and the mixtures were incubated for 30 min on ice in the dark. Rhodamine-labeled phalloidin (10 μM; Sigma) was then added to all samples, and the mixtures were incubated for 1 hour on ice in the dark. The samples were washed three times with buffer A and extracted overnight at 4°C with 0.1 N NaOH. An excitation wavelength of 550 nm and an emission wavelength of 580 nm were used to measure the fluorescence of rhodamine-phalloidin. The fluorescence of the extract that had been treated first with unlabeled phalloidin was subtracted from the fluorescence of the sample not treated with unlabeled phalloidin, and the difference was ascribed to filamentous actin. The rhodamine-phalloidin concentrations in the extracts were calculated from a standard curve generated by exposing phalloidin at a range of concentrations to the same treatment as the sputum samples.

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13. The viscosity measurements were made in a torsion pendulum [P. A. Janmey, *J. Biochem. Biophys. Methods* **22**, 41 (1991)], and values were computed from the rate of creep of the sample when it was subjected to a constant stress of 125 mPa (*6*). Uniform plugs of 0.25 ml were cut from coherent masses of sputum expectorated by CF patients and layered onto round cover slips. Buffer A (10 μl), with or without additives, was transferred to the sputum-covered cover slips, which were pressed against the suspended plate of the torsion pendulum for viscosity measurements. By this method, viscosity values of serial, untreated (buffer A alone) samples cut from the same patient's sputum did not vary over the course of the experiments.
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16. The elastic shear modulus was measured either in the torsion pendulum or in a Bohlin rheometer (Bohlin Co., East Brunswick, NJ) device in parallel plate geometry as described (*6*). The strain measurements were made at an angular frequency of 10 rad s^{-1}, and the strain rate measurements were made at a maximum strain of 1%.
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Kinetics of Molecular Chaperone Action

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Molecular chaperones of the Hsp70 type transiently sequester unfolded segments of proteins and promote their correct folding. Target peptides were labeled with an environmentally sensitive fluorophore so that their binding to the molecular chaperone DnaK of *Escherichia coli* could be followed in real time. The two-step process was characterized by relaxation times of 27 seconds and 200 seconds with 2 μM DnaK and 0.1 μM ligand at 25°C. In the presence of adenosine triphosphate, the formation of the complex was greatly accelerated and appeared to be a single-exponential process with a relaxation time of 0.4 second. The binding-release cycle of DnaK thus occurs in the time range of polypeptide chain elongation and folding and is too fast to be stoichiometrically coupled to the adenosine triphosphatase activity of the chaperone (turnover number, 0.13 per minute at 30°C).

The molecular chaperone DnaK of *Escherichia coli* belongs to the 70-kD heat shock protein (Hsp70) family (*1*, *2*). These proteins were first identified as part of a set of specific proteins that are induced during the cellular response to heat shock. Most of them are constitutively expressed and are essential for cell viability under normal growth conditions. Constitutive Hsp70s are thought to interact transiently with hydrophobic segments of newly synthesized proteins and thus to assist in folding and translocation of the proteins through membranes. DnaK, like its eukaryotic homologs, consists of an evolutionarily variable COOH-terminal peptide recognition domain and a conserved NH_2-terminal adenosine triphosphatase (ATPase) domain (*3*), which has been reported to trigger the release of a bound ligand upon adenosine triphosphate (ATP) hydrolysis (*4*, *5*). The rate of binding of a protein or peptide ligand has not been determined to date, because no kinetic assay for chaperone action has been available. We used peptide ligands labeled with an environmentally sensitive fluorophore, together with a stopped-flow spectrofluorimeter, to determine the kinetics of peptide binding to DnaK and the effects of ATP on peptide binding.

The targeting sequence of the precursor of mitochondrial aspartate aminotransferase is tightly bound by DnaK (*6*). Here, a synthetic 21-residue prepiece was used as a ligand for DnaK (Fig. 1). It was synthesized with an additional cysteine at the NH_2-terminus for the covalent attachment of the fluorescent probe acrylodan (a dimethylaminonaphthalene fluorophore with an acryl group) (*7*). The binding site of BiP, the Hsp70 in the endoplasmic reticulum, accommodates a peptide of seven amino acid residues. It selects for aliphatic residues and excludes charged residues at all positions with the exception of position 7, where positively charged residues are slightly preferred (*8*). If DnaK has a similar binding specificity, we expect that the prepiece contains two preferential sites for binding to DnaK: residues 1 to 7 and 8 to 14 (Fig. 1). On binding to DnaK, the fluorescence spectra of the acrylodan-labeled prepiece (a-pp) and the shorter acrylodan-labeled peptide a-p1 (corresponding to residues 1 to 7 of a-pp) underwent a blue shift; the emission maximum shifted from 525 to 505 nm. Binding to DnaK increased the fluorescence intensity at 500 nm to 185% (a-pp) and 300% (a-p1) of that of the unbound ligand (Fig. 1). The changes in fluorescence arise from an increased hydrophobicity of the microenvironment of the label (*9*). The dissociation constants (K_d), as determined from the hyperbolic binding curves (*10*) obtained by titration of a constant concentration of ligand (50 nM) with increasing concentrations of DnaK, were 63 nM for a-pp and 1.4 μM for a-p1. For the experiments below, a-pp, which bound more tightly, was used as the ligand if not stated otherwise.

Complex formation followed double-exponential kinetics. At 0.1 μM a-pp and 2 μM DnaK, the reaction equilibrated with τ_1 = 27 s and τ_2 = 200 s (Fig. 2). The pseudo–first-order rate constant (k_{obs}) of the first phase of the reaction was linearly proportional to DnaK concentration and corresponded to a bimolecular reaction with a second-order binding rate constant (k_{+1}) of 9400 M^{-1} s^{-1} and a dissociation rate constant (k_{-1}) of 0.004 s^{-1}. The k_{obs} value of the second, slower phase of complex formation increased on raising the concentration of DnaK, which suggested that the formation of an encounter complex might be followed by its isomerization.

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The ability to bind and hydrolyze ATP is common to all Hsp70s. The cycle of ligand binding and release by the chaperones was believed to be stoichiometrically coupled to the hydrolysis of ATP (*4, 5*). The ATP changes the conformation of DnaK, as reflected by altered spectroscopic properties (*11, 12*), a different trypsin digestion pattern, and a reduced affinity for target proteins (*5*). We confirmed that preincubation of DnaK with MgATP decreased its affinity for a-pp. In the presence of ATP, the K_d of the complex was 2.2 μM—that is, 36 times higher than without ATP. However, the most conspicuous effect of ATP was an acceleration of complex formation, necessitating stopped-flow measurements (Fig. 2). The reaction corresponded to a single exponential process and, at 0.1 μM a-pp and 2 μM DnaK, equilibrated with $\tau = 0.4$ s, amounting to a rate 540 times faster than the second, rate-limiting step of complex formation in the absence of ATP. The k_{obs} values were linearly proportional to DnaK concentration, corresponding to $k_{+1} = 4.5 \times 10^5\ M^{-1}\ s^{-1}$ and $k_{-1} = 1.8\ s^{-1}$. Thus, k_{+1} was increased 47-fold and k_{-1} 440-fold compared with the rates of the first, fast phase of complex formation in the absence of ATP. These changes are consistent with the decreased affinity for the ligand.

In the presence of the nonhydrolyzable ATP analog ATP-γ-S (adenosine-5′-[γ-thio]triphosphate), complex formation followed double-exponential kinetics with $\tau_1 = 3$ s and $\tau_2 = 16$ s at 0.1 μM a-pp and 2 μM DnaK (Fig. 2). Thus, the mere binding of ATP seems to bring about a 10-fold acceleration of both reaction steps in complex formation. The ATP-γ-S did not change the affinity of DnaK for the ligand; apparently, it accelerates binding and release by the same factor. Another nonhydrolyzable ATP analog, AMP-PCP (β,γ-methyleneadenosine-5′-triphosphate), had the same effect as ATP-γ-S. Adenosine diphosphate (ADP) (1 mM) was without effect. Complex formation with the peptide a-p1 showed analogous kinetic changes on incubation of DnaK with ATP or ATP-γ-S. To determine the time needed for ATP to take effect, we mixed DnaK in the stopped-flow apparatus with a solution of ligand and ATP (Fig. 3). The reaction curve showed a lag phase of ~0.5 s.

In conclusion, ATP and its nonhydrolyzable analogs bring about kinetic changes in the mode of action of DnaK (Fig. 4). In the presence of ATP-γ-S, which simulates the binding of ATP, the rates of binding and release of the peptide ligand increase to the same extent, with the affinity for the ligand remaining unchanged. Cleavage of ATP further increases the rate of formation of the complex and increases even more the rate of its dissociation, resulting in a decreased affinity for the ligand. DnaK has a weak ATPase activity and is autophosphorylated under certain conditions (*13*). The turnover number (k_{cat}) of the ATPase activity of DnaK was 0.13 min^{-1} at pH 7.0

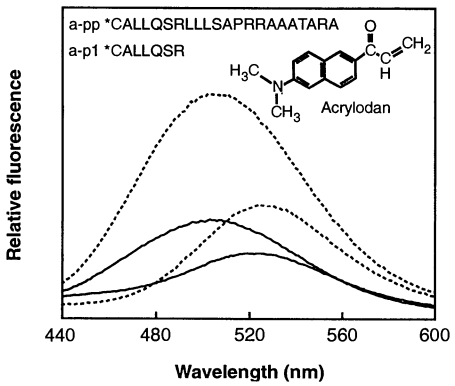

Fig. 1. Fluorescence spectra of free and DnaK-bound acrylodan-labeled peptides. The vinyl group of 6-acryloyl-2-dimethylaminonaphthalene (acrylodan) served to attach the fluorophore (denoted by an asterisk) covalently to the sulfhydryl groups of the cysteine residues of the peptides (*17*). The peptides (50 nM) a-pp (solid lines) and a-p1 (dotted lines) were incubated with 5 μM DnaK (*18*) for 10 min at 25°C in 2.0 ml of 25 mM Hepes (pH 7.0) and 100 mM KCl (assay buffer). The lower lines represent the spectra of the peptides in the absence of DnaK. The acrylodan-mercaptoethanol adduct served as a control; no changes in its fluorescence spectrum were observed on addition of DnaK. The spectra were recorded on a Spex Fluorolog spectrofluorimeter (SPEX Industries, Edison, New Jersey). The excitation wavelength was 370 nm. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; L, Leu; P, Pro; Q, Gln; R, Arg; S, Ser; and T, Thr.

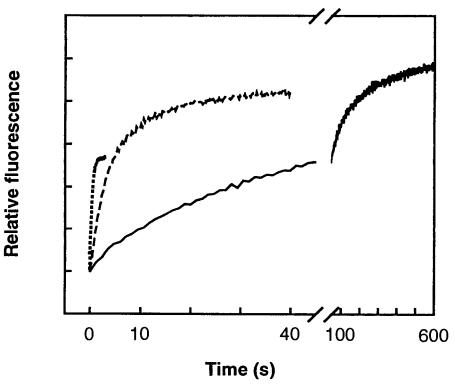

Fig. 2. Effect of ATP and of a nonhydrolyzable ATP analog on the time course of complex formation of DnaK with a-pp. DnaK in assay buffer (see Fig. 1) was kept for 10 min at 25°C without nucleotides (solid line), with ATP (dotted line), or with ATP-γ-S (dashed line). The reaction was started by addition of a-pp in assay buffer. The final concentrations after mixing of equal volumes were as follows: DnaK, 2 μM; a-pp, 0.1 μM; ATP and ATP-γ-S, 1 mM; and $MgCl_2$, 5 mM. The assays were done at 25°C with an SF-61 stopped-flow spectrofluorimeter (HI-TECH Scientific, Salisbury, United Kingdom) with a dead time of 1 ms. The excitation wavelength was set at 370 nm and the emission light passed through a GG455 cutoff filter (HI-TECH Scientific). The decreased final fluorescence in the presence of ATP is due to a decreased affinity of DnaK for the ligand.

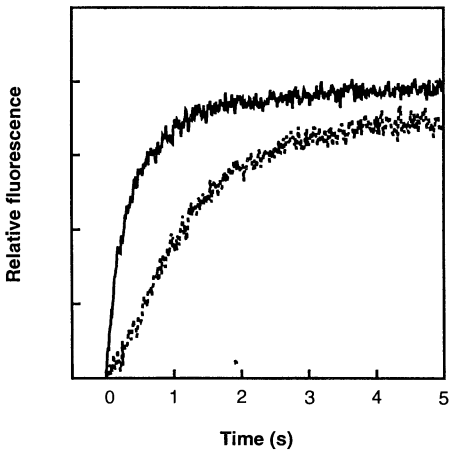

Fig. 3. Delay in the effect of ATP on complex formation. The solid line represents the reaction curve obtained with the stopped-flow spectrofluorimeter on mixing a-pp with a solution of DnaK that had been incubated with MgATP for 10 min at 25°C. The dotted line is the reaction curve obtained on mixing untreated DnaK with a solution of a-pp and MgATP. The final concentrations in assay buffer were the same in both experiments: DnaK, 1 μM; a-pp, 50 nM; ATP, 10 μM; and $MgCl_2$, 10 mM. At a concentration of 10 μM, the effect of ATP is maximal.

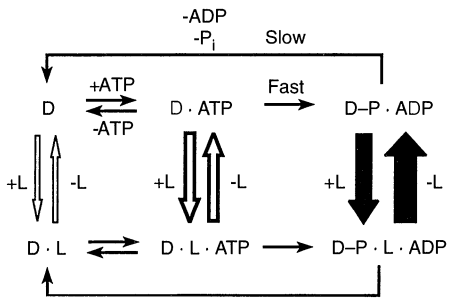

Fig. 4. Hypothetical functional states of DnaK (D) and the control of their interconversion by ATP binding and hydrolysis. The thickness of the vertical arrows indicates the relative rates of binding and release of the ligand L. The figure is simplified in that complex formation of D and D·ATP (as simulated with nonhydrolyzable analogs) with a ligand was actually found to proceed in two phases. D–P denotes Dnak with (possibly covalently) bound phosphate. The binding-release cycle predominant in the presence of ATP and in the absence of modulatory proteins is denoted by solid arrows.

and 30°C and was independent of the presence of the ligand a-pp (*14*). Thus, the binding-release cycle of DnaK appears not to be stoichiometrically coupled with the hydrolysis of ATP. In the presence of ATP, the binding-release cycle is at least 830 times faster than ATP hydrolysis. Furthermore, nonhydrolyzable ATP analogs also accelerate both binding and dissociation, although less effectively than ATP by one order of magnitude. The following observations suggest that the rate-limiting step for ATP hydrolysis is the release of ADP and inorganic phosphate (P_i) rather than ATP cleavage. The effect of ATP is evident after a delay of only 0.5 s (Fig. 3) although the overall ATPase activity is much slower. However, the effect does not appear to be due to the mere binding of ATP because it is clearly different from the effect of its nonhydrolyzable analogs. In the presence of ATP (Fig. 4), most DnaK molecules may thus be assumed to contain a phosphate group perhaps covalently attached to Thr^{199}, which has been shown to be phosphorylated during incubation of DnaK with ATP in vitro (*15*).

What is the physiological significance of the very weak ATPase activity of DnaK if it is not stoichiometrically coupled to the binding-release cycle? This study shows that ATP and its turnover control the kinetics of the chaperone action. The binding-release cycle is much faster in the presence of ATP—that is, under physiological conditions—than was previously thought and it occurs in the same time range as polypeptide chain elongation and folding. Tuning of the rates of ATP cleavage and release of ADP and P_i by the accessory Hsp's DnaJ and GrpE of *E. coli* (*16*) might thus shift the proportions of the three functional states of DnaK (Fig. 4) and adapt the kinetics of its chaperone action to the changing requirements of protein biogenesis.

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17. The peptides were synthesized with an ABI 430A Peptide Synthesizer (Applied Biosystems, Foster City, CA) by means of the orthogonal fluorenyl methoxycarbonyl protection strategy [G. Barany, N. Kneib-Cordonier, D. G. Mullen, *Int. J. Pep. Protein Res.* **30**, 705 (1987)]. Acrylodan was purchased from Molecular Probes (Eugene, OR). For labeling, acrylodan (final concentration, 1 mM) was freshly dissolved in acetonitrile and added to the peptide (0.25 mM) in 20 mM Hepes (pH 7.5). The mixture was incubated overnight at room temperature. Unreacted acrylodan was removed by gel filtration. Reversed-phase high-performance liquid chromatography with an acetonitrile–water–trifluoroacetic acid gradient removed unreacted peptide. The concentration of the labeled peptide was determined photometrically (molar extinction coefficient ϵ_{360} = 12,900 M^{-1} cm^{-1}) (*7*) and by amino acid analysis.
18. The plasmid pTTQ19*dnaK*$^+$ was used to produce DnaK. The expression is under the control of the *tac* promoter, which is inducible by isopropyl-thio-β-D-galactoside. The *E. coli* strain JM83 was used for expression. The collected cells were resuspended in 20 mM tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT) (buffer A), and sonicated. After ultracentrifugation (100,000*g* for 30 min), the supernatant was dialyzed against buffer A and loaded onto an anion-exchange column (Q Sepharose Fast Flow, Pharmacia). DnaK was eluted at the end of a linear gradient of 50 to 500 mM NaCl. The DnaK-containing fractions were pooled and dialyzed against 20 mM sodium acetate (pH 5.5), 0.1 mM EDTA, and 0.1 mM DTT (buffer B), and passed over a 5-ml column of ATP-agarose (C_8 linkage, Sigma). The column was washed with 1 M NaCl in buffer B, and DnaK was then eluted with 5 mM ATP, 7 mM $MgCl_2$, and 10 mM KCl in buffer B. Fractions containing DnaK were concentrated by ultrafiltration and loaded onto a Superose 12 fast protein liquid chromatography column (HR 10/30, Pharmacia) in 25 mM Hepes (pH 8.0), 10 mM 2-mercaptoethanol, and 1 mM EDTA. Fractions with pure DnaK, as analyzed by SDS–polyacrylamide gel electrophoresis were collected and stored in 10% glycerol at −20°C.

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Role of NO Production in NMDA Receptor–Mediated Neurotransmitter Release in Cerebral Cortex

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L-Glutamate and norepinephrine are examples of a major excitatory neurotransmitter and a neuromodulator in the cerebral cortex, respectively. Little is known of how chemical signaling between the anatomically distinct chemical pathways occurs. Specific activation of the *N*-methyl-D-aspartate (NMDA) class of glutamate receptor in synaptosomal preparations from guinea pig cerebral cortex caused release of both of these chemicals, and this release was blocked by agents that inhibit nitric oxide (NO) production or remove NO from the extracellular space. Furthermore, neurotransmitter release correlated with cortical NO production after NMDA receptor stimulation. These results suggest that NO production and its extracellular movement may be links in the pathway from NMDA receptor activation to changes in chemical signaling in surrounding synaptic terminals in the cerebral cortex.

Activation of the NMDA glutamate receptor on neurons in the vertebrate central nervous system is important for excitatory synaptic transmission (*1*, *2*), developmental and synaptic plasticity (*2*, *3*), and neurotoxicity (*4*) and can lead to the production of the membrane-permeant gas NO (*5*). Production of NO may contribute to neurotoxicity (*6*), possibly by means of the formation of peroxynitrite (ONOO) from NO and the superoxide ion (*7*). Further, NO may participate in synaptic plasticity at glutamatergic synapses in the hippocampus (*8*) [carbon monoxide (CO), another membrane-permeant gas made in the brain, also may play a role in this plasticity (*9*, *10*)]. However, the mechanisms underlying these actions remain to be elucidated.

In the cerebral cortex, the role of NO in synaptic transmission and plasticity is murkier. Nitric oxide and other potential neuromodulators such as norepinephrine (*11*) are likely to be important in both transient (*12*) and more long-lasting (*9*, *13*) alterations in functional forebrain circuitry. Pharmacologi-

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