ATP lowers the activation enthalpy barriers to DnaK-peptide complex formation and dissociation

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Abstract The role of nucleotide in controlling the pre-steady-state kinetics of peptide binding to the *Escherichia coli* 70-kDa molecular chaperone DnaK was investigated using stopped-flow fluorescence. The peptide used in this study, fVSV13 (representing amino acids 490–502 of the vesicular stomatitis virus glycoprotein), was dansylated specifically at its N-terminus. We found that (i) between 17 and 35°C in the presence of ATP the second-order rate constant (k_{on}) for fVSV13 binding to DnaK exhibited almost no dependence on temperature and did not deviate significantly from $3.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. In contrast, over the same temperature range in the presence of ADP, k_{on} increased by a factor of 32 (7.3×10^4 to $2.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$); and (ii) ATP increased the apparent first-order rate constant for the release of fVSV13 from preformed DnaK-fVSV13 complexes by several orders of magnitude relative to ADP. The activation energy parameters for fVSV13 binding to and dissociation from DnaK are compared to the activation parameters for the binding of an unrelated peptide to DnaK and are also discussed in terms of an open-to-closed equilibrium in the polypeptide-binding domain. On the basis of this comparison, it is suggested that the activation entropy term $\Delta S^\ddagger$, which is related to the structure of the DnaK-bound peptide or the degree of solvation of the peptide, is a controlling factor in the kinetics of peptide binding to DnaK.

INTRODUCTION

Molecular chaperones of the 70-kDa family, both stress induced and constitutively expressed, utilize free energy from ATP binding and hydrolysis to selectively bind and release segments of partially unfolded polypeptides. This cycle of polypeptide binding and release, which is coupled to conformational changes in the chaperone, promotes a variety of protein–protein interactions, such as the stabilization of nascent and partially unfolded proteins, the translocation of substrate proteins, and the assembly and disassembly of protein complexes. (Georgopoulos and Welch 1993; Hendrick and Hartl 1993; Becker and Craig 1994; Bucherberger and Bukau 1997; Fink and Goto 1998; Welch et al. 1998). The molecular mechanism that enables a single chaperone

molecule to interact with many different target sequences is the subject of intense investigation.

Molecular chaperones (70-kDa) consist of two functional domains. ATP binding and hydrolysis occur in the NH_2 -terminal domain, whereas selective substrate binding and release occur in the COOH-terminal domain. While the 3-dimensional structure of a 70-kDa molecular chaperone is not known, the structures of the separate functional domains have been determined. The NH_2 -terminal domain of Hsc70, which is similar to the structure of both hexokinase and actin (Flaherty et al. 1990; Flaherty et al. 1991), contains two lobes that are separated by a cleft in which Mg-ATP or Mg-ADP binds. The structure of a genetically engineered, truncated fragment of the COOH-terminal domain (residues 389–607) of DnaK complexed with a short peptide was recently reported (Zhu et al. 1996). The polypeptide binding portion of the fragment is a β -sandwich followed by α -helical segments, which appear to stabilize the bound peptide via an indirect interaction. Several biophysical studies have revealed that when Mg-ATP binds to the chaperone there is a structural change in the protein that results in a reduced affinity of substrates for the COOH-terminal

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domain (Palleros et al. 1993; Ha and McKay 1995; Shi et al. 1996). Conversely, peptide binding to the COOH-terminal domain must affect the global structure of the chaperone since polypeptide binding stimulates the rate of chaperone-catalyzed ATP hydrolysis (Flynn et al. 1989; Sadis and Hightower 1992; Blond-Elguindi et al. 1993).

In order to study how nucleotide controls the selection of polypeptides by 70-kDa molecular chaperones, the pre-steady-state kinetics of complex formation and dissociation between a short, positively charged, synthetic peptide, fVSV13¹, and the *Escherichia coli* 70-kDa molecular chaperone DnaK were investigated in the presence of ADP or ATP over a range of temperatures. The VSV13 peptide, also referred to as Peptide C, has been used as a substrate in a variety of studies on 70-kDa molecular chaperones (Flynn et al. 1989; Landry et al. 1992; Zhang and Walker 1996). The effect of these two nucleotides on the activation energy parameters for the initial step of DnaK-fVSV13 complex formation are dissected in this report.

MATERIALS AND METHODS

DnaK Purification

DnaK was purified at 4°C after being overproduced in the *E. coli* strain RLM893 (Zylicz and Georgopoulos 1984) as previously reported (Farr et al. 1995). Briefly, the supernatant from an *E. coli* cell lysate was passed over an ATP-agarose column and then a MONO Q column. Aliquots from the MONO Q column were dialyzed for 4 days (4 L/day) into the sample buffer (25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid/50 mM KCl/5 mM MgCl₂/5 mM 2-mercaptoethanol at pH 7.0). Protein concentration was determined by the BioRad Assay using bovine serum albumin as a standard and spectrophotometrically using $\epsilon_{280} = 15.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Montgomery et al. 1993). A sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis showed that the DnaK samples were >95% pure. After exhaustive 4-day dialysis following the MONO Q column step, DnaK preparations were assayed for endogenous nucleotide content by an HPLC assay as described in (Slepenkov and Witt 1998). This assay revealed that ≥94% of the DnaK molecules were free of nucleotide.

Peptide

The VSV13 peptide (KLIGVLSSSLFRPK) was synthesized and sequenced at the University of Kentucky Core Facility. The VSV13 peptide was specifically labeled at the NH₂-terminus with dansyl chloride as previously described (Farr et al. 1995), and the mass of this dansyl peptide (fVSV13) was verified with electrospray mass

spectrometry at the Louisiana State University Core Facility (New Orleans, LA). The concentration of the fVSV13 peptide was determined spectrophotometrically ($\epsilon_{335} = 4.63 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Characterization of the fVSV13 peptide revealed that the fVSV13 peptide bound to DnaK-MgADP complexes at 25°C with a $K_d = 0.5 (\pm 0.2) \mu\text{M}$ (data not shown); this equilibrium dissociation constant was determined by the method described by Stryer (Stryer 1965). Control experiments have shown that the dansyl label does not bind to DnaK (Farr et al. 1995), and that the fVSV13 peptide stimulates the ATPase activity of DnaK to nearly the same extent as the unlabeled peptide (data not shown).

High performance size exclusion chromatography

A Toso Haas size-exclusion column (G3000SW, 600 × 7.8 mm) and pre-column (80 × 7.8 mm) were connected to a Gilson two-pump high performance liquid chromatography system with UV-VIS detection (Gilson, Model 118). HPSEC experiments were conducted in a phosphate buffer mobile phase (25 mM KH₂PO₄-K₂HPO₄/50 mM KCl at pH = 7.0) at room temperature with a flow rate of 1 ml/min and 214 nm detection. The injection loop size was 20 μL . Bovine serum albumin (BSA) was used as a standard. The BSA monomer (66 kDa), dimer (132 kDa) and trimer (198 kDa) eluted at 16.0, 13.9, and 12.7 ml, respectively. The void volume, determined with thyroglobulin (660 kDa), was 11 ml.

Kinetic measurements

Stopped-flow experiments were performed with an Applied Photophysics, LTD. (Leatherhead, UK) SX18MV stopped-flow spectrophotometer equipped with a 150 W Xenon arc lamp. The instrument dead time was 1.4 milliseconds. An excitation monochromator was used to excite samples at 335 nm (3 nm band-pass), while emission was detected through a 399 nm long-pass filter. The sample compartment and the sample cell were maintained at the desired temperature ($\Delta T = \pm 0.2^\circ\text{C}$) via an external circulating water bath. For complex formation experiments, one syringe contained 0.2 μM fVSV13 and the other contained 3 to 30 μM DnaK (in the sample buffer) in the presence of 2 mM ADP or ATP. Samples were degassed before filling the sample syringes. After a 10 min preincubation period at the indicated temperature, the experiments were performed by rapidly mixing together 75 μL of sample from each syringe. In some cases, data (1000 points) were acquired logarithmically over the indicated time scale. For complex dissociation experiments, one syringe contained preformed DnaK-fVSV13 complexes, prepared by incubating DnaK (4 μM) and fVSV13 (1 μM) for 30–45 min at room temperature,

while the other syringe contained nucleotide (2 mM) and unlabeled VSV13 (60 μ M). Stopped-flow traces are the average of from four to ten individual traces. No correction was made for the slight variation in pH of the sample buffer caused by changing the temperature.

Curve fitting

Kinetic traces representing DnaK-fVSV13 complex formation or dissociation were fitted to exponential functions using an Applied Photophysics curve fitting program that employs a Marquardt algorithm based on the program Curfit given in Bevington (Bevington 1969). Arrhenius plots were analyzed using KaleidaGraph (Synergy Software, Reading, PA). The reported rate constants or rate parameters, which are functions of several individual rate constants, are the average of two to three determinations.

RESULTS

The oligomerization state of DnaK

Large chaperone concentrations, low temperatures, low salt concentrations, and large concentrations of Tris buffer promote the reversible self-association or oligomerization of molecular chaperones (Palleros et al. 1993; Benaroudj et al. 1995; Schönfeld et al. 1995; Gao et al. 1996; Shi et al. 1996), whereas ATP or substrates shift the equilibrium towards the monomeric form (Schmid et al. 1985; Carlino et al. 1992; Blond-Elguindi et al. 1993; Palleros et al. 1993; Toledo et al. 1993; Benaroudj et al. 1994; Gao et al. 1996; Shi et al. 1996). Because the rapid mixing experiments in this study used fairly large concentrations of DnaK, the amount of oligomerization at these large concentrations of DnaK was assessed over a range of temperatures (17–35°C) using HPSEC. Figure 1 shows results from experiments conducted at 25°C. DnaK (40 μ M) was incubated for 1 h with excess ADP (1 mM) and then analyzed using HPSEC. Two major species (14.7 and 12.3 ml) and one minor species (11.5 ml) were separated (Fig. 1), where the apparent molecular masses of these fractions correspond to 103 kDa, 220 kDa, and 284 kDa, respectively. The effect of ATP on the distribution of DnaK species was examined by incubating DnaK (40 μ M) with excess ATP (1 mM) for 15 min. ATP abolished the 284 kDa species, significantly reduced the amount of the 220 kDa species, and increased the amount of the 103 kDa species (Fig. 1). A chromatogram is also shown for a very dilute sample of DnaK (1 μ M) that had been incubated with excess ATP; a single species eluted at 14.8 ml. On the basis of the above results, we reasonably conclude that the species with the apparent molecular mass of 103 kDa is the DnaK monomer, and that the species with apparent molecular masses of 220 kDa and 284 kDa are DnaK dimers and

Fig. 1 High performance size exclusion chromatography of DnaK. Three chromatograms with DnaK are shown: (i) 40 μ M DnaK plus 1 mM ADP incubated for 1 h; (ii) 40 μ M DnaK plus 1 mM ATP incubated for 15 min; (iii) 1 μ M DnaK plus 1 mM ATP incubated for 15 min. This trace was multiplied by a factor of 5 to make it visible to the eye. Control: 1 mg/ml bovine serum albumin. Conditions: incubation and column temperatures = 25°C; injection volume = 20 μ L; 214 nm detection. The elution volumes of the two major forms of DnaK were 14.7 and 12.3 ml.

trimers, respectively. Such assignments agree with assignments made in a previous study of the oligomerization of DnaK (Schönfeld et al. 1995). Because DnaK is dumbbell-shaped (Shi et al. 1996) it is not surprising that its elution volume is different from a globular protein such as bovine serum albumin, which has a molecular mass of 66 kDa (Fig. 1). In summary, we conclude that at the high concentrations of DnaK used in this study greater than 95% of DnaK is monomeric in the presence of ATP, whereas 20–23% of DnaK is dimeric in the presence of ADP, depending on the temperature. DnaK dimerization is one source of error in the kinetic measurements performed in the presence of ADP.

Pre-steady-state kinetics of fVSV13 binding to DnaK in the presence of excess ADP

The addition of DnaK to a solution of the fVSV13 peptide results in a rapid increase in dansyl fluorescence coupled with 10 nm blue-shift in the emission maximum to 555 nm (Farr et al. 1995); both spectral features are consistent with the binding of the fVSV13 peptide to DnaK. In the following work, the pre-steady-state kinetics of DnaK-fVSV13 complex formation and dissociation were followed by monitoring the change in fluorescence of the fVSV13 peptide. The fluorescence signal is referred to as the DnaK-fVSV13 signal.

For these stopped-flow experiments, the concentration of DnaK was varied at a fixed concentration of peptide. DnaK (3–30 μ M) and ADP (2 mM) were sequestered in one syringe, fVSV13 (0.2 μ M) was sequestered in the

Fig. 2 Kinetics of DnaK-fVSV13 complex formation in the presence of ADP. (A) Complex formation between fVSV13 and 13 μM DnaK was followed using stopped-flow fluorescence spectrophotometry. The decay portion of the trace has been discussed (Farr and Witt 1997). The trace followed double exponential kinetics between 0 and 15 s, with rate parameters equal to 4.2 s^{-1} and 0.40 s^{-1} . The inset shows the change in fluorescence at early times. Final concentrations after mixing were 1.2, 7.8 or 13.0 μM DnaK, 0.1 μM fVSV13, and 1 mM ADP; temperature = 25°C. (B) Plot of the rate (■, λ_1 ; ●, λ_2) versus [DnaK]. The slope (k_{on}) of the λ_1 versus [DnaK] plot is $3.2 \pm 0.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$.

other. Upon mixing, fVSV13 bound to DnaK-ADP complexes. A representative kinetic trace, acquired at 25°C, is shown in Figure 2A. The trace is characterized by three main features: (i) a burst in the fluorescence signal occurs in the first second (inset) that accounts for 27% of the total change in fluorescence; (ii) an increase in fluorescence signal occurs over 5–20 that accounts for 73% of the change in fluorescence; and (iii) when [DnaK] > 5 μM, a decrease in fluorescence signal occurs at long times ($t > 25$ s). This decrease has been interpreted to be the result of a peptide-induced oligomerization of DnaK (Farr and Witt 1997). We chose to fit the DnaK-fVSV13

complex formation traces out to a point that included the fluorescence maximum but avoided the fluorescence decay portion of the trace. The 13 μM DnaK trace was fitted to a single-, double-, and triple-exponential function. In all cases, the traces were poorly fit by a single-exponential function but reasonably fit by a double-exponential function; significant improvement in the fits was not obtained when a triple-exponential function was used. The trace obtained at 13 μM followed $F(t) = -0.072 \exp(-4.2 \text{ s}^{-1} t) - 0.164 \exp(-0.4 \text{ s}^{-1} t)$ between 0 and 15 s, with the rate parameters λ_1 and λ_2 equal to 4.2 s^{-1} and 0.40 s^{-1} , respectively.

The rate parameters, obtained from fits of the data acquired at 25°C, are plotted against [DnaK] in Figure 2B. No correction was made for the presence of DnaK dimers. The rate (λ_1) from the fast phase of fluorescence change increases linearly with increasing concentrations of DnaK. Assuming a two-step sequential binding reaction (Scheme 1), the slope of this plot ($3.2 \pm 0.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) is the apparent second-order rate constant for fVSV13 binding to DnaK-ADP. Values for this constant at other temperatures are given in Table 1. The rate parameter (λ_2) from the slow phase of fluorescence change also increases with increasing concentrations of DnaK (Fig. 2B).

Pre-steady-state kinetics of fVSV13 binding to DnaK in the presence of excess ATP

These experiments were conducted the same way as those described above. DnaK (3–30 μM) and ATP (2 mM) were sequestered in one syringe, fVSV13 (0.2 μM) was sequestered in the other syringe. Upon mixing, fVSV13 bound to DnaK-ATP (low affinity) complexes. A representative kinetic trace, acquired at 25°C, is shown in Figure 3A. The trace is characterized by two main features: (i) a rapid burst of fluorescence occurs in the first 200 ms that accounts for approximately 15% of the total change in fluorescence (inset); and (ii) a slow increase in fluorescence occurs over 500 ms that accounts for 85% of the total change in fluorescence. Traces were fit to single-, double- and triple-exponential functions. In all cases, kinetic traces were poorly fit by a single-exponential function, and instead a triple-exponential function gave the best fit, although in some cases it was difficult to justify a triple-exponential fit over a double-exponential fit. The trace obtained at 14 μM followed $F(t) = -0.038 \exp(-6.94 \text{ s}^{-1} t) - 0.076 \exp(-0.10 \text{ s}^{-1} t) - 0.16 \exp(-0.0066 \text{ s}^{-1} t)$, with the rate parameters λ_1 , λ_2 , and λ_3 equal to 6.94 s^{-1} , 0.10 s^{-1} , and 0.0066 s^{-1} , respectively. In general, there is approximately 10% error associated with these parameter values associated with the second and third phases, whereas, due to the small amplitude of the first phase, there is approximately 20–25% error associated with the parameters associated with the first phase.

Fig. 3 Kinetics of DnaK-fVSV13 complex formation in the presence of ATP. (A) Complex formation between fVSV13 and 14 μM DnaK was followed using stopped-flow fluorescence spectrophotometry. The trace followed triple exponential kinetics, with rate parameters equal to 6.9 s^{-1} , 0.10 s^{-1} , and 0.0066 s^{-1} . The inset shows the change in fluorescence at early times. Final concentrations after mixing were 1.5, 8.4 or 14.0 μM DnaK, 0.1 μM fVSV13, 1 mM ATP; temperature = 25°C . (B) Plot of the rate ($\blacksquare$, λ_1) versus [DnaK]. The slope of the plot is $3.8 \pm 0.5 \times 10^5\text{ M}^{-1}\text{ s}^{-1}$. (C) Plot of the rate ($\bullet$, λ_2 ; $\blacktriangle$, λ_3) versus [DnaK].

The rate parameters, obtained from fits of the data acquired at 25°C , are plotted against [DnaK] in Figure 3B&C. The rate parameter (λ_1) from the fast phase of DnaK-fVSV13 signal change increases linearly with increasing concentrations of DnaK (Fig. 3B). Assuming a sequential binding reaction, the slope of this plot ($k_{\text{on}} =$

$3.8 \pm 0.5 \times 10^5\text{ M}^{-1}\text{ s}^{-1}$) is the apparent second-order rate constant for fVSV13 binding to a ATP-DnaK complex. Values for k_{on} at other temperatures are given in Table 1. The rate parameters λ_2 and λ_3 exhibit almost no dependence on [DnaK] (Fig. 3C); the asymptote of the plot of λ_2 versus [DnaK] equals 0.1 s^{-1} , whereas the asymptote of the plot of λ_3 versus [DnaK] equals 0.006 s^{-1} .

Dissociation kinetics

The effect of nucleotide on the dissociation of fVSV13 from preformed DnaK-fVSV13 complexes was also investigated by stopped-flow fluorescence. The experiments were conducted by rapidly mixing preformed DnaK-fVSV13 complexes with nucleotide and excess unlabeled VSV13, which prevents the rebinding of the fVSV13 peptide. Representative kinetic traces acquired at 35°C with ADP or ATP are shown in Figure 4. In the presence of ADP (1 mM), the fVSV13 peptide dissociated from preformed DnaK-fVSV13 complexes with double-exponential kinetics, according to $F(t) = 0.027 \exp(-42\text{ s}^{-1} t) + 0.054 \exp(-0.021\text{ s}^{-1} t) - 0.0098$, where the dissociation rate parameters k_α and k_β equal 0.42 s^{-1} and 0.021 s^{-1} , respectively. Note that the fVSV13 peptide dissociated from preformed DnaK-fVSV13 complexes with single-exponential kinetics at 15°C , but with double-exponential kinetics between 25 and 42°C . In the presence of ATP (1 mM), the fVSV13 peptide dissociated from preformed DnaK-fVSV13 complexes with single-exponential kinetics, according to $F(t) = 0.0731 \exp(-27\text{ s}^{-1} t) + 0.0033$, where the apparent first-order rate constant k_α equals 27 s^{-1} . The fVSV13 peptide dissociated from preformed DnaK-fVSV13 complexes with single-exponential kinetics over the temperature range 15 – 35°C . The rate constants obtained at other temperatures are given in Table 1.

Activation parameters

Eyring plots of the apparent on-and off-rate constants from Table 1 are shown in Figure 5. Data were fit to the equation $\ln k/T = (\ln k_B/h + \Delta S^\ddagger/R - \Delta H^\ddagger/RT)$, where k_B and h are the Boltzman and Planck constants, $\Delta S^\ddagger$ and $\Delta H^\ddagger$ are the activation entropy and enthalpy, and R is the universal gas constant. The slope and the y-intercept of each plot were used to compute values for $\Delta H^\ddagger$ and $\Delta S^\ddagger$. The plots reveal that ATP lowers the activation enthalpy for DnaK-fVSV13 complex formation and dissociation (Fig. 5A&B and Table 2). Others aspects of these results are discussed below.

DISCUSSION

This semi-quantitative kinetic study has compared the effect of nucleotide on the kinetics of DnaK-fVSV13

Fig. 4 Comparison of the effects of ADP and ATP on the kinetics of fVSV13 dissociation from preformed DnaK-fVSV13 complexes. The dissociation of fVSV13 from preformed complexes in the presence of ADP was followed by stopped-flow spectrophotometry. The trace followed double exponential kinetics (solid line), with dissociation rate parameters equal to 0.42 s^{-1} and 0.021 s^{-1} . The *inset* shows the dissociation of fVSV13 from preformed DnaK-fVSV13 complexes in the presence of ATP. The trace followed single exponential kinetics (solid line), with the apparent first-order rate constant equal to 27 s^{-1} . In each case, final concentrations after mixing were $2 \text{ }\mu\text{M}$ DnaK, $0.5 \text{ }\mu\text{M}$ fVSV13, 1 mM nucleotide, and $30 \text{ }\mu\text{M}$ VSV13; temperature = 35°C .

complex formation and dissociation. At relatively short times ($0\text{--}15 \text{ s}$), the fVSV13 peptide binds to DnaK-ADP complexes with biphasic kinetics (Fig. 2), consistent with a two-step sequential reaction where the initial binding step, i.e. the formation of a collisional complex, is followed by an isomerization step such as a rearrangement of the peptide or the protein or both (Scheme 1). In support of such a mechanism, the peptide dissociation experiments (Fig. 4) revealed that fVSV13 dissociates from preformed DnaK-fVSV13 complexes with biphasic kinetics. A mechanism similar to Scheme 1 was proposed recently to account for the biphasic kinetics of peptide binding to Hsc70-ADP complexes (Takeda and McKay

1996). The two-step mechanism given below represents only the early stage of peptide binding, because at longer times the fluorescence signal decays, especially at elevated temperatures and high concentrations of DnaK. This decay phase has been interpreted to arise from peptide-induced oligomerization of DnaK (Farr and Witt 1997), which was shown to occur even when $[\text{fVSV13}] < [\text{DnaK}]$. Relatedly, Montgomery and co-workers have suggested that the binding of VSV13 to DnaK at high temperatures induces the formation of oligomers (Montgomery et al. 1993).

Scheme 1

The pre-steady-state kinetics of fVSV13 binding to DnaK-ATP complexes were also probed. The fVSV13 peptide binds to DnaK-ATP complexes in three discrete phases, consistent with a three-step sequential reaction, where an increase in dansyl fluorescence occurs in each step. The third phase is probably a rearrangement of the peptide or the protein or both.

Dissociation

The effect of ATP on the rate of fVSV13 dissociation from preformed complexes is evident from the experiments conducted at 35°C . The fVSV13 peptide dissociated from preformed complexes in the presence of ATP with an apparent first-order rate constant $k_{\alpha} = 27 \text{ s}^{-1}$. This is 1285 times faster than the slow phase ($k_{\beta} = 0.021 \text{ s}^{-1}$) of fVSV13 dissociation and 69 times faster than the fast phase ($k_{\alpha} = 0.39 \text{ s}^{-1}$) of fVSV13 dissociation from preformed complexes in the presence of ADP (Table 1). In terms of the activation parameters, ATP reduced the apparent activation enthalpies of both dissociation steps (29 to $39 \text{ kcal mol}^{-1} \rightarrow 22 \text{ kcal mol}^{-1}$), relative to ADP, as well as the

Table 1 Kinetic constants

	+ADP			+ATP	
	On		Off	On	Off
	$k_{\text{on}} (\text{M}^{-1} \text{ s}^{-1})$	$k_{\alpha} (\text{S}^{-1})$	$k_{\beta} (\text{S}^{-1})$	$k_{\text{on}} (\text{M}^{-1} \text{ s}^{-1})$	$k_{\alpha} (\text{S}^{-1})$
Temperature ($^\circ\text{C}$)					
15			$9.1 (\pm 0.3) \times 10^{-4}$		2.3 ± 0.3
17	$7.3 (\pm 4.8) \times 10^4$			$3.4 (\pm 0.4) \times 10^5$	
25	$3.2 (\pm 0.3) \times 10^5$	$1.5 (\pm 0.1) \times 10^{-2}$	$2.1 (\pm 0.8) \times 10^{-3}$	$3.8 (\pm 0.5) \times 10^5$	7.1 ± 0.9
35	$2.3 (\pm 0.3) \times 10^6$	$3.9 (\pm 0.3) \times 10^{-1}$	$2.1 (\pm 0.02) \times 10^{-2}$	$4.1 (\pm 1.3) \times 10^5$	27 ± 4
42		$5.1 (\pm 0.2) \times 10^{-1}$	$5.6 (\pm 0.1) \times 10^{-2}$

Fig. 5 Temperature dependence of the kinetic constants. (A) Formation rate constant (Table 1): $\circ$, k_{on} (+ADP), $\Delta H^\ddagger = 34 \pm 1 \text{ kcal mol}^{-1}$ and $\Delta S^\ddagger = 79 \pm 4 \text{ cal mol}^{-1} \text{ K}^{-1}$; $\bullet$, k_{on} (+ATP), $\Delta H^\ddagger = 1 \pm 0.3 \text{ kcal mol}^{-1}$ and $\Delta S^\ddagger = -29 \pm 1 \text{ cal mol}^{-1} \text{ K}^{-1}$. (B) Dissociation rate parameters (Table 1): $\square$, k_a (+ADP; fast phase), $\Delta H^\ddagger = 39 \pm 7 \text{ kcal mol}^{-1}$ and $\Delta S^\ddagger = 64 \pm 23 \text{ cal mol}^{-1} \text{ K}^{-1}$; Δ , k_b (+ADP; slow phase), $\Delta H^\ddagger = 29 \pm 3 \text{ kcal mol}^{-1}$ and $\Delta S^\ddagger = 25 \pm 9 \text{ cal mol}^{-1} \text{ K}^{-1}$; $\blacksquare$, k_a (+ATP), $\Delta H^\ddagger = 22 \pm 2 \text{ kcal mol}^{-1}$ and $\Delta S^\ddagger = 16 \pm 3 \text{ cal mol}^{-1} \text{ K}^{-1}$. Solid lines, fits to the equation $\ln k/T = \ln k_b/h + \Delta S^\ddagger/R - \Delta H^\ddagger/RT$, where k_b is the Planck constant, h is the Boltzman constant, and R is the universal gas constant. In many cases, error bars are obscured by the symbols.

apparent activation entropies ($25 \text{ to } 64 \text{ cal mol}^{-1} \text{ K}^{-1} \rightarrow 16 \text{ cal mol}^{-1} \text{ K}^{-1}$) (Table 2). Taken together, these kinetic results are consistent with a substantial conformational change in the DnaK protein induced by ATP binding. Others have come to the same conclusion (Palleros et al. 1993; Schmid et al. 1994). A possible origin of these large activation energy barriers is given below.

Note that the value for $k_a(25^\circ\text{C})$ of 0.015 s^{-1} (Table 1), determined for the dissociation of fVSV13 from pre-formed DnaK-fVSV13 complexes upon rapid mixing with

ADP and unlabeled peptide, does not equal the value of the y-intercept ($\sim 0.2 \text{ s}^{-1}$) of the plot of λ_1 versus [DnaK] (Fig. 2B). For experiments conducted in the presence of ATP, $k_a(25^\circ\text{C})$ (7.1 s^{-1}) does not equal the y-intercept ($\sim 1 \text{ s}^{-1}$) of the plot of λ_1 versus [DnaK] (Fig. 3B). A possible reason for these discrepancies is that the direct dissociation experiments using preformed DnaK-fVSV13 complexes, which were incubated for 30 min prior to use, probe the dissociation of fVSV13 from the complex or complexes that form after the collisional complex (Scheme 1). In contrast, the fast phase of the association experiments probes the formation and dissociation of the collisional complex. Thus, the value of the y-intercept of the plots of the rate of the fast phase versus [DnaK] probably approximates the first-order rate constant k_{off} in Scheme 1. Unfortunately, because of variation in the y-intercept values in these plots we could not determine accurate values for k_{off} .

Association

The apparent second-order rate constant (k_{on}) for fVSV13 binding to DnaK in the presence of ATP is essentially constant ($3.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) between 17 and 35°C (Table 1). In contrast, k_{on} for fVSV13 binding to DnaK in the presence of ADP increase ~ 32 -fold (7.3×10^4 to $2.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) upon increasing the temperature from 17 to 35°C (Table 1). ATP reduced both the activation enthalpy ($34 \text{ kcal mol}^{-1} \rightarrow 1 \text{ kcal mol}^{-1}$) and entropy ($79 \text{ cal mol}^{-1} \text{ K}^{-1} \rightarrow -29 \text{ cal mol}^{-1} \text{ K}^{-1}$) for fVSV13 binding to DnaK (Table 2), relative to ADP; however, since these parameters have opposite effects on the rate, fVSV13 peptide does not bind any faster to DnaK in the presence of ATP than in ADP at 25°C . We conclude that ATP binding to DnaK has a dual effect on peptide binding: the enthalpic barrier to peptide binding is eliminated, while an entropic barrier to peptide binding is imposed. The net result: the rate of collisional complex formation (ATP-E + P $\rightleftharpoons$ ATP-EP*) has almost no temperature dependence over the range 17– 35°C in the presence of ATP.

The above results should be compared to previous studies. Schmid and co-workers found that ATP significantly increased the magnitude of the on-rate constant k_{on} for the binding of a synthetic 21-mer (a-pp) to DnaK. For example, at 25°C k_{on} increased from $9400 \text{ M}^{-1} \text{ s}^{-1}$ in the absence of ATP to $4.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of ATP. On the other hand, Takeda and McKay found that ATP decreased the magnitude of the on-rate constant for a short synthetic peptide (faf1) binding to recombinant bovine Hsc70. For example, at 25°C k_{on} decreased from $1210 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of ADP to $730 \text{ M}^{-1} \text{ s}^{-1}$ in the presence of ATP. Regarding our results, at 15°C the ratio:

Table 2 Thermodynamic constants

Formation kinetics			
Nucleotide	$\Delta H^\ddagger$ (kcal mol ⁻¹)	Frequency prefactor (M ⁻¹ s ⁻¹)	$\Delta S^\ddagger$ (cal mol ⁻¹ K ⁻¹)
+ADP	34 ± 1	4.1 × 10 ^{30±1}	79 ± 4
+ATP	1 ± 0.3	6.4 × 10 ^{6±0.2}	-29 ± 1
Dissociation kinetics			
Nucleotide	$\Delta H^\ddagger$ (kcal mol ⁻¹)	Frequency prefactor (s ⁻¹)	$\Delta S^\ddagger$ (cal mol ⁻¹ K ⁻¹)
+ADP (fast phase)	39 ± 7	2 × 10 ^{27±5}	64 ± 23
+ADP (slow phase)	29 ± 3	5 × 10 ^{18±2}	25 ± 9
+ATP	22 ± 2	6.3 × 10 ^{16±6}	16 ± 3

$$\frac{k_{\text{on}}(+\text{ATP})}{k_{\text{on}}(+\text{ADP})} = \frac{3.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}}{7.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}} = 4.7$$

whereas at 35°C:

$$\frac{k_{\text{on}}(+\text{ATP})}{k_{\text{on}}(+\text{ADP})} = \frac{4.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}}{2.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}} = \frac{1}{5.6}$$

One could argue that the results in this report are consistent with both previous studies. More studies using different peptides are needed before the kinetics of peptide binding to these heat shock proteins are fully understood.

A value of ~35 kcal mol⁻¹ is the activation enthalpy for fVSV13 binding to and dissociation from an DnaK-ADP complex. Interestingly, similar $\Delta H^\ddagger$ values have been determined for several of the reactions between DnaK as well as Hsc70 with their respective substrates. First, consider results on the Cro peptide (representing amino acids 1–12 of the cro repressor protein, MQERITLKDYAM). In the absence of nucleotide, the activation enthalpy barriers to DnaK-fCro complex formation and dissociation equal 26 ± 8 kcal mol⁻¹ and 34.6 ± 1.2 kcal mol⁻¹ (Farr et al 1995), respectively. Second, the cationic DNA-binding protein protamine (PRRRRSSRPVR-RRRRPRVSRRRRRRGRRRR) dissociates from pre-formed DnaK-protamine complexes with $\Delta H^\ddagger$ equal to 32 ± 4 and 28 ± 1 in the absence of nucleotide and in the presence of ADP (Witt et al. 1997), respectively. Third, the $\Delta H^\ddagger$ values for the second step of ATP binding to both DnaK and bovine brain Hsc70 are 34 kcal mol⁻¹ (Slepenkov and Witt 1998) and 40 kcal mol⁻¹ (Ha and McKay 1995), respectively. These combined results lead to the conclusion that in the absence of nucleotide or in the presence of ADP there is a significant physical and/or electrostatic barrier to the binding and release of peptides to and from DnaK, and that the barrier is eliminated by the action of ATP. This barrier may be related to the

opening of the α -helical lid that tops the deep polypeptide-binding channel (Zhu et al. 1996).

Let us assume that ATP binding induces the α -helical lid that tops the deep polypeptide-binding channel of DnaK to open. One would then expect that the fVSV13 peptide, or any other peptide, would bind to the open binding site on a ATP-DnaK complex with an on-rate constant equal to ~10⁷ M⁻¹ s⁻¹ – which is an on-rate constant typical for a diffusion-controlled reaction. Instead, the fVSV13 peptide binds to ATP-DnaK with $k_{\text{on}} = 3.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. We suggest that this k_{on} value is less than the diffusion-controlled value because the fVSV13 peptide molecule must adopt a specific conformation in order to bind to an ATP-DnaK complex. The $\Delta S^\ddagger$ value of -29 cal mol⁻¹ K⁻¹, determined for fVSV13 binding to ATP-DnaK, is consistent with this idea. On the other hand, we cannot rule out the possibility of a fast, undetected initial binding step in the reaction between the fVSV13 peptide and an ATP-DnaK complex.

Lastly, it is intriguing that the fVSV13 and the fCro peptides, which probably bind to the same site on DnaK (Witt et al. 1997), have such different on-rate constants. For example, fCro peptide binds to DnaK in the absence of nucleotide at 25°C with k_{on} equal to 18 ± 5 M⁻¹ s⁻¹, whereas the fVSV13 peptide binds to DnaK in the presence of ADP with k_{on} equal to 3.2 ± 0.3 × 10⁵ M⁻¹ s⁻¹. Insight into why these on-rate constants are so different can be obtained by examining the activation parameters for these two reactions. The activation parameters for fCro binding to DnaK are $\Delta H^\ddagger = 26 \pm 8 \text{ kcal mol}^{-1}$ and $\Delta S^\ddagger = 22 \pm 2 \text{ cal mol}^{-1} \text{ K}^{-1}$ (Farr et al. 1995) versus $\Delta H^\ddagger = 34 \pm 2 \text{ kcal mol}^{-1}$ and $\Delta S^\ddagger = 79 \pm 4 \text{ cal mol}^{-1} \text{ K}^{-1}$ for fVSV13 binding to ADP-DnaK complexes (Table 2). The $\Delta H^\ddagger$ values are the same within the experimental uncertainty, but the $\Delta S^\ddagger$ values are quite different. We suggest that the difference in these on-rate constants is related to the activation entropy term, $\Delta S^\ddagger$. Note that the $\Delta S^\ddagger$ value of 79 cal mol⁻¹ K⁻¹ is

entirely consistent with an extended conformation for fVSV13 when it is bound to an ADP-DnaK complex (Landry et al. 1992). By comparison, the relatively small $\Delta S^\ddagger$ value of $22 \text{ cal mol}^{-1} \text{ K}^{-1}$ indicates that in contrast to fVSV13 the free fCro peptide either adopts a more constrained conformation when bound or more intermolecular contacts are established between its residues and the residues in the DnaK binding site. These very different $\Delta S^\ddagger$ terms for the Cro and VSV13 peptides may also reflect differences in the amount of solvation of the two peptides.

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