The polypeptide binding and release cycle of the molecular chaperone DnaK (Hsp70) of *Escherichia coli* is regulated by the two co-chaperones DnaJ and GrpE. Here, we show that the DnaJ-triggered conversion of DnaK-ATP (T state) to DnaK-ADP-P$_i$ (R state), as monitored by intrinsic protein fluorescence, is monophasic and occurs simultaneously with ATP hydrolysis. This is in contrast with the T→R conversion in the absence of DnaJ which is biphasic, the first phase occurring simultaneously with the hydrolysis of ATP (Theyssen, H., Schuster, H.-P., Puckschies, L., Bukau, B., and Reinstein, J. (1996) *J. Mol. Biol.* 263, 657–670). Apparently, DnaJ not only stimulates ATP hydrolysis but also couples it with conformational changes of DnaK. In the absence of GrpE, DnaJ forms a tight ternary complex with peptide-DnaK-ADP-P$_i$ ($K_d = 0.14$ μM). However, by monitoring complex formation between DnaK (1 μM) and a fluorophore-labeled peptide in the presence of ATP (1 mM), DnaJ (1 μM), and varying concentrations of the ADP/ATP exchange factor GrpE (0.1–3 μM), substoichiometric concentrations of GrpE were found to shift the equilibrium from the slowly binding and releasing, high-affinity R state of DnaK completely to the fast binding and releasing, low-affinity T state and thus prevent the formation of a long lived ternary DnaJ-substrate-DnaK-ADP-P$_i$ complex. Under *in vivo* conditions with an estimated chaperone ratio of DnaK:DnaJ:GrpE = 10:1:3, both DnaJ and GrpE appear to control the chaperone cycle by transient interactions with DnaK.

Heat shock proteins of the Hsp70 family are involved in essential cellular processes such as protein folding (1), the translocation of proteins through biological membranes (2), the oligomeric assembly of proteins (3) and their degradation (4). Hsp70s exert their function in conjunction with cycles of binding and hydrolysis of ATP which for their part are regulated by proteinaceous cofactors.

DnaK, the Hsp70 homolog of *Escherichia coli*, is controlled by the two co-chaperones DnaJ (41 kDa) and GrpE (22 kDa), which if acting together increase the weak ATPase activity of DnaK by 2 orders of magnitude (5–7). DnaJ accelerates the rate of γ-phosphate cleavage of DnaK-bound ATP (5, 6), whereas GrpE promotes the release of ADP (5). Several model cycles for the DnaK/DnaJ/GrpE molecular chaperone machinery and their interaction with target polypeptides have been proposed. In two of those models (8, 9), DnaJ rather than DnaK interacts first with the substrate polypeptide and targets it for DnaK. Following DnaJ-induced hydrolysis of DnaK-ATP to DnaK-ADP-P$_i$, a ternary DnaJ-substrate-DnaK-ADP-P$_i$ complex is formed. The ternary complex is thought to be responsible for the chaperone effect by sequestering the substrate protein and thereby preventing it from aggregation (8). This mechanism requires equimolar concentrations of DnaK and DnaJ for every turn of the chaperone cycle. In another model (6), the role of DnaJ is to convert the low-affinity DnaK-ATP form to the high-affinity DnaK-ATP-P$_i$ form, thereby locking the chaperone onto the target polypeptide. In this model, DnaJ may act in a catalytic manner and fulfill its action without forming a stable ternary complex with peptide-DnaK-ATP. Such a catalytic effect of DnaJ has been directly shown in the binding of the α$^{32}$P heat shock transcription factor to DnaK; DnaJ promotes the binding without becoming itself part of the DnaKα$^{32}$P complex (10).

By measuring the kinetics of the individual steps of the DnaK/DnaJ/GrpE chaperone cycle, we have recently shown that at intracellular concentrations of chaperone and co-chaperones, the rate-limiting step of the cycle is the DnaJ-triggered conversion of the ATP form of DnaK (T state) with low affinity for target peptides to its ADP form (R state) with high affinity for target peptides. The ratio of the rates of the T→R and R→T conversions indicates, that the R state is only a minor form of DnaK (7). Since DnaK in the presence of ATP and the absence of the co-chaperones has no or only a minor chaperone effect (11) and since the peptide segment bound to the R state of DnaK is in an extended conformation (12) and thus represents the result of any disaggregating and disentangling action, the power stroke, i.e. the step which underlies the chaperone effect of DnaK, must be the slow ATP-driven T→R conversion. ATP hydrolysis might provide energy for performing conformational work on the peptide during the T→R conversion. In this hypothetical chaperone cycle both the ternary DnaJ-polypeptide-DnaK-ADP-P$_i$ complex and the polypeptide-DnaK-ADP-P$_i$ complex are only short-lived intermediates that are not directly responsible for the chaperone effect (7). The present study investigates the dependence of the chaperone cycle on the concentrations of the co-chaperones. The results support the notion that substoichiometric amounts of DnaJ and GrpE suffice to control the DnaK chaperone cycle.

**MATERIALS AND METHODS**

**Proteins**—DnaK was purified and prepared for experimentation as described (7, 13). Protein concentration was determined photometrically with a molar absorption coefficient of $\varepsilon_{280} = 14500$ M$^{-1}$ cm$^{-1}$ (14). Purified DnaK contained $<0.1$ mol of ADP per mol of DnaK (15). The concentrations of DnaK stock solutions were in the range of 60 to 115 μM. All concentrations of DnaK and the co-chaperones refer to their protomers. GrpE and DnaJ were prepared as described previously (16,
DnaK was incubated with [2,5-9,8-3H]ATP (*ATP; Amersham) for 10 min counting (5). The concentrations of the stock solutions of α-p5 were 24–34 mM nonaphthalene; *ATP, [2,5-9,8-3H]ATP. 0.7 M LiCl. *ATP and *ADP were quantified by liquid-scintillation counting (5).

Fluorescence Spectra—Fluorescence spectra of the acrylodan-labeled peptide were recorded with a Spex Fluorolog spectrofluorimeter in a 0.2-cm cuvette using excitation slits of 4.6/4.6 nm and emission slits of 18.5/9.2 nm. The excitation wavelength was set at 370 nm. Spectra of the intrinsic fluorescence of DnaK were either recorded with the SPEX spectrofluorimeter with excitation slits of 0.9/0.9 nm and emission slits of 18.5/9.2 nm (experiment of Fig. 1) or a Perkin-Elmer LS 50B spectrofluorimeter in a 1-cm cuvette using excitation and emission slits of 5 nm (experiment of Fig. 2). Excitation wavelength was set in both cases at 290 nm.

Kinetic Measurements—For measuring changes in the fluorescence of the acrylodan-labeled peptides, emission spectra were recorded at 580 nm. For following changes in the intrinsic fluorescence of DnaK, emission was recorded at 340 nm. Single-turnover experiments were measured with the SPEX fluorometer with excitation slits of 0.9/0.9 nm and emission slits of 18.5/9.2 nm (experiment of Fig. 3B) or the Perkin-Elmer spectrofluorimeter, with excitation and emission slits of both 5 nm (experiment of Fig. 3A) using a 0.4-cm cuvette with a magnetic stirrer. To ensure rapid mixing, the reactant by which the reaction was started was injected with a syringe. The dead time was thus reduced to approximately 2 s. The experiments of Figs. 6–8 were performed with a SF-61 stopped-flow spectrofluorimeter (Hi-Tech Scientific, Salisbury, UK) with a dead time of 1 ms. For measuring the fluorescence of labeled target peptides, the excitation wavelength was set at 370 nm and the emission light was passed through a GG455 cut-off filter. The kinetic data obtained with the spectrofluorometers were fitted with the program Sigma Plot (Jandel Scientific), which provides the asymptotic standard deviation of the calculated parameters for each evaluation. In the case of the stopped-flow experiments, the average of 3–10 measurements was fitted to a single or double exponential decay or rise function.

Results and Discussion

By monitoring changes in the tryptic digestion pattern, three different conformational states of DnaK can be detected, the nucleotide-free state of DnaK and the ADP- and ATP-ligated states (19). Another possibility to discern the different conformational states of DnaK is by following the intrinsic tryptophan fluorescence of DnaK upon addition of nucleotides. DnaK contains a single tryptophan residue (Trp-102) in its ATPase domain. By this technique, Theyssen et al. (19) recently confirmed the existence of three different conformational states of DnaK. We obtained virtually the same results, which among others are reproduced in Fig. 1 to facilitate understanding of the experiments below. While addition of ATP to nucleotide-free DnaK (Fig. 1, spectrum a) caused a blue shift from 349 to 345 nm and led to a 18% decrease of the relative fluorescence at 349 nm (spectrum b), hydrolysis of DnaK-bound ATP to ADP and P, reversed the blue shift and increased the fluorescence again to a value 4% higher than that of the nucleotide-free state (spectrum c). In this experiment, a slight excess of ATP over DnaK was used (DnaK, 1 μM; ATP, 1.4 μM).

The DnaJ-induced Change in Intrinsic Fluorescence of DnaKATP Is Due to Hydrolysis of ATP and Not to DnaK-DnaJ Complex Formation—Since both DnaJ and GrpE are devoid of tryptophan (20, 21), effects of the co-chaperones on the tryptophan fluorescence of DnaK can easily be observed. Addition of DnaJ to DnaK in the presence of excess ATP (1 mM) increased the fluorescence at 349 nm from 82% of that of the nucleotide-free state to 90% and reversed the ATP-induced blue shift, resulting in a fluorescence spectrum (Fig. 1, spectrum d) intermediary to the spectra of nucleotide-free and ATP-ligated DnaK (spectra a and b). In a recently published report suggesting a model of the DnaK/DnaJ/GrpE reaction cycle, DnaJ was also found to increase the fluorescence of DnaK, which contains no tryptophan residues (20), was negligible at wavelengths above 325 nm.

Fig. 1. Influence of ATP and DnaJ on the intrinsic tryptophan fluorescence of DnaK. a, DnaK (1 μM) in assay buffer containing 5 mM MgCl2. DnaK contained <0.1 μM of ADP per mol (15). b, DnaK after addition of 1.4 μM ATP and 10 min of incubation. With a Kd for ATP of 7.3 μM (19), DnaK is saturated to 98%. Addition of excess ATP (1 mM) to DnaK had the same effect on the intrinsic fluorescence of DnaK (not shown). c, DnaK-ADP-Pi, produced by hydrolysis from DnaK-ATP (spectrum b). The solution was incubated for 4 h. d-f, DnaK was incubated with assay buffer containing 5 mM MgCl2 and 1 mM ATP for 5 min (d), with 36 μM ATP for 4 min (e), and with 1.4 μM ATP for 10 min (f). After binding of ATP to DnaK, 1 μM DnaJ was added to convert DnaK-ATP to the ADP-state. After having reached the steady state or equilibrium, respectively, spectra d, e, and f were recorded. The excitation wavelength for all spectra was 290 nm. Under the conditions used, the tyrosine fluorescence of DnaJ, which contains no tryptophan residues (20), was negligible at wavelengths above 325 nm.

1 The abbreviations used are: acrylodan, 6-acryloyl-2-dimethylaminonaphthalene (acyrloylan); *ATP, [2,5-9,8-3H]ATP.

2 ADP and P, bind to DnaK with Kd values of 0.13 and 0.45 μM, respectively (19). They are thus assumed to be exchanged together against ATP.
DnaJ complexes has proven most difficult (23). After the other. In accordance with this notion, the isolation of (see below). Thus, the action of DnaJ is that of a catalytic DnaK with presumably high on and off rates and low affinity completely from the ATP- to the ADP-liganded state. This chiometric concentrations of DnaJ suffice to convert DnaK formation in the presence of 0.2 μM DnaJ, a correspondingly smaller increase in fluorescence of DnaK (1 μM) would ensue. Under single-turnover conditions, substoichiometric concentrations of DnaJ suffice to convert DnaK completely from the ATP- to the ADP-liganded state. This finding implies that DnaJ forms a short-lived complex with DnaK with presumably high on and off rates and low affinity (see below). Thus, the action of DnaJ is that of a catalytic trigger switching on the ATPase activity of one DnaK molecule after the other. In accordance with this notion, the isolation of DnaK-DnaJ complexes has proven most difficult (23).

The DnaJ-triggered Conformational Change T→R of DnaK Occurs Concomitantly with ATP Hydrolysis—Recently, the change in intrinsic fluorescence accompanying the spontaneous conversion of DnaK-ATP to DnaK-ADP-Pγ in the absence of DnaJ was shown to be a two-step process with \( k_1 = 0.0018 \text{s}^{-1} \) and \( k_2 = 0.007 \text{s}^{-1} \) (19). Since the rate \( k_1 \) was virtually identical to the rate of ATP hydrolysis \( (k_{\text{hyd}} = 0.0015 \text{s}^{-1}) \), the first step was ascribed to the hydrolysis of ATP and the second step to a subsequent structural change. We have shown previously that the T→R conversion of DnaK (1 μM), triggered by DnaJ (1 μM) and measured under single-turnover conditions, is a monophasic process with \( k_{\text{obs}} = 0.044 \text{s}^{-1} \). The tryptophan spectrum of the isolated ATPase domain (DnaK1–385) shows only minor changes upon binding of ATP and does not undergo the characteristic ATP-induced blue shift. Therefore, it was concluded that the ATP-induced changes in intrinsic fluorescence of the complete DnaK molecule derive from interaction of the exposed Trp residue (Trp-102) in the ATPase domain with the peptide-binding domain (18). According to this notion, the DnaJ-triggered changes in fluorescence of DnaK reflect the T→R conversion of DnaK. Because the affinity for peptide ligands is low in T-state DnaK and is enhanced in the R state, the slow T→R transition ought to be observable also by following the binding of a target peptide. We followed the T→R transition under single-turnover conditions by intrinsic fluorescence and compared its rate with that of complex formation between DnaK and the fluorescence-labeled peptide a-p5 in the course of the T→R conversion (Fig. 3A). The nonapeptide p5 (for its sequence, see “Materials and Methods”) corresponds to the main binding site for DnaK of the 23-residue prepiece of mitochondrial aspartate aminotransferase; a-p5 is a high-affinity ligand for DnaK with \( K_d \) values of 5.1 μM and 0.07 μM in the presence of ATP and ADP plus Pγ, respectively (7). The change in intrinsic fluorescence and the binding of the peptide proceeded with virtually the same rate of 0.003 s−1. Under the conditions used, 16 and 93% of a-p5 are expected to be bound to T- and R-state DnaK, respectively. Indeed, during the T→R transition, the overall reaction amplitude was 5.3-fold expanded compared with the amplitude for the reaction a-p5 + DnaK-ATPγS before the addition of DnaJ.

To explore the temporal relationship between ATP hydrolysis and the T→R conversion, we followed the DnaJ-stimulated single-turnover ATP hydrolysis under the same experimental conditions as for measuring the DnaJ-induced conformational change T→R (Fig. 3B). Under these conditions, ATP was hydrolyzed with a turnover number of 0.05 (± 0.01) s−1. Thus, ATP hydrolysis occurred with virtually the same rate as the conformational switch T→R (Ref. 7). Apparently, in the presence of DnaJ unlike in its absence (see above), the conformational changes of DnaK are tightly coupled with ATP hydrolysis. Binding of DnaJ to DnaK has been proposed to stabilize the α-helical lid of the peptide-binding domain of DnaK, thereby favoring the ADP state over the ATP state and promoting hydrolysis (12).

Stimulation of the Rate of γ-Phosphate Cleavage Is Due to Formation of a Transient DnaK-DnaJ Complex—A low concentration of DnaJ (1.2 μM; DnaJ, 22 nM) was reported previously to shorten the half-life of DnaK-bound ATP from 9.1 to 1.8 min, indicating that DnaJ stimulates the ATPase activity of DnaK in a catalytic manner (10). We measured the DnaJ-induced acceleration of the single-turnover rate of ATP hydrolysis as a function of the concentration of DnaJ (Fig. 4). The stimulation depended linearly on the concentration of DnaJ. At concentrations of DnaK and DnaJ of 1 and 0.1 μM, respectively, DnaJ stimulated the ATPase approximately 20-fold. This degree of acceleration is of physiological relevance since the concentrations of DnaK and DnaJ in E. coli are in the micromolar.

\[^3\] Gisler, E. V., Pierpaoli, and P. Christen, manuscript in preparation.
range (20, 24), and their ratio is approximately 10:1 (20). At a concentration of DnaJ of 2 μM, a >900-fold acceleration was reached. The plot of the rate of single-turnover ATP hydrolysis as a function of the concentration of DnaJ indicates the $K_d$ value for DnaJ in the DnaK-ATP-DnaJ complex to be considerably higher than 2 μM (Fig. 4B), which agrees with the notion of a relatively weak DnaK-ATP-DnaJ interaction.

In the Absence of GrpE, DnaJ Forms a Ternary Complex with Peptide-DnaK-ADP-P$_i$. DnaJ is known to form ternary complexes with peptide-DnaK in the presence of ATP (8, 9, 25, 26). To examine whether DnaJ also forms a ternary complex with peptide a-p5 and DnaK in the presence of ADP and P$_i$, a-p5-DnaK-ADP-P$_i$ complex was preformed under conditions such that all peptide was bound. Addition of DnaJ to the preformed complex indeed led to a further increase in fluorescence in the region from 440 to 500 nm and was accompanied by a blue shift from 500 to 484 nm, indicating an additional interaction between the label of DnaK-bound peptide and DnaJ (Fig. 5). From the titration of a-p5-DnaK-ADP-P$_i$ with DnaJ, a $K_d$ value of 0.14 μM was calculated.

The $K_d$ value in the presence of a target peptide is at least 15-fold lower than the estimated $K_d$ value of the DnaK-ATP-DnaJ complex ($K_d > 2 \mu M$; see Fig. 4B). The latter high $K_d$ value apparently reflects the low affinity between DnaJ and T-state DnaK, whereas the $K_d$ value of 0.14 μM corresponds to the affinity of DnaJ for the peptide-liganded R state. Since peptide a-p5 is short (9 residues), a large portion of the peptide chain (5 to 7 residues; Ref. 12) can be assumed to interact with the peptide binding domain of R-state DnaK and to be inaccessible for direct binding of DnaJ.

DnaJ has been shown to trigger the conversion of DnaK from the fast one-step-binding, low-affinity T state to the slow two-step-binding, high-affinity R state by stimulating hydrolysis of DnaK-bound ATP to ADP-P$_i$ (6, 7). To test the effect of varying concentrations of DnaJ on peptide binding of DnaK, we measured the rate of complex formation between a-p5 (50 nM) and DnaK (1 μM) in the presence of excess ATP (1 mM) and varying concentrations of DnaJ (0–3 μM). The fast one-step reaction for the a-p5-DnaK complex formation in the absence of DnaJ was transformed to a slow two-step process already at 0.1 μM DnaJ, indicative of conversion of DnaK from the T to the R state (Fig. 6). At all concentrations of DnaJ from 0.2 to 3 μM, the rate $k_{obs1}$ of the faster first phase was reduced 20-fold compared with that of complex formation in the absence of DnaJ. As expected

![Fig. 3. DnaJ-stimulated conversion of DnaK-ATP (T state) to DnaK-ADP-P$_i$ (R state). A, for following the T→R conversion under single-turnover conditions, ATP (final concentration 1.4 μM) was added to 1 μM DnaK in assay buffer containing 5 mM MgCl$_2$. Intrinsic fluorescence at 340 nm was recorded until the ATP-induced decrease had leveled off after about 100 s. DnaJ (0.075 μM) was then added at time zero and the increase in fluorescence recorded. The excitation wavelength was 290 nm. To follow the binding of the peptide to DnaK during the T→R conversion, 1 μM DnaJ was incubated in assay buffer containing 5 mM MgCl$_2$ for 5 min. ATP (final concentration 1.4 μM) was added as above. After binding of ATP to DnaK (~ 100 s), peptide a-p5 (final concentration 50 nM) was added. Under these conditions, the complex formation of a-p5 and DnaK is very fast (completed after ~ 2 s; Ref. 7). After an interval of 35 s, 0.075 μM DnaJ was added at time zero, and the increase of the acrylodan fluorescence was followed. Excitation wavelength was 370 nm. The two fluorescence time courses have been normalized. Fitting to single exponential functions gave $k_{obs} = 0.003 (\pm 0.00001) \text{s}^{-1}$ and $k_{obs} = 0.004 (\pm 0.00002) \text{s}^{-1}$ for the change in intrinsic fluorescence and the binding of the peptide, respectively. B, for determining the stimulation of the single-turnover ATPase activity of DnaK (●), DnaK with bound *ATP in assay buffer containing 5 mM MgCl$_2$, was separated from excess *ATP at 4 °C with a NICK column and reincubated at 25 °C. The reaction of DnaK-*ATP (1 μM) was started by addition of DnaJ (1 μM). Aliquots were taken at the indicated times, and the reaction was stopped by precipitation of the proteins in 13% formic acid. *ADP was separated from *ATP by thin layer chromatography. For experimental details, see "Materials and Methods." The T→R conversion (○) was followed as described in panel A except that 1 μM DnaJ was used. The experimental points were fitted to single exponential functions.](http://www.jbc.org/)

![Fig. 4. Stimulation of the single-turnover ATPase activity of DnaK by DnaJ. A, single-turnover hydrolysis of ATP by DnaK (DnaK-ATP complex, 1 μM) in the absence of DnaJ (●), in the presence of 0.1 μM DnaJ (■), 1 μM DnaJ (○), 2 μM DnaJ (▲). The experiments were carried out as described in the legend of Fig. 3B. B, to determine the turnover rates $k_{obs}$ the experimental points of the corresponding reaction curves in Fig. 4A were fitted to single-exponential functions.](http://www.jbc.org/)
Control of the DnaK Chaperone Cycle by DnaJ and GrpE

The aim of this study was to gain more detailed insight into the regulation of the DnaK chaperone cycle by the co-chaperones DnaJ and GrpE. In particular, the question was addressed whether the co-chaperones participate in a stoichiometric manner in the chaperone cycle as proposed in several hypothetical models or whether they rather serve as regulatory catalysts.

The DnaJ-overriding effect of GrpE can also be observed by the tryptophan fluorescence of DnaK (7). Addition of stoichiometric concentrations of GrpE to DnaK-ADP-P₆ produced by DnaJ-triggered hydrolysis of DnaK-ATP in the presence of 1 mM ATP decreased the fluorescence exactly to that of DnaK in the presence of ATP alone, indicating that GrpE converted DnaK from the R state back to the T state. Almost the same effect was obtained using substoichiometric concentrations of GrpE, confirming the notion of GrpE acting in a catalytic manner.

Conclusion—The aim of this study was to gain more detailed insight into the regulation of the DnaK chaperone cycle by the co-chaperones DnaJ and GrpE. In particular, the question was addressed whether the co-chaperones participate in a stoichiometric manner in the chaperone cycle as proposed in several hypothetical models or whether they rather serve as regulatory catalysts.

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Fig. 6. DnaJ-induced conversion of T- to R-state DnaK as a function of the concentration of DnaJ. Solutions of a-p5 and the proteins were each prepared in assay buffer containing 1 mM ATP and 5 mM MgCl₂ and incubated for 10 to 15 min in the syringes of the stopped flow apparatus as indicated by the following brackets: [DnaK + ATP] + [a-p5 + ATP]. After mixing equal volumes of both solutions, kinetics of the a-p5-DnaK complex formation were measured as described under “Materials and Methods.” Final concentrations were: a-p5, 50 nM; DnaK, 1 μM; and DnaJ, 0–5 μM. The reaction curves were fitted to a single exponential function in the absence of DnaJ and to double exponential functions in its presence. The values of kₒᵇ of the first phases of the reaction curves (●) were plotted as a function of the concentration of DnaJ.

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The rate kₒᵇ of the first phase of complex formation between a-p5 and DnaK to that in the absence of GrpE.

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from previous experiments (see above), substoichiometric concentrations of DnaJ suffice to convert DnaK to the slow binding- and releasing-, high-affinity R state. The data should not be taken to indicate that, under the conditions used (1 mM ATP), all DnaK is converted to the R state; particularly at low DnaJ concentrations, the rate of spontaneous ADP/ATP exchange will suffice to maintain a fraction of the chaperone in the T state (see above).

Substoichiometric Concentrations of GrpE Prevent Formation of the Ternary DnaJ-Peptide-DnaK-ADP-P₆-Complex—At equimolar concentrations of DnaK and the two co-chaperones, GrpE reverses the DnaJ-induced conformational switch of DnaK from the T to the R state, indicating that the GrpE-mediated ADP/ATP exchange is faster than the DnaJ-triggered hydrolysis of DnaK-bound ATP (7). To titrate this effect of GrpE, the formation of the a-p5-DnaK complex in the presence of ATP was followed at constant concentrations of DnaK and DnaJ (1 μM each) and varying concentrations of GrpE (Fig. 7A). The rate kₒᵇ, in the case of a single-step process, or kₒᵇ1, in the case of a double-step process, and the total reaction amplitudes were determined. The rate for the first phase increased at least 110-fold at 0.5 μM GrpE compared with the reaction without GrpE (Fig. 7B). Apparently, catalytic concentrations of GrpE maintain DnaK in its fast-binding-and-releasing T state. A further increase in the concentration of GrpE decelerated the binding reaction.

This observation was supported by analysis of the amplitudes of the reaction curves. The reaction amplitudes corre-
In several studies, ternary DnaJ-polypeptide-DnaK complexes have been isolated. In the presence of ATP, denatured luciferase coelutes with DnaK and DnaJ in a higher molecular mass complex of ~250 kDa on size-exclusion chromatography (8). DnaK and DnaJ also form ternary complexes with RepA (26, 27), aP-protein (28) and s32 (9, 25), reduced carboxymethylated lactalbumin, and denatured rhodanese (29). The DnaJ-s32-DnaK complex is quite stable, the Kd value of DnaJ for s32-DnaK having been estimated to be below 1 μM (9). The dissociation constant of 0.14 μM for DnaJ and a-p5-DnaK-ADP (Fig. 5) also indicates a tight interaction. Based on the existence of such complexes, formation of the ternary complex was proposed to be the key step of the DnaK/DnaJ/GrpE-chaperone cycle; in the complex, the polypeptide-substrate is assumed to be shielded from aggregation (8).

The present data show, however, that substoichiometric concentrations of GrpE in the presence of ATP suffice to dissociate such ternary complexes. The GrpE-induced dissociation of the peptide-DnaKADP-Pi complex in the presence of excess ATP was already apparent at concentrations of DnaK/DnaJ/GrpE of 1 μM/1 μM/0.2 μM, respectively, and is not only observed with a-p5 but also with a set of other peptides. Obviously, transient interaction of GrpE with DnaK suffices for GrpE to become effective. In E. coli, the concentrations of the chaperone and the co-chaperones are in the micromolar range, and the molar ratio of DnaK:DnaJ:GrpE is approximately 10:1:3 (20, 24, 30). Under these conditions, the formation of ternary DnaJ-peptide-DnaKADP-Pi complexes seems highly unlikely, at least for polypeptides that themselves are not bound by DnaJ.

In a study on the regulation of the DnaK/DnaJ/GrpE chaperone cycle by DnaJ and GrpE, solutions of a-p5 and proteins were each prepared in assay buffer containing 1 mM ATP and 5 mM MgCl2 and incubated for 10 min in the syringes of the stopped flow apparatus as indicated by the following brackets: (a) [DnaK + DnaJ + ATP] + [a-p5 + ATP], and (b–f) [DnaK + DnaJ + GrpE + ATP] + [a-p5 + ATP]. The final concentrations of GrpE are as indicated in the figure. The final concentrations of the other reactants were: a-p5, 50 nM; and DnaK, DnaJ, 1 μM (constant in all experiments). After mixing equal volumes of both solutions, kinetics of the a-p5-DnaK complex formation were measured as described under “Materials and Methods.” The obtained reaction curves were fitted to double exponential functions in the experiments with [GrpE] = 0–0.8 μM (reaction progress curve at 0.8 μM not shown) and to single-exponential functions in the experiments with [GrpE] = 1 and 3 μM. The values of kobs of the first phase of the binding process (●) and the total signal amplitude (■) were plotted against the concentration of GrpE.
Control of the DnaK Chaperone Cycle by DnaJ and GrpE

Fig. 8. Effect of DnaJ on the complex formation of DnaK with a-p5 in the presence of GrpE and ATP. Solutions of a-p5 and proteins were each prepared in assay buffer containing 1 mM ATP and 5 mM MgCl₂ and incubated for 10 min in the syringes of the stopped flow apparatus as indicated by the brackets: [DnaK + DnaJ + GrpE + ATP] + [a-p5 + ATP]. After mixing equal volumes of both solutions, kinetic studies of the formation of the a-p5-DnaK complex were measured as described under "Materials and Methods." Final concentrations were: a-p5, 50 nM; DnaK, GrpE, 1 mM, and DnaJ, 0–3 mM. The obtained binding curves were fitted to a single exponential function in the experiments with [DnaJ] = 0–1 mM and to double exponential functions in the experiments with [DnaJ] = 2–5 μM.

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Control of the DnaK Chaperone Cycle by Substoichiometric Concentrations of the Co-chaperones DnaJ and GrpE
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