Reactions of protamine with the molecular chaperone DnaK

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Abstract Molecular chaperones of the 70 kDa family mediate protein–protein interactions by selectively binding to partially unfolded segments of other proteins in an ATP-dependent activity cycle. Previous investigations of chaperone substrate selectivity have shown that chaperones have a propensity to bind to partially unfolded segments of polypeptides that contain bulky hydrophobic residues. However, recent investigations have shown that 70 kDa chaperones such as DnaK, which is expressed by *Escherichia coli*, also bind short basic peptides and even polycations. We report here that DnaK specifically binds to the polycation protamine when [protamine]/[DnaK] is near unity, whereas protamine induces the aggregation of DnaK when [protamine]/[DnaK] ≥ 20 . Complexes between DnaK and protamine were detected using fluorescently labeled protamine (protamine*) in conjunction with high performance size exclusion chromatography. We found that: (i) an unlabeled peptide of known affinity for DnaK partially inhibited the formation of DnaK-protamine* complexes; (ii) Mg-ATP (and Mg- γ -S-ATP) significantly reduced the affinity of protamine* for DnaK; and (iii) the rate of DnaK-protamine* complex dissociation is highly temperature-sensitive, with apparent activation enthalpies (ΔH^*) equal to 32 ± 4 and 28 ± 1 kcal mol⁻¹ in the absence of added nucleotide and in the presence of ADP, respectively. The results are consistent with the specific binding of protamine* at the (poly)peptide binding site of DnaK. A model is proposed to account for the protamine-induced aggregation of DnaK.

INTRODUCTION

Molecular chaperones promote a variety of essential protein–protein interactions within cells (Gething and Sambrook 1992; Georgopoulos and Welch 1993; Hendrick and Hartl 1993; Becker and Craig 1994). Constitutive 70 kDa chaperones (Hsc70) promote the folding, translocation and assembly of other proteins, whereas stress-inducible 70 kDa chaperones (Hsp70) protect other proteins from the denaturation that may occur as a consequence of stressful conditions. The common feature of these disparate reactions is that chaperones utilize free energy derived from ATP binding and hydrolysis to drive the chaperone molecules through an activity cycle consisting of repeated binding and release of hydrophobic, flexible segments of polypeptides.

There is emerging evidence that members of the 70 kDa family of molecular chaperones also bind to charged, basic peptide sequences in addition to hydrophobic peptide sequences (Fourie et al 1994; Gragerov et al 1994; Takenaka et al 1995; deCrouy-Chanel et al 1996; Endo et al 1996). For example, it was recently reported that purified bovine Hsc70 selects short peptides that are enriched with basic amino acids from a phage display random peptide library (Takenaka et al 1995). These basic peptides that are selected by Hsc70 resemble organellar targeting sequences. It was proposed that, consistent with both its function in chaperoning proteins to organelles and with its role in assisting protein folding, Hsc70 recognizes two different types of peptide sequences, a basic organellar targeting sequence (NIVRKKK-like sequences) and a hydrophobic protein folding sequence (FYQLALT-like sequences). Regarding Hsp70s, the bacterial chaperone DnaK selects peptides from a combinatorial peptide library which contain a hydrophobic core that is flanked on either end by arginine residues (Gragerov et al 1994). A recent report has

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revealed that DnaK possesses a binding site for arginine/lysine and that DnaK can bind polylysine and polyarginine (deCrouy-Chanel et al 1996).

We have been investigating the ability of DnaK to bind polycations. Here we report that DnaK, but not purified bovine Hsc70, specifically binds to the DNA-binding protein protamine from salmon (salmine), with the following sequence:

The specificity of DnaK-protamine complex formation and the kinetics of DnaK-protamine complex dissociation are characterized in this report.

MATERIALS AND METHODS

Cell culture and DnaK purification

DnaK was harvested from the *E. coli* strain RLM893 (Zylicz and Georgopoulos 1984). DnaK was purified as described previously (Farr et al 1995) and stored in a N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) sample buffer (25 mM HEPES/50 mM KCl/5 mM MgCl₂/5 mM 2-ME at pH = 7.0).

Peptides and fluorophores

All reagents were of the highest purity and were purchased from Sigma Chemical Company (St Louis, MO), unless stated otherwise. Bovine brain Hsc70 was purchased from StressGen (#SPP-750, Victoria, Canada). The protamine-fluorescein conjugate was prepared by the addition of a two-fold molar excess of fluorescein isothiocyanate (#F-143, Molecular Probes, Eugene, OR) in 50 μ l of dimethylsulfoxide to approximately 5 mg of salmon protamine (#P 4005, Sigma) which was dissolved in 1.0 ml of bicarbonate buffer (25 mM NaHCO₃ at pH = 8.3) at room temperature. A three-fold molar excess of the organic base diethylisopropylamine was also added, and the reaction was allowed to proceed at room temperature with vigorous shaking shielded from the light. After 2 h, 200 μ l aliquots were removed and injected onto a C₄ reverse phase column (#214TP1010, Vydac, Hesperia, CA) that was initially equilibrated in water/trifluoroacetic acid (99.9/0.1%), and the labeled protamine was eluted with a linear gradient of acetonitrile/trifluoroacetic acid (99.9/0.1%). The one main fraction was collected and lyophilized. The HPLC-purified fluorescein-protamine conjugate, which is referred to in this report as protamine*, was characterized using an SDS polyacrylamide gel electrophoresis recipe which enables the resolution of low molecular weight proteins (Schägger and von Jagow 1987). Protamine* migrated to a similar position on the gel as unlabeled protamine. This result is consistent

with one fluorescein molecule per protamine molecule since the gel cannot resolve unlabeled protamine (MW = 4100) from singly-labeled protamine (MW = 4489). The concentration of the protamine* stock solutions were determined spectrophotometrically ($\epsilon_{495} = 76\,000\text{ M}^{-1}\text{ cm}^{-1}$) (Haugland 1992).

A conjugate of ethanolamine and fluorescein was prepared by reacting excess ethanolamine with fluorescein isothiocyanate at room temperature with vigorous shaking for 1 h. The conjugate was then purified using a reverse phase C₄ column as described above. The concentration of the ethanolamine* stock solutions were determined spectrophotometrically ($\epsilon_{495} = 76\,000\text{ M}^{-1}\text{ cm}^{-1}$).

In addition to unlabeled protamine, the Cro peptide (representing residues 1 to 12 of the cro repressor protein, MQRITLKDYAM) and the VSV5 peptide (representing amino acids 498 to 502 of the New Jersey strain of vesicular stomatitis virus glycoprotein, LFRPK) were used in the competition assay (Hsiang et al 1977; Farr et al 1995).

High-performance size exclusion chromatography

A Phenomenex size-exclusion column (Bio-Sep-S, 300 $\times$ 7.8 mm) and pre-column (80 $\times$ 7.8 mm) were connected to a Gilson two-pump high-performance liquid chromatography system. The column run-through passed through a fluorescence detector (Gilson, Model 121) and a UV-VIS detector (Gilson, Model 118) in series. The fluorescence detector was equipped with a 480 nm excitation filter and a 520 nm emission filter; the UV-VIS detector was set at 280 nm. The high-performance size exclusion chromatography (HPSEC) column experiments were conducted in a phosphate buffer mobile phase (25 mM KH₂PO₄-K₂HPO₄/50 mM KCl/5 mM 2-ME/0.06% (w/v) sodium azide at pH = 7.0) at room temperature with a flow rate of 0.70 ml/min. Bovine serum albumin (BSA) was used as a control. The BSA monomer (66 kDa) and dimer (132 kDa) elute at 15.8 and 14.5 ml, respectively.

Competition experiments

The HPSEC column assay was used to detect complexes between DnaK and protamine* (DnaK-protamine*). When unlabeled Cro and unlabeled VSV5 were used as competitors, the ratio of [unlabeled peptide]/[protamine*] was varied at a fixed concentration of DnaK by mixing 96 μ l of a DnaK stock solution, 1.5 μ l of a protamine* stock solution, and a variable amount of concentrated unlabeled peptide to a total volume of 150 μ l. In each sample, the final concentrations of DnaK and protamine* were both 2.0 μ M. Samples were incubated in a heating block for 15 min at 37 °C, and 120 μ l aliquots were injected on the HPSEC column. When unlabeled protamine was

used as the competitor, the ratio of [unlabeled protamine]/[protamine*] was varied at a fixed concentration of DnaK by mixing 827 μ l of a DnaK stock solution, 3.3 μ l of a protamine* stock solution, and a variable amount of concentrated unlabeled protamine to a total volume of 1.2 ml. In each sample, the final concentrations of DnaK and protamine* were 2.0 and 0.4 μ M, respectively. Samples were incubated in a heating block for 15 min at 37°C, and 1.0 ml aliquots were injected on the HPSEC column. For all of the competition experiments, the order of the addition of reagents was as follows: the unlabeled competitor was added to an aliquot of DnaK, and then the aliquot of protamine* was rapidly added (within 10 sec). Similar results were obtained when the unlabeled competitor and protamine* were mixed together first and then added to DnaK. All reagents were dissolved in the HEPES sample buffer. Samples were contained in 1.5 ml siliconized polypropylene Eppendorf tubes. For each sample injected, both the fluorescence signal (F , which is proportional to [DnaK-P*]) at 15.0 ml and the absorbance signal (A , which is proportional to [DnaK-P*] + [DnaK]) at 15.0 ml were integrated. The relative concentration of complexes is defined as $(F_c/A_c)/(F_o/A_o)$, where F_c and A_c represent the integrated intensities of the fluorescence and absorbance signals at a competitor concentration c , and F_o and A_o represent the integrated signal intensities in the absence of unlabeled competitor.

Dissociation kinetics

Preformed DnaK-protamine* complexes were isolated by incubating DnaK (14 μ M) with protamine* (5 μ M) at 37°C for 15 min and then injecting the sample onto the HPSEC column. The fraction that contains DnaK-protamine* complexes was collected in a sterile 15 ml polystyrene Falcon tube that was immersed in an ice bath. Since the mobile phase was a phosphate buffer, the DnaK-protamine* complexes, once collected, were also in the phosphate buffer. Complex dissociation kinetics were initiated within 30 min of collection by the addition of excess unlabeled Cro peptide to inhibit the rebinding of protamine* (protamine* + DnaK $\rightarrow$ DnaK-protamine*), and the sample was transferred to a thermostatted water bath. For each injection, the peak at 15.0 ml in the fluorescence-detected chromatogram was integrated and then divided by the integrated intensity of the zero time point [$F(t)/F(t=0)$]. The dissociation kinetic curves were analyzed using KaleidaGraph (Synergy Software, Reading, PA).

Spectroscopy

A Beckman Model DU-70 was used to monitor the protamine-induced aggregation of DnaK at 340 nm. The sample

was contained in a thermostatted quartz cuvette and was continuously stirred during the course of the absorbance measurement.

Data analysis

The activation enthalpy (ΔH^*) and frequency prefactor ($\omega \exp\{\Delta S^*/R\}$) were obtained from the slope and y-intercept of the Arrhenius plot, respectively. The activation entropy (ΔS^*) was obtained from the y-intercept of a plot of $\ln(k_{\text{off}}/T)$ versus $1/T$. Uncertainties in these thermodynamic parameters were calculated according to Bevington (1969).

RESULTS

In vivo, protamine binds to DNA at a specific stage in the maturation of the sperm cells of some vertebrates (Ando et al 1973). In vitro, protamine is often used to precipitate DNA from cell lysates. It was observed that under certain conditions protamine induces the aggregation of DnaK, and that the protamine-induced DnaK aggregation depends on the temperature, ionic strength, and the concentrations of both protamine and DnaK. The effect of the concentration of protamine on DnaK aggregation is shown below. In the experiments described in this report, DnaK was stored in the HEPES sample buffer.

Light scattering

The protamine-induced aggregation of DnaK was monitored by light scattering (Fig. 1). At a fixed concentration of DnaK (1.0 μ M) at 30°C, the addition of protamine to a final concentration of 40 μ M resulted in reproducible biphasic absorbance changes at 340 nm. With continuous stirring, there was an initial rapid increase in absorbance from 0 to ~ 0.12 A which was followed by a slow decrease in absorbance. This biphasic absorbance versus time profile revealed that the rapid aggregation of DnaK was followed by the slow dissolution of the aggregates. We also found that an approximate 20-fold molar excess of either poly-L-arginine (69 residues) or poly-L-lysine (422 residues) over DnaK induced the aggregation of DnaK, leading to similar absorbance changes at 340 nm as described above (data not shown). An alternative explanation for the gradual reduction in absorbance at 340 nm is that aggregated DnaK slowly drops out of solution. However, based on visual inspections of the samples after the 100 min of incubation, there was nearly a complete absence of turbidity, and there was no precipitate on the bottom of the cuvette. In contrast, there was no detectable aggregation of DnaK when only 2 μ M protamine was added to a solution of DnaK (1.0 μ M). From these light-scattering experiments we determined the

Fig. 1 Protamine-induced aggregation of DnaK followed by light scattering at 340 nm. The concentration of protamine was varied at a fixed concentration of DnaK (1.0 μ M) and the absorbance changes at 340 nm were monitored. $\bullet$, 2 μ M protamine; $\blacksquare$, 40 μ M protamine. The sample was stirred continuously during the experiment. Temperature = 30°C.

conditions where there was no detectable aggregation of DnaK; however, the assay could not discern whether DnaK-protamine complexes form at low concentrations of protamine. A fluorescein-protamine conjugate was prepared in order to determine whether DnaK-protamine complexes form at low concentrations of protamine ([protamine] < 3 μ M).

DnaK-protamine* complexes

Fluorescence- and absorbance-detected HPSEC was used to detect DnaK-protamine* complexes. The fluorescence-detected chromatograms show the amount of DnaK-protamine complexes (Fig. 2), and the absorbance-detected chromatograms, which were obtained simultaneously, show the total amount of DnaK (Fig. 2, inset). When DnaK (5.4 μ M) was incubated with protamine* (2.0 μ M) for 15 min at 37°C and then injected on the HPSEC column, there was an intense signal at 15.0 ml in the fluorescence-detected chromatogram (Fig. 2 A). Since DnaK with no added protamine eluted at the same position in the absorbance-detected chromatogram (inset, d), and since 5 μ M DnaK is predominantly monomeric (Schönfeld et al 1995), the intense fluorescence signal at 15.0 ml was consistent with the formation of fairly long-lived, monomeric DnaK-protamine* complexes. We note that under identical experimental conditions there was no detectable binding of protamine* to bovine brain Hsc70 (data not shown). Mg-ATP significantly reduced the affinity of protamine* for DnaK. For example, when DnaK (5.4 μ M) was incubated with protamine* (2.0 μ M) for 15 min at 37°C in the presence of 1 mM Mg-ATP and

Fig. 2 Detection of DnaK-protamine* complexes using fluorescence-detected HPSEC. DnaK was incubated with protamine* and then injected on the HPSEC column; parallel absorbance- and fluorescence-detected chromatograms were obtained for each injection. Fluorescence traces A–C: (A) Trace obtained after incubating DnaK (5.4 μ M) and protamine* (2.0 μ M) for 15 min at 37°C. (B) Trace obtained after incubating DnaK (5.4 μ M) and protamine* (2.0 μ M) with Mg-ATP (1.0 mM) for 15 min at 37°C. (C) Trace obtained after incubating DnaK (5.4 μ M) and ethanolamine* (2.0 μ M) for 15 min at 37°C. Inset: Absorbance traces a–c were obtained from the same samples as A–C. An injection of DnaK only is shown in trace d. The vertical scale bar in the inset depicts 0.008 absorbance units. Conditions: Flow rate = 0.7 ml/min; HPSEC column temperature = 25°C.

then injected on the HPSEC column, a weak fluorescence signal appeared at 15.0 ml (Fig. 2B). As a negative control, DnaK (5.4 μ M) was incubated with a fluorescein-ethanolamine conjugate (2.0 μ M) for 15 min at 37°C and then injected on the HPSEC column (Fig. 2C). The control experiment demonstrated that there was no non-specific binding of fluorescein to DnaK. The inset (a–d) shows the population of DnaK for each injection described above. DnaK was predominantly monomeric (V_e = 15.0 ml) with a small subpopulation of DnaK dimer (V_e = 13.4). Dimer formation was prevented when DnaK was co-incubated with ATP, as can be seen by the absence of the dimer subpopulation in trace b. On the basis of the results in Figure 2, we conclude that relatively long-lived, monomeric DnaK-protamine* complexes form at equimolar concentrations of DnaK and

Fig. 3 Effect of added nucleotide on the relative amount of DnaK-protamine* complexes. Samples of DnaK (5.4 μ M) and protamine* (2.0 μ M) were incubated with or without added nucleotides for 15 min at 37°C and then injected on the HPSEC column. The peak at 15.0 ml in the fluorescence-detected chromatograms was integrated, and the integrated intensity of the sample with no added nucleotide was used as the reference sample.

protamine, and the affinity of protamine* for DnaK is significantly reduced by Mg-ATP.

Figure 3 shows the relative amounts of DnaK-protamine* complexes formed in the presence of no-added nucleotide, added ADP, γ -S-ATP (non-hydrolyzable ATP), and ATP. These results demonstrate that added nucleotide affected the equilibrium binding constants for protamine* binding to DnaK. Since γ -S-ATP reduced the amount of DnaK-protamine* complexes to almost the same extent as ATP, we conclude that the binding of ATP is sufficient to destabilize the DnaK-protamine* complexes.

Competition assay

Competition experiments were conducted using the HPSEC assay to assess the specificity of DnaK-protamine* complex formation (Fig. 4). Three unlabeled species were tested for their ability to compete with labeled protamine: (i) VSV5, which does not bind to DnaK (Farr et al 1995); (ii) the Cro peptide, which binds to DnaK (Sherman and Goldberg 1991; Gragerov et al 1994); and (iii) salmon protamine. As expected, unlabeled VSV5 did not compete to a significant degree with protamine* since there was only a 10% reduction in the number of DnaK-protamine* complexes when $[VSV5]/[protamine^*] = 80$. Unlabeled Cro competed with protamine*, as evidenced by 50% and 70% reductions in the number of DnaK-protamine* complexes when $[Cro]/[protamine^*] = 25$ and 80, respectively. About 30% of the DnaK-protamine* complexes were not subject to competition with Cro. Unlabeled protamine competed with protamine*, with a ~50% reduction in the number of DnaK-protamine complexes occurring when $[protamine]/[protamine^*] \approx 10$.

Fig. 4 Inhibition of DnaK-protamine* complex formation by unlabeled peptides. Unlabeled peptides were tested for their ability to inhibit DnaK-protamine* complex formation. The concentrations of the unlabeled peptides (□, VSV5; ○, Cro; and ◇, protamine) were varied at fixed concentrations of DnaK and protamine*. For the experiments with unlabeled VSV5 and unlabeled Cro as competitor peptides, $[DnaK] = [protamine^*] = 2.0 \mu$ M. For the experiments with unlabeled protamine as the competitor, $[DnaK] = 2.0 \mu$ M and $[protamine^*] = 0.4 \mu$ M. Samples were incubated for 15 min at 37°C, and aliquots were injected on the HPSEC column. HPSEC column temperature = 25°C. (Dashed lines = guide)

Complete inhibition occurred at a large molar excess of protamine. In Figure 4, there is an apparent 10% increase in the number of DnaK-protamine* complexes upon the addition of approximately equimolar amounts of protamine* and unlabeled protamine. A possible explanation for this phenomenon is given below. The inability of VSV5 to compete with protamine* coupled with the ability of both Cro and protamine to compete with protamine* are consistent with the specific binding of protamine* to DnaK.

DnaK-protamine* complex dissociation kinetics

The kinetics of DnaK-protamine* complex dissociation were investigated using simultaneous absorbance- and fluorescence-detected HPSEC. Complexes were first isolated using HPSEC, and then the dissociation reaction was initiated as described above. In the absence of added nucleotide, DnaK-protamine* complex dissociation at 15°C followed single exponential kinetics $F(t)/F(t=0) = \alpha \exp[-k_a t]$, where the amplitude α and the apparent first-order rate constant k_a equal 0.97 and $1.1 \times 10^{-4} \text{ s}^{-1}$, respectively (Fig. 5). The half-time ($t_{1/2}$) for the dissociation was 1.8 h. The DnaK absorbance signal was also integrated for each injection. The plot of the normalized absorbance signal $[A(t)/A(t=0)]$ was a straight line with zero slope which proves that the population of DnaK molecules was constant throughout the incubation.

Fig. 5 Kinetics of DnaK-protamine* complex dissociation at 15°C. Absorbance- and fluorescence-detected HPSEC was used to monitor complex dissociation (DnaK-protamine* $\rightarrow$ DnaK + protamine*) in the absence of added nucleotide. Complexes were isolated as described in Materials and Methods. Samples were incubated in a 15°C temperature bath, and 200 μ l aliquots were removed and injected on to the HPSEC column at the indicated times. Both the normalized fluorescence signal [$F(t)/F(t=0)$] (■) and the normalized absorbance signal [$A(t)/A(t=0)$] (□) are plotted. The normalized fluorescence trace was fitted to the equation $F(t) = 0.97 \exp(-1.1 \times 10^{-4} s^{-1} t)$ (solid line). (Dashed line = guide).

Therefore, the reduction in the DnaK-protamine* fluorescence signal in Figure 5 is a consequence of complex dissociation (DnaK-protamine* $\rightarrow$ DnaK + protamine*) rather than an artifact.

To determine the activation parameters for protamine dissociation from preformed DnaK-protamine* complexes, the kinetics of DnaK-protamine* dissociation were investigated over a limited range of temperatures (5–25°C) in both the absence and presence of added Mg-ADP (Fig. 6A,B). At all temperatures, protamine* dissociation from preformed DnaK-protamine* complexes followed single exponential kinetics. In the absence of added nucleotide (Panel A), the $t_{1/2}$ values for protamine* dissociation were 33.2, 2.2, and 0.84 h at 5, 15, and 25°C, respectively. In the presence of added Mg-ADP (Panel B), the $t_{1/2}$ values for protamine* dissociation were 26.4, 3.4, and 0.84 h at 5, 15, and 25°C, respectively. The apparent first-order rate constants are given in Table 1. Co-incubation of preformed DnaK-protamine* complexes with Mg-ATP resulted in the very rapid release of protamine* ($t_{1/2}$ [15°C] = 9.6 min).

Fig. 6 Effect of temperature on the kinetics of DnaK-protamine* dissociation. (A) DnaK-protamine* dissociation kinetics were investigated at 5–25°C in the absence of added nucleotide using fluorescence-detected HPSEC as described. (B) DnaK-protamine* complex dissociation in the presence of added Mg-ADP (1.0 mM) at 5–25°C. Each data set was fitted to the single exponential function $F(t)/F_0 = \alpha \exp(-k_{off} t)$ (solid lines), where α and k_{off} are the amplitude and the apparent first-order rate constant. The k_{off} values are given in Table 1.

Arrhenius plots of the rate constants in Table 1 are shown in Figure 7. An activation enthalpy ($\Delta H^\ddagger$) and frequency prefactor ($\omega \exp\{\Delta S^\ddagger/R\}$) were obtained from the slope and y-intercept of each $\ln k$ versus $1/T$ plot, respectively. The thermodynamic parameters obtained from the Arrhenius plots are given in Table 2.

DISCUSSION

This work was undertaken to examine the ability of DnaK, which is a highly negatively-charged protein at

Table 1 Effect of added nucleotide on the rate of protamine* dissociation from preformed DnaK-protamine* complexes

Temperature (°C)	No nucleotide	+ADP	+ATP
	$k_{off} (s^{-1})^\dagger$	$k_{off} (s^{-1})$	$k_{off} (s^{-1})$
5	$5.2 (\pm 0.6) \times 10^{-6}$	$7.2 (\pm 0.1) \times 10^{-6}$	nd
15	$8.2 (\pm 2.1) \times 10^{-5}$	$5.3 (\pm 0.4) \times 10^{-5}$	$1.2 (\pm 0.2) \times 10^{-3}$
25	$2.31 (\pm 0.04) \times 10^{-4}$	$2.28 (\pm 0.03) \times 10^{-4}$	nd

[†]Values represent the mean (and s.e.m.) from either duplicate or triplicate experiments.

Figure 7 Arrhenius plots ($\ln k_{\text{off}}$ versus $1/T$) for DnaK-protamine* complex dissociation. (A) no added nucleotide ($n = 7$). (B) added Mg-ADP ($n = 6$). Each data set was fitted to the equation $\ln k = -\Delta H^*/RT + \ln \omega + \Delta S^*/R$ (solid lines). The activation parameters derived from the plots are given in Table 2. Due to the small uncertainty in the k_{off} values obtained in the presence of Mg-ADP, panel B appears to contain one $\ln k_{\text{off}}$ value for each of the three temperatures, but actually two $\ln k_{\text{off}}$ values are plotted for each temperature.

physiological pH, to bind polycations. We found that the polycation protamine induces the aggregation of DnaK when the polycations are in an approximate 20-fold molar excess over DnaK. However, when protamine and DnaK are at equimolar concentrations specific DnaK-protamine complexes are formed. We discuss below various aspects of the interactions of protamine with DnaK.

Protamine-induced aggregation of DnaK

A model for protamine-induced aggregation of DnaK is shown in Figure 8. As depicted in the illustration, at physiological pH DnaK is a highly negatively-charged protein since its pI equals 5.1 (Zylicz and Georgopoulos 1984). The model shows that at equimolar concentrations of DnaK and protamine DnaK-protamine complexes form, and, in such complexes, protamine is bound at the (poly)peptide binding site of DnaK. However, when excess protamine is present, it is proposed that more than one molecule of protamine binds per molecule of DnaK, resulting in charge neutralization on the surface of the protein and subsequent aggregation. Note that in addition to the (poly)peptide binding site, DnaK has a large number of aspartic acid and glutamic acid residues in its COOH-terminal domain between residues 477 to 556 (Bardwell and Craig 1984) which might interact with protamine. The model in Figure 8 is consistent with our experimental findings: DnaK-protamine complexes formed at equimolar concentrations of DnaK and protamine, and, under those conditions, there was no detectable aggregation; whereas, considerable aggregation occurred when $[\text{protamine}] / [\text{DnaK}] > 20$.

It has been reported that both polyarginine (75 residues) and polylysine (75 residues) stimulate the ATPase activity of DnaK and that these polycations competitively inhibit the binding of bovine pancreatic trypsin inhibitor (BPTI) to DnaK (deCrouy-Chanel et al 1996). While such experiments were conducted up to a 100-fold molar excess of polycation over DnaK, no aggregation of DnaK was reported. In contrast, we have observed that poly-L-arginine (69 residues) and poly-L-lysine (422 residues), in addition to protamine, induce the aggregation of DnaK at a relatively low molar excess of polycation over DnaK ($[\text{polycation}] / [\text{DnaK}] > 20$). The absence of polycation-induced aggregation of DnaK in the work of deCrouy-Chanel may be a consequence of slightly different buffer conditions. On the other hand, since the population of DnaK molecules monitored via UV-absorbance during the course of their HPLC gel filtration competition experiments was not reported, it is possible

Table 2 Apparent activation parameters for DnaK-protamine* dissociation

Experiment	ΔH^* (kcal mol ⁻¹)	$\omega \exp(\Delta S^*/R)$ (s ⁻¹)	ΔS^* (cal mol ⁻¹ K ⁻¹)
DnaK-protamine* off kinetics			
(No added nucleotide)	32 ± 4	$4 \times 10^{19 \pm 3}$	32 ± 14
DnaK-protamine* off kinetics			
(added Mg-ADP)	28.0 ± 1.0	$1.4 \times 10^{17.0 \pm 0.7}$	18.1 ± 3.4
DnaK-Cro* off kinetics			
(No added nucleotide) [†]	34.6 ± 1.2	$2 \times 10^{21 \pm 1}$	38 ± 4

[†]The activation entropy terms (ΔS^*) were calculated from a plot of $\ln(k_{\text{off}}/T)$ versus $1/T$ using $\omega = k_{\text{B}}T/h$, where k_{B} and h are the Boltzman and the Planck constants, respectively.

[†]Farr et al., 1995.

Fig. 8 Model for polycation-induced aggregation of DnaK.

that DnaK aggregated upon the addition of excess polycation. In such a scenario, a combination of polycation-induced aggregation of DnaK and polycation competition may explain the reduction in the number of DnaK-BPTI complexes reported in their competition assay. The results of the competition experiments for this study (Fig. 4) were corrected for the reduction in the population of DnaK monomers due to protamine-induced aggregation (see Materials and Methods).

It was found that γ -S-ATP partially inhibited the binding of protamine* to DnaK (Fig. 3). This result is consistent with a previous report which showed that γ -S-ATP partially inhibited the binding of carboxymethylated α -lactalbumin (CLMA) to DnaK (Palleros et al 1993). In contrast, it was reported that γ -S-ATP either promoted or inhibited the binding of CLMA to DnaK, depending on whether the nucleotide was present in the sample buffer only or in both the sample buffer and the HPSEC column buffer (Wawrzynów and Zylisz 1995). Our interpretation of these combined results is that the ability of γ -S-ATP, and nucleotides in general, to stabilize or destabilize the binding of a substrate to DnaK is remarkably sensitive to the buffer composition (K^+ or Na^+) and the amino acid composition of the substrate.

Competition

Two explanations are offered as to why a 10-fold excess of unlabeled protamine over protamine* is required to achieve 50% inhibition (Fig. 4). One explanation is that protamine* binds to DnaK with a higher affinity than unlabeled protamine, and the fluorescent tag is responsible for altered affinity. We note, however, that the fluorescein conjugate of ethanolamine does not bind to DnaK. Another explanation involves the phenomenon of self-quenching. Self-quenching can occur if a subpopulation of DnaK has two molecules of protamine* bound per molecule of DnaK (P^* -DnaK- P^*) (see Fig. 8). The apparent 10% increase in the number of DnaK-protamine* complexes (Fig. 4) at near equimolar concentrations of protamine and protamine* results from the replacement of one DnaK-bound protamine* molecule with a molecule of protamine (P -DnaK- P^*) which results in the relief of self-quenching and an increase in fluorescence. Such self-quenching can also explain why a 50% reduction in the number of DnaK-protamine* complexes occurred when $[protamine]/[protamine^*] > 1$.

It is interesting that protamine binds to the bacterial Hsp70 (DnaK) but not to the mammalian Hsc70 from bovine brain. Such a differential reactivity between members of the heat shock protein family and a cationic peptide ligand has been previously reported. Fourie and co-workers (1994) investigated the binding of the cationic peptide S7 (KKLMFKT) to DnaK, Hsc70, and BiP and showed that S7 binds with highest affinity to DnaK, with intermediate affinity to Hsc70, and with negligible affinity to BiP and concluded that the differences in relative affinities are related to sequence differences between the three proteins. DnaK clearly has the greatest propensity to bind short basic peptides relative to Hsc70 and BiP (DnaK > Hsc70 >> BiP). In the extreme case presented here, DnaK but not Hsc70 reversibly binds protamine, where 21 of the 32 residues are arginine. It is thought that the hydrophilic substrate binding site on bovine brain Hsc70 cannot accommodate protamine because of its high density of positively-charged residues; whereas, the hydrophilic substrate binding site on DnaK is large enough or sufficiently charged to accommodate protamine. Thus, the difference in reactivity between the two heat shock proteins and protamine is related to sequence differences.

Kinetics of DnaK-protamine* complex dissociation

Protamine* dissociated from DnaK with single exponential kinetics at all of the temperatures we investigated which indicates that the dissociation experiments probed a predominantly homogeneous population of DnaK-protamine* complexes. The rate of dissociation of protamine from DnaK in both the absence of added nucleotide and with added Mg-ADP is highly dependent on temperature, with

apparent activation enthalpies of 32 ± 4 and 28.0 ± 1.0 kcal mol⁻¹, respectively. It is intriguing that the kinetics of protamine* dissociation from DnaK in the absence of added nucleotide exhibit nearly the same temperature dependence as the dissociation of dansyl-Cro from preformed DnaK-dansyl-Cro complexes (Farr et al 1995). Specifically, the apparent activation enthalpy for dansyl-Cro dissociation from preformed DnaK-dansyl-Cro complexes was 34.6 ± 1.2 kcal mol⁻¹ (Table 2). Since Cro competes with protamine* for binding to DnaK (Fig. 4), and because the activation enthalpies for the release of both dansyl-Cro and protamine* from preformed DnaK complexes are nearly identical, the simplest interpretation is that Cro and protamine interact with DnaK at the same binding site.

One other, albeit less likely, interpretation of the kinetic data for the release of protamine* from DnaK is suggested. Suppose protamine* binds to DnaK at the DnaJ binding site, which is in close proximity to but is spatially distinct from the (poly)peptide binding site (Wawrzynów and Zylicz 1995). It is possible that, upon the incubation of preformed DnaK-protamine* complexes, a slow conformational change occurs at the (poly)peptide binding site – even in the absence of bound peptide – which induces the rapid release of protamine* from the DnaJ site. In this model, since the rate determining step is the conformational change, the kinetics of protamine* dissociation will actually reflect the kinetics of the slow conformational change. In other words, the activation enthalpy of 32 kcal/mol for the putative dissociation of protamine* from DnaK may actually be the activation enthalpy for a slow conformational change in the protein. In this model, at low temperatures, in the absence of a peptide bound in the (poly)peptide binding site of DnaK, the α -helical lid-like subdomain (Zhu et al 1996), which is in close proximity to the peptide binding site, may act as an internal ligand and partially occupy the (poly)peptide binding site. When the sample temperature is raised, the α -helical lid slowly dissociates from the site. Such a slow conformational change could also induce the release of a substrate occupying the DnaJ site.

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