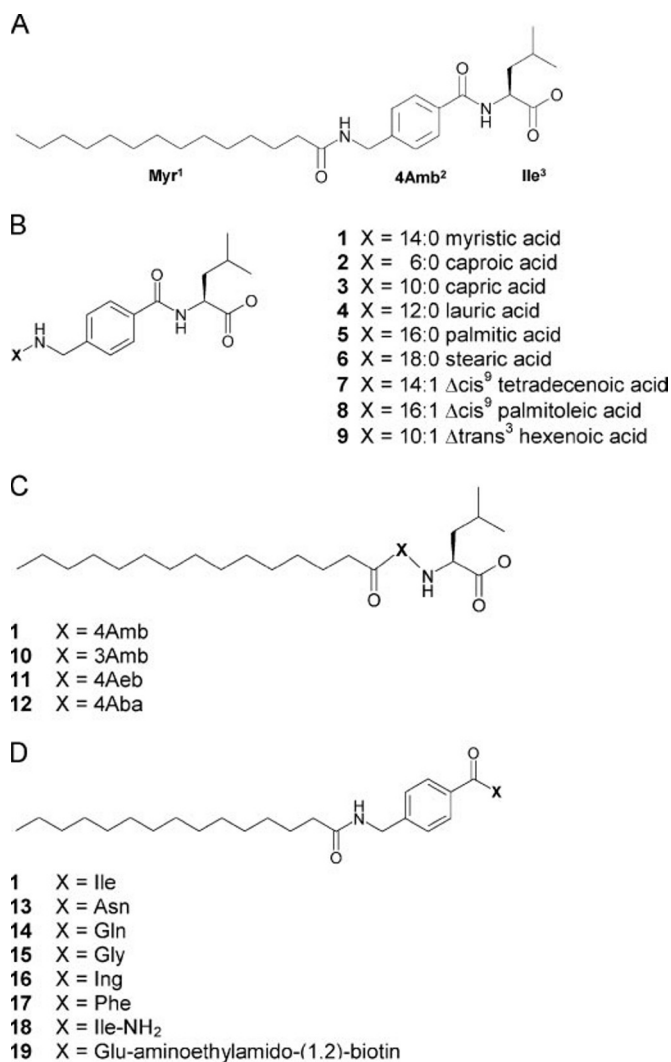


FIGURE 1. Various *N*^α-[fatty-acyl-(4-aminoalkylbenzoyl)]-L- α -amino acids used in this work. The peptidomimetic compound structure (A) is composed of three building blocks, a fatty acyl residue (B), a linker consisting of a benzamido derivative (C), and an amino acid (D). 4Amb, 4-aminoethylbenzoyl; 3Amb, 3-aminoethylbenzoyl; 4Aeb, 4-aminoethylbenzoyl; 4Aba, 4-aminobenzoyl; Ing, indanylglycine.

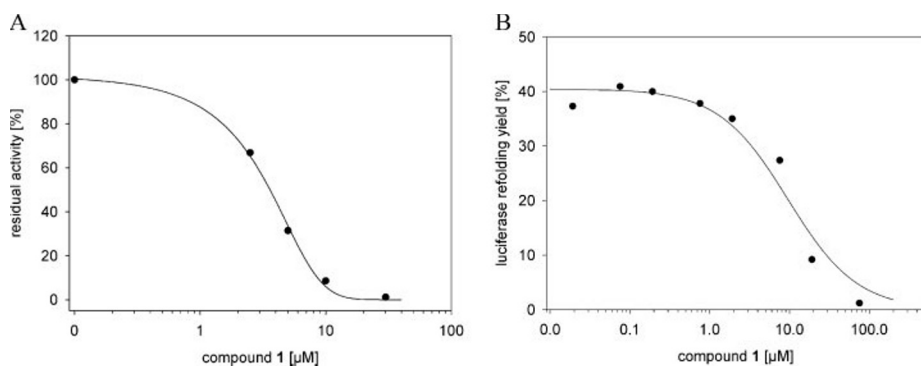


FIGURE 2. Fatty acylated benzamido derivative 1 inhibits APIase activity of DnaK and DnaK/DnaJ/GrpE-assisted firefly luciferase refolding. A, residual activity of the rate acceleration of the Tyr³⁸-Ala³⁹ *trans* to *cis* isomerization limited refolding of the RNase T1 P39A variant by 2 μ M DnaK in the additional presence of different concentrations of compound 1. The drawn line was calculated using an IC₅₀ of 2.6 μ M. B, inhibitory effect of compound 1 on the reactivation of guanidinium chloride-denatured firefly luciferase by DnaK (436 nm), GrpE (436 nm), and DnaJ (160 nm). Refolding was initiated by dilution of 2.08 μ M unfolded luciferase into refolding buffer containing the helper proteins. The drawn line was calculated using an IC₅₀ of 9.5 μ M.

RNase T1 P39A variant and the luciferase refolding assay. In both assays, compound 2 up to concentrations of 20 μ M and 200 μ M, respectively, had no effect.

Reversibility of inhibition was examined by dialysis of the DnaK-1 complex formed by incubation of DnaK (0.56 μ M) and 1 (10.5 μ M). The refolding activity of DnaK completely recovered from the inhibited enzyme when dialyzed against 10 mM MOPS buffer, pH 7.8, 50 mM KCl, 5 mM MgCl₂, regardless of whether the incubation time was prolonged or the concentration of 1 was further increased.

Next we analyzed if 1 binds to DnaK at the peptide binding site of DnaK, which constitutes a channel defined by loops from the beta sandwich structure of the C-terminal substrate-binding domain (28). Therefore, our competition assay correlated with the decrease in fluorescence at 460 nm in the presence of increasing amounts of 1 when administered to a solution of a DnaK-bound σ ³²-derived peptide (σ ³²-¹³²QRKLFFNLRK-TKQ¹⁴²-C-IAANS) that was labeled with the fluorescent probe IAANS. This 14-mer oligopeptide represented a segment of the transcription factor σ ³², which was shown to specifically bind to the peptide binding site of DnaK with a substantial increase in fluorescence emission upon DnaK binding (29). The concentration-dependent decrease of fluorescence signal upon addition of compound 1 indicated the ability of 1 to displace the fluorescent labeled peptide from the DnaK-peptide complex by competitive binding to the same site of the protein (Fig. 3, A and B). Competition by 1 revealed an IC₅₀ value of 29.1 μ M, which translates into a *K_d* value of 2.6 μ M for the DnaK-1 complex as was calculated according to the equation described by Craig (30). This value is close to the *K_i*-value directly measured using the APIase assay. Competition assays were repeated with the peptide NRRLLTG, controlling for method of σ ³²-Q132-Q144-C-IAANS displacement. Displacement of σ ³²-Q132-Q144-C-IAANS by NRRLLTG from DnaK resulted in an IC₅₀ of 1.3 μ M indicating a slightly higher affinity for DnaK of this peptide when compared with those of 1.

To determine whether the enzymatically inactive DnaK-1 complex is still able to serve as a holding chaperone and thus can sequester unfolded proteins, the influence of 1 on the formation of DnaK complexes with reduced carboxymethylated lactalbumin (RCMLA) was investigated. Already under native conditions this protein variant is in its unfolded state (2). Again, the peptide NRRLLTG was utilized as a control, because it was already shown by x-ray crystallography to cover a considerable part of the peptide binding cleft of DnaK (32). After incubating DnaK with RCMLA in the absence or presence of different concentrations of 1 (Fig. 3C) or NRRLLTG (Fig. 3D) at 37 °C, DnaK-RCMLA complexes were separated from free DnaK and free

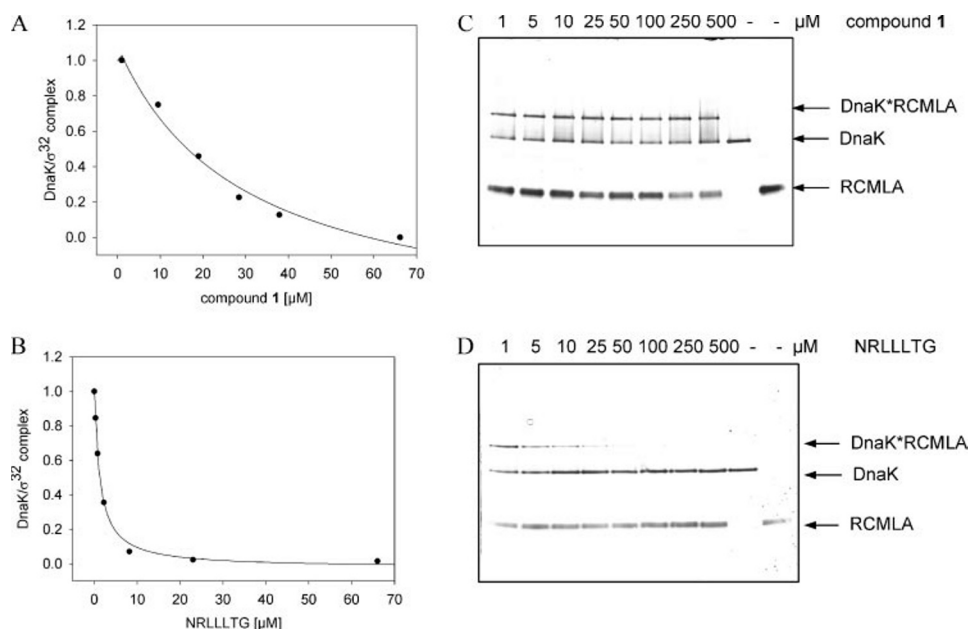


FIGURE 3. Fatty acylated benzamido derivative 1 competes with the DnaK substrates σ^{32} -peptide but not with RCMLA for DnaK binding. A and B, fluorescence response of an IAANS-conjugated peptide (σ^{32} -Q132-Q144-C-IAANS) in complex with DnaK upon addition of compound 1 (A) or the peptide NRLLLTG (B) shows release of the peptide from proteinaceous environment. The solid lines represent the calculated curve with an IC_{50} value of 29.1 μ M for compound 1 and 1.3 μ M for NRLLLTG. C and D, SDS-PAGE analysis of the DnaK-RCMLA complex in the presence of increasing concentrations of compound 1 (C) or the peptide NRLLLTG (D).

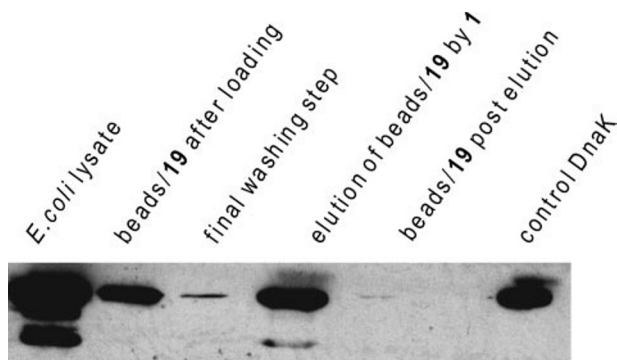


FIGURE 4. Fatty acylated benzamido derivatives 1 and 19 interact with DnaK in *E. coli* cell lysate. A mixture of biotinylated compound 19 and *E. coli* lysate was treated with streptavidin-agarose beads to bind compound 19-protein complexes as described under "Experimental Procedures." Specifically bound protein was eluted by using compound 1. Proteins were resolved by SDS-PAGE and immunoblotting identified DnaK.

RCMLA by native PAGE. The peptide NRLLLTG completely prevented DnaK-RCMLA complex formation at concentrations higher than 25 μ M. However, up to a 500 μ M concentration 1 was not able to inhibit the formation of this complex.

Attempts were made to directly identify the cellular targets of fatty acylated benzamido derivatives in cell lysate. Cell lysates were prepared from *E. coli* MC4100 cells grown at 42 °C and were incubated with compound 19, a biotinylated derivative of 1 in which the Ile residue was replaced by a C-terminally biotinylated Glu residue. Complexes of 19 with cell proteins were extracted by streptavidin beads, washed extensively, and analyzed by Western blotting with anti-DnaK antibody. Evidence for a DnaK-19 interaction in the cell was established by detec-

tion of DnaK in the eluate after washing the beads with a buffer solution containing 1.92 mM of 1 (Fig. 4).

Because under heat shock conditions DnaK was described to have important functions in preventing or reversing thermally induced protein damage (3, 9, 33), we analyzed the influence of 1 on bacterial growth at 42 °C. Antibacterial activity was detected characterized by a MIC of 380 ± 50 μ g/ml against *E. coli* MC4100 cells at 42 °C (Table 1). At 37 °C, bacterial growth was unaffected at concentrations up to 1.4 mg/ml. The analysis of the bactericidal effect of 1 at 30 °C revealed a MIC of 860 ± 100 μ g/ml using *E. coli* MC4100 Δ tig cells and a MIC $\gg 5$ mg/ml toward wild-type *E. coli* MC4100 cells at 30 °C. Uptake studies of 1 by reversed-phase HPLC separation of the cytosol combined with electrospray ionization mass spectrometry analysis verified its intracellular

availability. The inability of *E. coli* cells to regrow on agar plates after application of compound 1 in concentrations above MIC for 12 h in LB medium at 42 °C shows that the inhibitor is bactericidal for *E. coli*. To exclude membrane effects of 1 on *E. coli* cell viability, membrane permeability in the presence of 200 μ M 1 or 2 was assayed by measuring the activity of β -lactamase released to the medium in comparison to the total activity (supplemental Table S1). No significant difference in the amount of extracellular β -lactamase compared with the total β -lactamase activity was observed after growth of the cells in the presence of buffer or in the presence of compounds 1 or 2.

Inhibitor-related Viability after Severe Heat Shock—To judge whether different cellular levels of DnaK influence the antibacterial activity of compound 1, we have introduced the plasmid pTrc99A-DnaK expressing DnaK under the control of an IPTG-inducible promoter into *E. coli* MC4100 Δ dnaK cells (Fig. 5). We analyzed the restoration of thermotolerance of the Δ dnaK pTrc99A-DnaK cells (7). *E. coli* Δ dnaK cells are not able to survive severe heat shock at 57 °C for 210 s neither in the presence nor in the absence of 1 (Fig. 5A). However, induction of intracellular DnaK production by IPTG (Fig. 5B) allows the bacteria to survive unless 1 was present. The complementation of the temperature-sensitive phenotype of *E. coli* Δ dnaK cells at 0.2 mM IPTG was abolished in the presence of 1.35 mM of 1 (640 μ g/ml) during heat shock. Application of 1 immediately after heat shock resulted in bacterial survival at all IPTG concentrations. These data suggest that DnaK is indeed involved in the antibacterial action of 1 in *E. coli* cells.

Influence of Inhibitor on Protein Aggregation in Vivo—To probe how thermal aggregation of *E. coli* proteins *in vivo* responds to the administration of 1 we analyzed the extent of

TABLE 1

Antibacterial and hemolytic activities of compound 1 and its analogues with substituted fatty acids in position 1

| Compound | Fatty acid | Inhibition of assisted refolding IC ₅₀ ^a | Hemolytic activity EC ₅₀ ^b | MC4100 MIC ^c |
|----------|--|--|--|-------------------------|
| | | | μg/ml | |
| 1 | 14:0 Myristic acid | 4.9 ± 0.4 | 66 ± 2 | 380 ± 50 |
| 2 | 6:0 Caproic acid | >400 ^d | >1500 | 1000 ± 150 |
| 3 | 10:0 Capric acid | 40.4 ± 1.7 | >1000 | 490 ± 70 |
| 4 | 12:0 Lauric acid | 1.9 ± 0.1 | 400 ± 30 | 650 ± 90 |
| 5 | 16:0 Palmitic acid | 1.2 ± 0.0 | 50 ± 30 | 180 ± 30 |
| 6 | 18:0 Stearic acid | 2.3 ± 0.3 | 30 ± 1 | 3100 ± 400 |
| 7 | 14:1 Δ ^{cis} ⁹ -Tetradecenoic acid | 0.75 ± 0.2 | 350 ± 10 | 630 ± 90 |
| 8 | 16:1 Δ ^{cis} ⁹ -Palmitoleic acid | 3.6 ± 0.5 | 250 ± 80 | 1300 ± 200 |
| 9 | 10:1 Δ ^{trans} ³ -Hexenoic acid | 35.9 ± 5.8 | >1500 | 280 ± 90 |
| | Ampicillin | ND ^e | ND | 14 ± 3 |
| | Pyrrhocoricin | 25 ± 3 | ND | 1700 ± 200 |

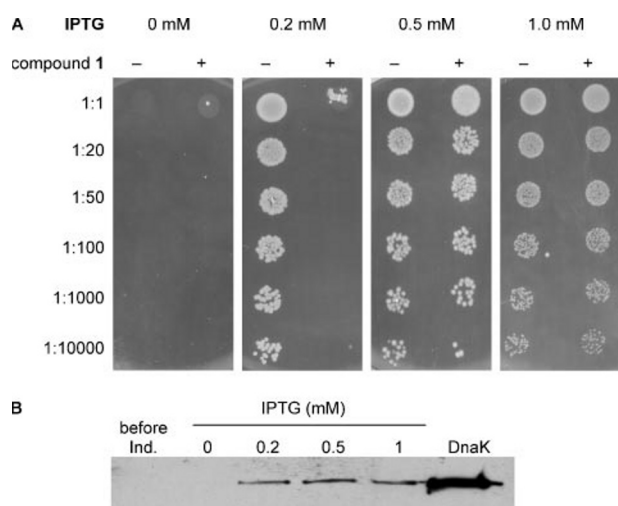
^a Compound concentrations causing 50% (IC₅₀) inhibition of assisted refolding of luciferase.^b Compound concentrations causing 50% (EC₅₀) hemolysis determined with fresh human erythrocytes.^c Minimal inhibitory compound concentrations causing cell death (MIC) in accordance to the NCCLS guideline M7-A2 of *E. coli* MC4100 cells (27).^d > indicates no measurable effect at the highest concentration of the compound that could be used dependent on the water solubility.^e ND, not determined.

FIGURE 5. Fatty acylated benzamido derivative 1 inhibits thermotolerance of *E. coli* cells. A, DnaK-deficient *E. coli* cells were thermosensitive against severe heat shock at 57 °C for 210 s. Expression of DnaK from the plasmid pTrc99A-DnaK after induction by different amounts of IPTG restored thermotolerance. The additional presence of compound 1 inhibited thermotolerance at lower IPTG concentrations. The thermotolerant *E. coli* cells were identified by spotting of different dilutions of the cell cultures on LB plates supplemented with the indicated IPTG concentrations and incubation at 30 °C for 12 h. B, Western blot analysis demonstrating expression of DnaK dependent from the IPTG concentrations. As positive control, 0.2 μg of recombinant DnaK was used.

heat-induced protein aggregation in *E. coli* cells. The cells were grown at 30 °C to logarithmic phase followed by incubation at 42 °C for 1 h and subsequent characterization of aggregated proteins by SDS-PAGE. As described previously, in wild-type cells only a minor portion of proteins underlies aggregation under these conditions (26). In contrast, wild-type cells treated with 1 under the same conditions show a strong increase in the fraction of aggregated proteins resembling the *E. coli* Δ*dnaK* strain in this respect (Fig. 6). As a less inhibitory structural analogue of 1, 2 did not enhance aggregation tendency when compared with wild-type cells (Fig. 6).

Effect of Inhibitor on the Transcriptional Activity of σ³²-Regulated Genes—It is well established that DnaK negatively regulates heat shock response as the lack of DnaK function leads to enhanced levels of heat shock proteins (34). We tested if 1 also influences the levels of induction of heat shock-inducible pro-

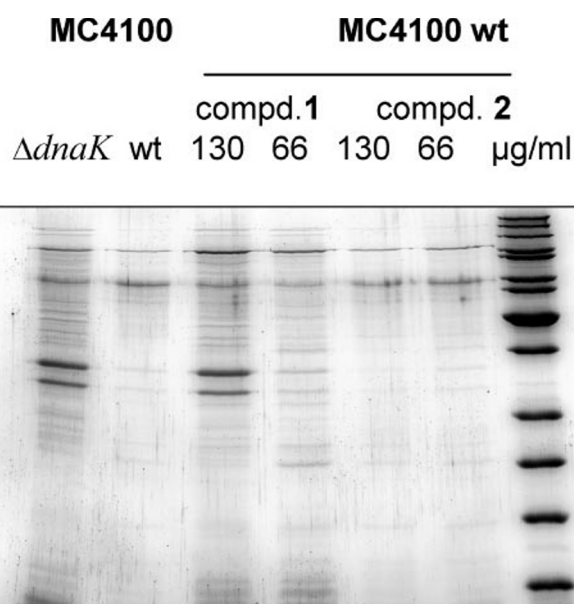


FIGURE 6. Fatty acylated benzamido derivative 1 induces temperature-dependent protein aggregation in wild-type *E. coli* MC4100 cells. SDS-PAGE gel showing the aggregated proteins isolated from *E. coli* lysate after heat shock. Wild-type *E. coli* MC4100 were subjected to heat shock at 45 °C in the presence of fatty acylated benzamido derivative 1 and after a recovery period at 30 °C aggregated proteins were isolated and visualized as described under "Experimental Procedures." Controls show protein aggregation in *E. coli* MC4100 cells lacking DnaK, and in wild-type *E. coli* cells without any supplementation and in the presence of control compound 2 after identical treatment.

motors of well known σ³²-regulated heat shock genes. Previously characterized isogenic strains, carrying LacZ fusions to heat shock promoters *rpoDP*h and *PgroESL*, were incubated at 30 °C or 42 °C in the presence of 200 μM 1, a concentration that does not affect the growth of bacteria significantly as compared with the related compound 2. Bacterial cultures were grown at 30 °C in M9 minimal medium without any supplementation. Equivalent amounts of exponentially growing cells at an A₆₀₀ of 0.1 were shifted to 42 °C in pre-warmed culture tubes. Approximately 2- to 3-fold increases in the activity from both the heat shock promoters after 12-h incubation in the presence of 1 was reproducibly observed as compared with cultures grown either in the presence of 2 or M9 minimal medium alone (Fig. 7). No significant effect in the promoter activity was observed as meas-

ured by β -galactosidase activity driven from heat shock promoters between the cells grown in M9 medium with the control compound **2**, and M9 medium alone at 42 °C with 12-h incubation. As a control for nonspecific effects of **1** on either the activity of β -galactosidase *per se* or due to some indirect effect on cellular physiology, we performed measurements of β -galactosidase activity of previously described bacterial strains carrying *PhtrL-lacZ* or *PhtrP-lacZ*, known non-heat shock promoter fusions in the same isogenic genetic background (Fig. 7). After the 12-h incubation at 42 °C the presence of 200 μ M **1** did not lead to any bacterial cell death, and a normal growth was observed. When the initiation of synthesis of β -galactosidase from the two heat shock promoters immediately after temperature upshift following 10, 15, 20, or 30 min incubation was measured, the addition of either **1** or **2** was comparable to normal heat shock induction (data not shown). Thus, the initiation of heat shock response within the transient shift to 42 °C is not altered under these conditions, but the level of heat shock promoter activity is elevated upon prolonged exposure to DnaK inhibitor. *E. coli* heat shock response is not induced by application of **1** at 30 °C (supplemental materials). To exclude that **1** activates *E. coli* RNA polymerase, we measured the effect of **1** on an *in vitro* transcription assay. The transcription of KoolTM NC-45TM universal RNA polymerase templates, which represent small circular single strand DNA molecules, by *E. coli* RNA

polymerase core enzyme was performed according to the manufacturer's protocol. At the concentration of 200 μ M **1**, which was also used to analyze the transcriptional activity of σ^{32} -regulated genes, we found a residual transcriptional activity of ~45% of the activity obtained in the absence of **1**. Thus, the activation of the transcriptional activity of σ^{32} -regulated genes is not related to the activation of *E. coli* RNA polymerase.

Structure-Activity Relationship of Fatty Acyl Benzamido Derivatives—To develop a clinically useful drug candidate, structure-activity relationship analysis can be exploited to increase the therapeutic index, namely, minimizing the hemolytic activity while maintaining high antibacterial activity. Structure variations within all building blocks of **1** revealed large changes of the IC₅₀ values for the assisted refolding of luciferase, for the EC₅₀ value of hemolytic activity on red blood cells, as well as for antibacterial activity. Table 1 contains compounds encompassing fatty acid substitutions in N ^{α} -[fatty-acyl-(4-aminomethylbenzoyl)]-L-isoleucine (Fig. 1B).

An intriguing result was obtained with the caproic substitution resulting in **2** that did not influence the assistance by DnaK of luciferase refolding and is less active as an antimicrobial. Because **1** did not affect *E. coli* growth at 37 °C, determinations of hemolytic and antibacterial activities were performed at 42 °C. Beside chain length subtle structural changes in the fatty acyl residue also affected the refolding capability of DnaK in that a 6-fold difference of IC₅₀ values was found between **1** and **7** in favor of a more effective inhibition by the unsaturated fatty acid. Of particular interest is the result of the introduction of Δ trans³-hexenoic acid **9** showing decreased hemolytic activity accompanied by good inhibition of growth of *E. coli* MC4100 cells. In general, fatty acid substitution in **1** does not indicate a quantitative correlation between DnaK inhibition and antibacterial activity.

Next, we analyzed the influence of linker building blocks differing in the benzoic acid substituents (Fig. 1C), as shown in Table 2. Again, replacement of the 4-aminomethyl benzamido residue in **1** with chemically rather different linker groups did not markedly affect DnaK-assisted refolding. However, positional changes of the aminomethyl function from para (**1**) to meta (**10**) resulted in an dramatic increase of MIC at retained value for hemolytic efficiency.

Considerable changes in antibacterial activity were achieved with N ^{α} -[tetradecanoyl-(4-aminomethylbenzoyl)]-L- α -amino acids (Fig. 1D), which vary in the nature of the amino acid (Table 3). Replacement of the C-terminal carboxylate with a carboxamide group weakened slightly antibacterial activity indicating that the anionic site is of some importance for the efficiency of the compounds. Hemolytic effects were found to be in the same order of magnitude for all compounds depicted in Table 3. With N ^{α} -[tetradecanoyl-(4-aminomethylbenzoyl)]-

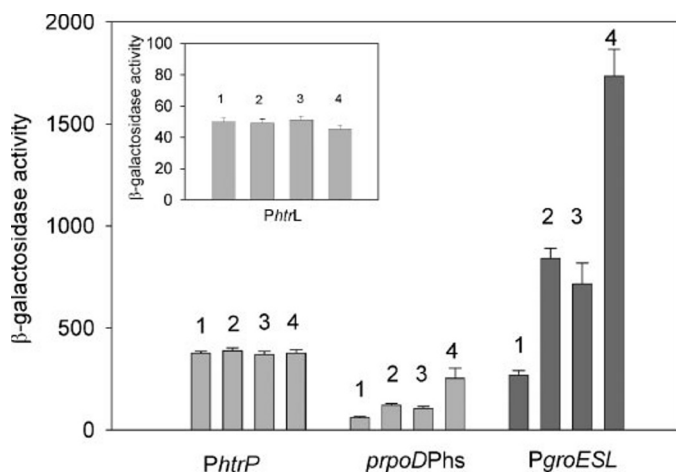


FIGURE 7. Increased activity of heat shock promoters in the presence of the fatty acylated benzamido derivative 1. The plot shows the β -galactosidase activity for three isogenic strains carrying individually single copy promoter fusions to the two heat shock promoters *prpoDPhs* and *PgroESL* and the control promoters *PhtrP* and *PhtrL* (inset). Equivalent amounts of exponentially grown cultures were grown at 30 °C in M9 minimal medium (lane 1) and shifted to 42 °C for 12 h without any supplementation (lane 2), with the addition of control non-inhibitory compound **2** (lane 3), and in the presence of compound **1** (lane 4). Increased β -galactosidase activity in the case of the heat shock promoters depicted in Miller units was observed after shift to 42 °C, which was further increased in the additional presence of compound **1**. Data represent the means of five independent experiments.

TABLE 2
Antibacterial and hemolytic activities of compound **1** and its analogues with substituted linkers in position 2

| Compound | Linker | Inhibition of assisted refolding IC ₅₀ | Hemolytic activity EC ₅₀ | MC4100 MIC |
|-----------|-----------------------------|---|-------------------------------------|--------------|
| | | | μ g/ml | |
| 1 | 4-(Aminomethyl)benzoic acid | 4.9 \pm 0.4 | 66 \pm 2 | 380 \pm 50 |
| 10 | 3-(Aminomethyl)benzoic acid | 7.0 \pm 0.7 | 84 \pm 12 | >2000 |
| 11 | 4-(Aminoethyl)benzoic acid | 2.6 \pm 0.4 | 35 \pm 8 | 640 \pm 90 |
| 12 | 4-Aminobenzoic acid | 1.1 \pm 0.1 | >10 | >60 |

TABLE 3

Antibacterial and hemolytic activities of compound 1 and its analogues with substituted amino acid residues in position 3

| Compound | Amino acid residue | Inhibition of assisted refolding IC ₅₀ | Hemolytic activity EC ₅₀ μg/ml | MC4100 MIC |
|----------|---------------------|---|--|------------|
| 1 | Ile | 4.9 ± 0.4 | 66 ± 2 | 380 ± 50 |
| 13 | Asn | 4.6 ± 0.3 | >10 | 100 ± 20 |
| 14 | Gln | 11.5 ± 0.3 | 520 ± 30 | 2800 ± 400 |
| 15 | Gly | 5.5 ± 0.0 | >50 | >500 |
| 16 | Ing ^a | 3.1 ± 0.3 | >10 | 400 ± 60 |
| 17 | Phe | 3.9 ± 0.1 | >200 | 1200 ± 200 |
| 18 | Ile-NH ₂ | 6.1 ± 0.7 | 100 ± 10 | 1200 ± 200 |

^a Ing, indanylglycine.

L-asparagine the most effective fatty acyl benzamido antibacterial was identified with a MIC of 100 ± 20 μg/ml. Ampicillin as the reference antibiotics showed a higher antibacterial activity of ~7-fold (Table 1). An increase in MIC has been already found for ampicillin if temperature is shifted from 37 °C to 42 °C (35, 36). In this respect the MIC of 14 ± 3 μg/ml compares well with the value of 7.5 μg/ml already described for ampicillin at 42 °C (36). In the luciferase refolding assay compounds 1 and 13–18 were almost equally inhibitory to DnaK (Table 3).

DISCUSSION

In this study we have used inhibition analyses to characterize a molecular pathway by which DnaK triggers protein folding in *E. coli* cells. Generally, enzyme inhibition proved to be a successful approach from mechanistic investigations to chemotherapeutic intervention. We previously hypothesized that the APIase activity of the hsp70 chaperone DnaK serves as an interventional strategy to incorrect folds of *de novo* synthesized proteins in bacterial cells (1). Obviously, the new enzyme nature of DnaK opens up the possibility to invent low molecular weight inhibitors of it. In the present study we evaluated the effect of newly developed small molecule inhibitors of the APIase activity of DnaK on the protein aggregation *in vitro* and *in vivo* and revealed that these compounds have bactericidal activity against *E. coli* MC4100 cells. Inhibitors were constructed as peptidomimetics corresponding to three structural portions mimicking the major determinants of the ground state binding properties of DnaK substrates (37). These portions include a hydrophobic tail attached to an aromatic ring with an α-amino acid head, and N^α-[fatty-acyl-(4-aminoalkylbenzoyl)]-L-α-amino acids were found to realize this design. A series of derivatives were also prepared that contained replacements of the three building blocks allowing for the evaluation of structure-activity relationships. We then demonstrated that N^α-[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine, with a molecular mass of 475 Da, inhibited in a dose-dependent manner the DnaK-assisted *cis/trans* isomerization of the Tyr³⁸–Ala³⁹ peptide bond in the RNaseT1 P39A variant. This peptidomimetic compound inhibited 50% of DnaK activity (IC₅₀) at the concentration of 2.7 μM. A similar inhibition profile was determined in the DnaK/DnaJ/GrpE/ATP-assisted refolding of denatured luciferase but at a slightly higher IC₅₀ value of 9.5 μM. Evidently, the slow *cis/trans* isomerization rates at low temperatures make enzyme catalysis an essential component of protein folding (Fig. 2). To assess the need for biocatalysis at elevated temperatures we utilized the refolding of the RNaseT1

P39A variant at 30 °C. We found that it is not only dependent on catalytic assistance by DnaK and thus prevents the formation of a native-like folding intermediate (1) but also indicates inhibition of the APIase activity by N^α-[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine. Similarly, the DnaK/DnaJ/GrpE/ATP-assisted formation of native luciferase from its guanidinium chloride-denatured state is inhibited by this compound in the whole temperature range from 15 to 30 °C (supplemental materials). These data substantiate that inhibition of the APIase activity of DnaK relates to the inhibition of protein folding assistance in the cell under normal growth conditions.

Inhibition was found to be reversible, and compounds did not influence the ATPase activity of DnaK. Because DnaJ-mediated stimulation of ATPase activity (38) could be also observed in the presence of the inhibitor, DnaJ is probably not an additional target of it. As we could show by competition experiments (Fig. 3A), the DnaK-inhibitor complex locates the inhibitor on the peptide binding cleft of the protein. Only a section of it will constitute the catalytic center of the APIase. Because the 14-mer σ³²-derived peptide (σ³²-Q132-Q144-C-IAANS) is displaced from its DnaK binding site by the inhibitor, its active site binding makes the remaining subsite interactions of the 14-mer energetically unfavorable. However, for unfolded polypeptides such as unfolded lactalbumin, a larger number of remote subsites might participate in DnaK-substrate interactions. Consequently, it appeared that N^α-[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine allowed for the separation of the holding function of a chaperone and its APIase-mediated folding function. In a traditional chaperone model, the reaction sequence of DnaK would be consistent with repetitive cycles of binding and release of unfolded segments of client proteins. Besides suppressing aggregation of a client protein (“holding”), this reaction sequence is thought to cause its proper folding to a native protein (“folding”) when used in conjunction with chain dissociation enhancing factors like nucleotides and co-chaperones (39). N^α-[Tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine did not prevent the binding of an unfolded protein to DnaK while the active site-directed peptide NRLLLTG did so (Fig. 3, C and D). The most likely explanation is that the inhibitor cannot extend over the complete array of remote protein substrate subsites leaving many of them unperurbed (40). As a result, the inability of the DnaK-inhibitor complex to act as a folding chaperone in the luciferase assay, despite its efficacy in sequestering unfolded chains, can be most easily explained by inhibition of the APIase function.

Alternatively, blocking the conventional general chaperone activity of DnaK must be considered because the length of the fully extended chain of N^{α} -[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine would suffice to block the complete binding region of NRRLLTGG on DnaK. Site-directed mutagenesis producing a DnaK variant by which a separation between the chaperoning and the APIase activity is possible, would allow a discrimination of the alternative mechanism of action.

Inhibition of heat shock proteins in cells by small molecules may also have direct relevance to the control of intracellular protein folding and thus to pharmacological approaches based on it (41, 42). As a precondition, the inhibitor must block the folding helper protein under cellular conditions. In this study we showed that N^{α} -[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine sequesters DnaK in *E. coli* lysates (Fig. 4). Consequently, the presence of a potent inhibitor in the growth medium caused protein aggregation upon heat shock while less effective DnaK inhibitors did not (Table 1 and Fig. 6). A similar increase in protein aggregation was induced by the lack of DnaK in a DnaK deletion strain (Fig. 6). In addition, DnaK deletion caused a decrease of cell regrowth after severe heat shock at 57 °C. This gene-based effect paralleled the inability of regrowth of cells containing chemically inhibited DnaK. However, regrowth occurred when intracellular DnaK production was enhanced by an inducible plasmid. Regulation of the level of expression of the target enzyme was already shown as an approach of evaluating the origin of the antibacterial activity of inhibitors. Thus, overexpression of peptide deformylase counteracts the growth inhibition in *E. coli* caused by peptide deformylase inhibitors (43). Taken together, the APIase activity of DnaK, which can be switched off by small molecule inhibitors, is likely to control protein aggregation in *E. coli*.

DnaK is known to play a critical role in regulating heat shock response by modulating the activity, stability and amounts of free σ^{32} (34). An increase in the level of DnaK represses the synthesis of σ^{32} -regulated heat shock proteins and in converse lack of DnaK function leads to a significant increase in the σ^{32} activity with constitutively elevated levels of heat shock proteins. In this study we found a direct effect by the prominent DnaK inhibitor N^{α} -[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine on the increase in the transcriptional activity of σ^{32} -regulated genes. The results reported here demonstrate that this effect is a reflection of decreased APIase activity of DnaK, thus making it limiting in the cell. Reduced APIase activity of DnaK will make cellular pools of σ^{32} more accessible to transcription machinery, because the DnaK will presumably be titrated out by virtue of increased accumulation of various substrate proteins that aggregate in the presence of inhibitor in agreement with titration model of heat shock response. These data provide additional support that N^{α} -[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine and its derivatives act at the physiological level involving the APIase site of DnaK, which in turn leads to elevated levels of heat shock gene transcription consistent with already mentioned effects on inducing cellular protein aggregation.

Accordingly, although severe protein aggregation is most likely under stress conditions, bacteria should not maintain viability in the presence of N^{α} -[fatty-acyl-(4-aminomethylben-

zoyl)]-L- α -amino acid derivatives. Indeed, N^{α} -[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine, as an example, was shown to have bactericidal activity, which became apparent under heat stress at 42 °C, leaving cells unaffected at normal growth temperatures of 37 °C. Because the combined deletion of DnaK and the ribosome associated peptidylprolyl-*cis/trans*-isomerase trigger factor in *E. coli* is lethal (4, 5), the susceptibility of MC4100 cells lacking trigger factor toward the inhibitor at 30 °C supports that the inhibition of DnaK forms the direct basis of the bactericidal activity.

MIC was in the range of 100 to $\gg 1000$ $\mu\text{g/ml}$ against MC4100 cells dependent on the constitution of the inhibitors. Under the same conditions ampicillin showed an antibacterial activity of up to 7-fold better. Membrane permeabilization by the inhibitor as origin of antibacterial activity was ruled out by determining the activity distribution of the periplasmic enzyme β -lactamase (44) after incubation of *E. coli* with 200 μM of N^{α} -[tetradecanoyl-(4-aminomethylbenzoyl)]-L-isoleucine for 24 h. The compound had no influence on membrane permeability expressed by the ratio of the extracellular to the total amount of β -lactamase activity in the incubation mixture. In actuality, in the series of benzamido derivatives the inhibitor effects on luciferase refolding *in vitro* did not well correlate with their MIC, in agreement with the notion that differences in the metabolic stability, cell attachment, and uptake of the compounds into *E. coli* cells might also contribute (45). The rather different hemolytic activities of the compounds gave an impression of the importance of the latter effects even for bacterial cells. Alternatively, two homologues of DnaK, HscA and HscC, could be also important for mediating antibacterial activity (31, 46).

In conclusion, the biological studies demonstrated that inhibition of DnaK in *E. coli* MC4100 cells resulted in massive protein aggregation and loss of viability under heat stress. Our study raises the question of whether further improvement of our peptidomimetics by optimization would lead to inhibitors with improved bactericidal activity against MC4100 and human pathogens containing DnaK homologues.

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