Substrate recognition by Clp chaperones is dependent on interactions with motifs composed of specific peptide sequences. We studied the binding of short motif-bearing peptides to ClpA, the chaperone component of the ATP-dependent ClpAP protease of *Escherichia coli* in the presence of ATPγS and Mg2+ at pH 7.5. Binding was measured by isothermal titration calorimetry (ITC) using the peptide, AANDENYALAA, which corresponds to the SsrA degradation motif found at the C terminus of abnormal nascent polypeptides in vivo. One SsrA peptide was bound per hexamer of ClpA with an association constant \( K_\text{a} \) of \( 5 \times 10^6 \) M\(^{-1}\). Binding was also assayed by changes in fluorescence of an N-terminal dansylated tide was bound per hexamer of ClpA with an association constant \( K_\text{a} \) of \( 5 \times 10^6 \) M\(^{-1}\). Similar results were obtained when ATP was substituted for ATPγS at 6 °C. Two additional peptides, derived from the phage P1 RepA protein and the *E. coli* HemA protein, which bear different substrate motifs, were competitive inhibitors of SsrA binding and bound to ClpA hexamers with \( K_\text{a} \) > \( 3 \times 10^7 \) M\(^{-1}\). DNS-SsrA bound with only slightly reduced affinity to deletion mutants of ClpA missing either the N-terminal domain or the C-terminal nucleotide-binding domain, indicating that the binding site for SsrA lies within the N-terminal nucleotide-binding domain. Because only one protein at a time can be unfolded and translocated by ClpA hexamers, restricting the number of peptides initially bound should avoid nonproductive binding of substrates and aggregation of partially processed proteins.

Molecular chaperones of the Clp/Hsp100 family are key components of cellular protein quality control systems and play an important role in homeostatic mechanisms governing the levels of global regulatory proteins, particularly in response to stress and other environmental signals (1). Clp chaperones have an ATP-dependent protein unfolding activity that is employed for disaggregation, structural remodeling, or degradation depending on the functional partners with which they interact (2–4). ClpB/Hsp104 proteins act primarily to disrupt protein aggregates and hand misfolded proteins over to the DnaK/Hsp70 chaperone system for refolding (5, 6), whereas ClpA and ClpX function primarily to unfold native proteins and deliver the unfolded proteins to the ClpP protease for degradation (7–9). ClpY (HslU) functions similarly to ClpA and ClpX, delivering substrates to the proteasome-like ClpQ (HslV) for degradation (10). The protein binding and enzymatic activities of Clp chaperones require assembly into homomeric rings of six subunits (2, 11). The rings are internally dynamic in response to nucleotide binding and hydrolysis, which provide a means of exerting the forces necessary to structurally disrupt and translocate protein substrates. The proteolytic holoenzyme complexes of ClpAP and ClpXP are barrel-like structures with a core composed of the double heptameric ring of ClpP, with hexameric rings of ClpA or ClpX stacked coaxially on each end of ClpP (12, 13). The center of ClpP is a hollow aqueous channel housing the proteolytic active sites (14). Substrates bind to ClpA or ClpX on the ends of the complex, are unfolded, and are then translocated into the ClpP chamber.

The specificity of interaction between Clp chaperones and their intended targets is a crucial aspect of their function in vivo and is best understood with the ClpAP and ClpXP systems of *Escherichia coli*. ClpA and ClpX each target a unique set of proteins by recognizing different specific-sequence motifs located in accessible regions of the target proteins, most often within 15 amino acids of the N or C terminus (15–19). A few motifs, such as the co-translationally added SsrA tag, are recognized by both ClpA and ClpX. Substrate recognition is further affected by adaptor proteins that bind to the target protein and to the Clp chaperone and mediate or modulate their interaction. Adaptors are specific for particular motifs or classes of proteins and appear to increase the specificity of interaction with a particular chaperone. For example, ClpA and ClpX can directly recognize SsrA-tagged proteins; however, interaction of SsrA-tagged proteins with ClpX is further promoted by the adaptor, SspB, with the consequence that SsrA-tagged proteins are primarily targeted by ClpXP *in vivo* (20, 21). Adaptor proteins also offer an additional level of cellular regulation, which can serve to govern targeting of substrates under specific physiological conditions, as in the case of the adaptor, RssB, which mediates degradation of the stationary phase sigma factor, RpoS, by ClpXP only when RssB is phosphorylated in response to specific environmental or intracellular signals (22, 23).

The substrate engagement and motif binding sites on ClpA and ClpX are not known with certainty. Studies with the substrate protein, RepA, indicate that approximately one RepA dimer binds per hexamer of ClpA (24), and electron microscopic imaging shows the protein bound at the apical ring surface of the chaperone in the vicinity of the axial channel (25–27). Tight substrate binding requires assembly of the hexamer for both ClpX and ClpA. Because each ClpA or ClpX subunit is identi-
cal, there are potentially six sites in the hexamer for interaction with sequence motifs present in substrates. Thus, several questions about peptide and protein interactions arise. Is binding limited strictly by steric constraints on accessibility to the peptide binding sites? Are all peptide sites identical and equally accessible? Are there separate sites for the different motifs, or are there alternate conformations of the same site that vary in specificity? Do substrate motifs and adaptor motifs bind at separate and distinct sites? Is there cooperativity in binding for identical or different motifs or between substrate and adaptor motifs?

To address these issues, we have studied the binding of several relatively small peptides bearing motifs recognized by either ClpA or ClpX. We show that hexameric ClpA and ClpX bind only one peptide with high affinity and that peptides with different motifs bind at the same site or at overlapping sites.

**EXPERIMENTAL PROCEDURES**

**Proteins and Peptides—**Unless otherwise specified, Buffer A containing 50 mM Tris/HCl, 0.3 mM KCl, 10 mM MgCl₂ and 10% (v/v) glycerol at pH 7.5 (25 °C) was used throughout. All solutions were prepared with deionized and filtered water from a Milli-Q UV plus system. ClpA and ClpX were prepared as described previously (13, 28) and stored frozen at −80 °C in 50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 0.2 M KCl. Green fluorescent protein-SsrA was purified as described previously (8).

ClpA-Δ153, which lacks the entire N-domain but retains both nucleotide binding domains (NBD),1 displays unfolded activity and assembles with ClpP and promotes ATP-dependent protein degradation, was prepared as described previously (29). ClpXAN lacking the N-terminal domain and ClpA-N438 lacking the second nucleotide binding domain (NBD),2 also were prepared as described (29). ClpA-D265K is mutated in the Walker B motif of NBD2, and retains both nucleotide binding domains (NBD),1 displays unfolded activity and assembles with ClpP and promotes ATP-dependent protein degradation.

The forward and back primers were 5'-GGACACTAACGTATCAGTTAATGATTCGCAAGAGATCCACATTACCGC and 5'-CCGATTATGGGTTGATCTCCTTCAAAACAGATAGTTGATGTC, respectively. ClpA-D564A, mutated in the Walker B motif of NBD2, was made by site-directed mutagenesis using a pBAD24 plasmid containing the ClpA gene under a modified arabinose promoter (pGB-2) as a template for PCR. MOPS, 4-morpholinepropanesulfonic acid.

**RESULTS**

**ClpA Has a Single High Affinity Binding Site per Hexamer**

ClpA-D564A, mutated in the Walker B motif of NBD2, was made by QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA).

The proteins were expressed and purified as described previously for the wild-type ClpA protein (8). The mutations were confirmed by DNA sequencing.

HemA-N18aa (MTLLALGINHKTAPVSLR), RepA-N18aa (MNQSFISDILYADIESKA), and BBA-SsrA (YTVdPSATF-LKKLEELLKKLEELKGKLEELLKKLA), were loaded into the syringe at concentrations of 400-600 nmol in 500-μl volumes at 400-600-s intervals into a 1.4-ml cell containing ClpA₆ protein (1.7-2.2 μM).

Data collection and analysis were performed with Origin software (OriginLab, Northampton, MA) using the model for a single class of binding sites.

**Fluorescence Measurements—**Fluorescence spectra were obtained using an AB2 (SLM Instruments, Urbana, IL) or a QM-68E (PTI, Long Island, NY) spectrofluorometer. Protein was diluted into Buffer A containing 1 mM ATP·S₆, and measurements were recorded in 100-μl quartz cells. Sample temperature was maintained at 28 °C unless stated otherwise, by circulating water through jacketed cuvette or with a Peltier holder. All spectral measurements were performed with Glan polarizers at magic angle conditions. For DNS fluorescence measurements, the excitation wavelength was set at 340 nm, and the emission wavelength was set at 400 nm. To calculate fluorescence intensities at 540 nm, fluorescence intensities at 540 nm were read from spectra and corrected for the fluorescence intensity of the probe in the absence of the protein to obtain ΔF. In titration experiments, measurements were corrected for sample dilution, which did not exceed 10%. Using Origin software (OriginLab), ΔF values were fitted to Equation 1 (31) assuming a single binding site,

\[ \text{ΔF} = K_{A}[L]/(1 + K_{A}[L]) \]  
(1)

In Equation 1, ΔFₘₐₓ is the maximum fluorescence signal change at saturating ligand concentration, and [L] is the free ligand concentration which can be determined from the Equation 2,

\[ [L]_0 = [L]_0 + K_{A} \]  
(2)

where [M]₀ and [L]₀ are total protein and ligand concentration, respectively.

Fluorescence anisotropy measurements were performed by simultaneous measurements of the two polarization components in two emission channels of the PTI spectrofluorometer. The same double detection setup was used to simultaneously monitor DNS fluorescence and light scattering intensity of the protein sample in oligomerization experiments. DNS photostability under experimental conditions was confirmed by a 3-h illumination with fluorescence monitoring without observed intensity change. Binding of dansyl alone or dansyl alanine to ClpA₆ was not detected. The fluorescence signal from dansylated peptides did not change upon addition of nucleotide or unlabeled ligands.

All steady-state fluorescence measurements were performed after time-dependent changes had occurred, usually >30 s.

For competition binding experiments using nonfluorescent ligands, 0.5 μM dansylated SsrA peptide was equilibrated with 0.5 μM ClpA₆ and 1–2 mM ATP·S₆ in buffer A at 28 °C. Unlabeled competitor peptide was then titrated into the cuvette, and the loss in dansyl fluorescence was monitored to follow DNS-SsrA displacement by the competitor. Fluorescence intensity values were corrected for sample dilution and contribution of the unbound fraction of DNS-SsrA at each point of the titration. Corrected data were normalized to the limiting fluorescence intensity values, where 0 corresponds to the fluorescence intensity of 0.5 μM DNS-SsrA in the absence of ClpA₆, and 1 to the fluorescence intensity of 0.5 μM DNS-SsrA saturated with protein. The saturation value was established in a separate experiment. The free competing ligand concentrations at half saturation \([C_{1/2}]\) were calculated from \([C_{1/2}]_{\text{DNS}}\) values obtained from data plots. The \([C_{1/2}]_{\text{DNS}}\) value was used to calculate the competing peptide affinity constant \(K_{A}\) from Equation 3,

\[ v = nK_{C}/(1 + K_{C} + K_{C}) \]  
(3)

where \(v\) is the average saturation of n binding sites with ligand.
ClpA Has a Single High Affinity Binding Site per Hexamer

Fig. 1. Isothermal titration calorimetry measurements of SsrA binding to ClpA. E. coli ClpA (~2 μM hexamer in the cell) was titrated with SsrA peptide in the presence of ATP·γS in buffer A at different temperatures (see “Experimental Procedures” and Table I). Titration at 10 °C (A) and 28 °C (B) in the presence of 2 mM ATP·γS with data (●) fitted to a model for a single class of binding sites (solid line); C, titration of SsrA into ClpA in the absence of nucleotide at 12 °C; D, control titration of SsrA into buffer at 12 °C; E, thermodynamic parameters for SsrA binding to ClpA in buffer A as a function of temperature. Apparent enthalpy changes (ΔH) were determined by ITC (filled circles); ΔG (open circles) were calculated from K_i obtained from ITC; ΔH^* and ΔG^* values were used to calculate −TΔS (filled triangles). Error bars on the points are from the fit errors for each parameter. Dashed lines are for ΔH and −TΔS values at 10 and 28 °C corrected for buffer proton effects. The slopes of the ΔH versus T plots give ΔC_p values (see text).

Results

The ClpA Hexameric Ring Binds One SsrA Peptide—A synthetic 11-amino acid peptide (AANDENYALAA), corresponding to the SsrA degradation tag co-translationally added to stalled nascent polypeptide chains in vivo (32), was used to monitor peptide binding to ClpA by isothermal titration calorimetry (ITC) (Fig. 1). Binding was measured in the presence of 10 mM MgCl_2, 2 mM ATP·γS, which promotes formation of ClpA hexamers ClpA_6 (33). Dynamic light scattering confirmed that ClpA_6 was assembled under these conditions and remained stable at temperatures from 4 to 40 °C. Peptide titrations were performed at several temperatures between 4 and 28 °C; representative ITC titrations at 10 and 28 °C are shown in Fig. 1, A and B, respectively. ITC data were fitted well to a model for a single class of binding sites. Higher molar ratios of SsrA to ClpA_6 than shown in Fig. 1 (A and B) gave no evidence for a second set of lower affinity binding sites. In each case, the results were consistent with tight binding of one SsrA peptide to ClpA_6, with an average K_i of 5 × 10^6 M^{-1} (K_i of 0.2 μM) (Fig. 1). The binding of SsrA to ClpA_6 in Tris buffer is an endothermic process at 10 °C and exothermic at 28 °C. Thermodynamic parameters obtained from the analysis of ITC titration data are presented in Table I.

Injections of SsrA into ClpA solutions in the absence of nucleotide (Fig. 1C) yielded ITC signals similar to those obtained when the peptide was titrated into Buffer A alone (Fig. 1D). In addition, in buffer alone or buffer with unassembled ClpA, the small signal accompanying each injection was proportional to the amount of peptide added up to a final concentration of 10 μM SsrA. The absence of any saturatable peptide concentration-dependent change in ITC signal is evidence against significant binding of SsrA to unassembled ClpA and suggests that SsrA binds tightly to ClpA only after assembly of the hexamer.

The thermodynamic parameters (ΔH, −TΔS, and ΔG') for binding of SsrA to ClpA_6 are shown as a function of temperature in Fig. 1E and Table I. Values of ΔH are small and decrease with increasing temperature, becoming slightly negative at −21 °C. To quantitate proton uptake or release upon binding of SsrA to ClpA, titrations were repeated at 10 and 28 °C using 20 mM MOPS buffer, which has a much lower heat of protonation than Tris (ΔΔH = −5.9 kcal/mol). At both temperatures, there were differences in the enthalpies of SsrA binding to ClpA_6 measured in MOPS or Tris buffer. The changes in binding enthalpy and in the temperature dependence of binding enthalpy obtained between the two buffers are consistent with a net uptake of two protons at 10 °C and a net release of one proton at 28 °C. The difference in proton uptake at the two temperatures suggests that ClpA is in different conformational states at 10 and 28 °C.

After correction for the heat of protonation of the buffer, the enthalpy of binding between SsrA and ClpA_6 at 10 and 28 °C were −7.3 and +6.0 kcal mol^{-1}, respectively (dashed line for ΔH, Fig. 1E). Using these values of ΔH and the ΔG' value derived from the average association constant of 5 × 10^6 M^{-1}, the calculated −TΔS values at 10 and 28 °C were −1.4 and −15.1 kcal K^{-1} mol^{-1}, respectively. Thus, the binding of SsrA to ClpA_6 is enthalpically controlled at 10 °C and entropically controlled at 28 °C. The crossover point at which both ΔH and −TΔS make equal contribution to ΔG' occurs at about 14 °C.
regardless of the order of addition of ClpA and ATP, fluorescence properties of the final complex were the same (Fig. 2). Thus, the hypsochromic shift (Fig. 2). Addition of DNS-SsrA to ClpA in the absence of nucleotide. Addition of excess ATP to ClpA, although there may be solvent rearrangement and burial of surface polar residues. The enthalpy of binding in Buffer A decreased with increasing temperature for several modified species of SsrA peptides as well (Table I), indicating that the binding interactions between the different peptides and ClpA₆ share similar characteristics.

Binding of Dansylated SsrA Peptide to ClpA and ClpX—To confirm the stoichiometry and other SsrA binding properties obtained in the ITC experiments, the SsrA peptide with a dansyl moiety attached to the N terminus was used to measure binding by fluorescence methods. Upon binding of DNS-SsrA to ClpA hexamers in the presence of ATPγS, the fluorescence intensity increased and the fluorescence spectrum underwent a hypsochromic shift (Fig. 2). Addition of DNS-SsrA to ClpA in the absence of ATPγS produced only small changes in fluorescence, consistent with poor binding to unassembled ClpA. When ATPγS was added to a mixture of DNS-SsrA and ClpA to promote formation of ClpA hexamers, DNS-SsrA bound to ClpA as indicated by the increase in fluorescence intensity and a shift of the fluorescence emission spectrum (Fig. 2). Thus, the fluorescence properties of the final complex were the same regardless of the order of addition of ClpA and ATPγS. These data indicate that, as seen for unmodified SsrA, tight binding of DNS-SsrA can only occur with the assembled ClpA hexamer. No change was observed when DNS-SsrA was added to nucleotide solutions in the absence of ClpA. Binding to ClpA was dependent on the peptide portion of DNS-SsrA, because no significant changes in fluorescence were observed when either free dansyl chloride or dansylated alanine was added (data not shown). The small fluorescence changes seen with DNS-SsrA and unassembled ClpA were similar to those seen in control experiments with DNS-Ala, indicating that the dansyl moiety itself has some tendency to interact weakly with ClpA.

Additional evidence also supports the conclusion that high affinity binding of DNS-SsrA requires the assembled ClpA hexamer. DNS-SsrA binding monitored by changes in fluorescence intensity or anisotropy followed the same time course as ClpA oligomerization, which occurred in <20 s after addition of ATPγS (Fig. 3, A and B). The increase in fluorescence due to DNS-SsrA binding was reversed by addition of EDTA to chelate Mg²⁺, which causes disassembly of ClpA hexamers (Fig. 3A). The decrease in fluorescence intensity occurred on the same time scale as disassembly of the ClpA hexamers and remained at the level seen in the absence of nucleotide. Addition of excess unlabeled SsrA to the complex of ClpA and DNS-SsrA led to a decrease in fluorescence intensity (Fig. 3B). DNS-SsrA was not simply bound in a different conformation, because fluorescence anisotropy also decreased, indicating that the DNS-SsrA was displaced from the high molecular weight complex and confirming that the SsrA peptide portion of DNS-SsrA was necessary for binding.

At 28 °C, DNS-SsrA bound ClpA₆ with a $K_A$ of $1.1 \times 10^7$ M⁻¹ (Fig. 4). Binding of DNS-SsrA to ClpA₆ was also monitored by direct ITC measurements giving the same $K_A$ value as the fluorescence titration (Table I). Competition between unmodified SsrA and DNS-SsrA indicates that both species interact with ClpA₆ at the same binding site (Fig. 3B, and see Fig. 7 below), although the higher $K_A$ for DNS-SsrA binding compared with unmodified SsrA might reflect an additional inter-
action between the dansyl moiety and ClpA. Other N-terminal modifications to SsrA, such as the extended helical peptide in SsrA, also contribute additional binding energy (Table I).

A Job plot based on the method of continuous variations (34) of DNS-SsrA peptide binding to ClpA6 shows a maximum at a molar ratio of protein to peptide of 0.45, indicating that approximately 1 mol of DNS-SsrA peptide binds to the 1 mol of ClpA hexamer (Fig. 3A). The small deviation from the 0.5 ratio expected for 1:1 stoichiometry can be attributed to a slight underestimation of the DNS-SsrA concentration used in our experiment (see “Experimental Procedures”). The 1 to 1 peptide to ClpA6 stoichiometry was also determined in an independent fluorescence titration end point experiment conducted at a high, 2.4 M concentration of ClpA6 under which conditions the DNS-SsrA added was mostly bound (Fig. 3B).

In vivo, SsrA-tagged substrates are primarily targeted by ClpX, which binds SsrA motifs present in peptides and proteins in vitro. To determine the stoichiometry of DNS-SsrA binding to ClpX, a fluorescence titration was performed with 1.9 M ClpX6. DNS-SsrA binding reached saturation at a 1:1 ratio of peptide to ClpX6 (Fig. 3B). This result is consistent with a previous report in which fluorescence anisotropy changes upon binding of BODIPY-SsrA were best fit assuming a 1 to 1 ratio of peptide to hexameric ClpX (20). Thus it appears that both ClpA and ClpX hexamers have a similar mode of substrate binding that limits high affinity interactions to a single peptide.

Binding of DNS-SsrA to ClpA in the Presence of ATP—DNS-SsrA binding to ClpA was tested in the presence of ATP, because ATP is continuously hydrolyzed by ClpA and the hexamer is expected to be in a more dynamic state during cycles of ATP hydrolysis. To avoid excessive ATP hydrolysis during the long incubations required to allow full equilibration of the DNS-SsrA with ClpA, the titrations were performed at 6 °C. Interestingly, DNS-SsrA binding in the presence of ATP occurred with the same stoichiometry of one peptide per ClpA hexamer (Fig. 5A). The small deviation from the 0.5 ratio expected for 1:1 stoichiometry can be attributed to a slight underestimation of the DNS-SsrA concentration used in our experiment (see “Experimental Procedures”). The 1 to 1 peptide to ClpA6 stoichiometry was also determined in an independent fluorescence titration end point experiment conducted at a high, 2.4 M concentration of ClpA6 under which conditions the DNS-SsrA added was mostly bound (Fig. 3B).
hexamer and with nearly the same affinity as observed in the presence of ATP$\gamma$S (Fig. 6). The increase in fluorescence intensity upon binding to ClpA hexamers in the presence of ATP was about 80% of that observed when ATP$\gamma$S was present. The smaller fluorescence enhancement indicates that the peptide binding site is in a less protected, possibly in a more dynamic region that is more exposed to solvent. The modest change in binding affinity despite the apparent greater exposure of the binding site might indicate that both on and off rates for SsrA binding are affected by cycles of ATP hydrolysis. At higher temperatures in the presence of ATP, DNS-SsrA addition to ClpA was accompanied by an increase in fluorescence during the first minute or so, followed by a decline over the next 10 or more minutes (data not shown). The more rapid release of bound DNS-SsrA might reflect depletion of ATP and an unfavorable ratio of ADP to ATP after several minutes of hydrolysis by high concentrations of ClpA at the higher temperatures. To determine if hydrolysis of ATP at NBD1 or NBD2 affects DNS-SsrA binding, we titrated ClpA-D285K and ClpA-D584A with DNS-SsrA in the presence of either ATP or ATP$\gamma$S (Fig. 6). These Walker B mutants bind nucleotide and allow assembly of ClpA hexamers, but are much less active for ATPase activity (data not shown). For both mutants, as well as for wild type ClpA, a smaller fluorescence yield for bound DNS-SsrA was seen in the presence of ATP than in the presence of ATP$\gamma$S, suggesting that a different conformational change promoted by ATP binding compared with ATP$\gamma$S binding influences the peptide binding site and increased exposure does not require ATP hydrolysis in either site. There was no difference between ClpA-D584A and wild type ClpA in their relative affinity for DNS-SsrA, irrespective of the nucleotide used, suggesting that any changes in nucleotide binding properties or induced conformational changes in NBD2 did not affect peptide binding. However, ClpA-D285K had reduced affinity compared with wild type ClpA in the presence of both ATP and ATP$\gamma$S (−2-fold lower $K_a$). This effect is most likely due to the importance of NBD1 for stability of the hexamer and peptide binding (see below). For all titrations in Fig. 6, however, affinity constants were in the range of 1–7 $\times$ 10$^5$ M$^{-1}$ for binding DNS-SsrA.

**Competitive Binding of Mutant SsrA Peptides to ClpA**—Unlabeled SsrA competes with DNS-SsrA bound to ClpA, displacing it and reversing the fluorescence increase observed for the protein-bound fraction of the fluorescent ligand (Figs. 3 and 7). Mutational analysis of the residues needed for binding to ClpA suggest that SsrA residues 1, 8, and 9, numbering from the most N-terminal residue in the 11-residue peptide (AAN-DENYALAA), are the most critical ones for recognition by ClpA (35). We used synthetic peptides in which Ala-1, Ala-8, and Leu-9 were changed to aspartate and tested the ability of these altered SsrA peptides to compete with DNS-SsrA for ClpA binding. Consistent with the mutational data, peptides in which Ala-8 and Leu-9 were changed had greatly reduced affinity for ClpA (Table II). Less than 50% inhibition of DNS-SsrA binding was observed with either peptide added in >20-fold excess over DNS-SsrA. These data indicate that the binding properties of the small peptides faithfully reflect those of the SsrA degradation motif attached at the C terminus of a physiological substrate. The Ala-1 residues had less effect on binding of the SsrA peptide, although substitution of aspartate did lead to a slight decrease in binding and substitution of leucine led to a slight increase in affinity. It is possible that aspartate in this position of an 11-amino acid peptide has a different effect on the binding interaction than it does in a much larger tagged protein. A contribution from the hydrophobic side chain of leucine at the N terminus is consistent with the higher affinity observed for the N-dansylated peptide and suggests that the region around that end of the binding pocket on ClpA might have a site that accommodates hydrophobic or aromatic residues.

Truncation of the N-terminal residues of SsrA peptide progressively weakens the competition; a peptide comprising the last five C-terminal amino acids of the SsrA tag (YALAA)
exhibited weak competition with DNS-SsrA, and no competition was observed with a shorter peptide consisting of just the three C-terminal amino acids (LAA) (data not shown).

**Competition between SsrA and Other Peptide Motifs for Binding to ClpA**—We used a similar competition assay to study the interactions of several other peptides, including extended derivatives of SsrA and peptides containing different motifs recognized by ClpA$_6$. In the experiments presented in Fig. 7 and Table III only 66% of the protein was saturated with DNS-SsrA in the absence of competitor, leaving 34% of the protein free. Half-saturation values $C_{1/2}$ were derived from the concentration giving a relative DNS-SsrA fluorescence intensity of 0.5 (Fig. 7). The free ligand concentration at half-saturation $C_{1/2}$ was calculated from $C_{1/2} = \frac{C_{total}}{2}$ and substituted into Equation 3 to obtain the apparent affinity constant for competitor binding (Table III). Direct measurements of SsrA and $\alpha$-SsrA binding by ITC (see Table I below) yielded higher estimates of the affinity constants.

SsrA conjugated to model peptides, forming either a three-stranded $\beta$ sheet or a regular $\alpha$-helix, competed with DNS-SsrA more effectively than SsrA itself. The extended peptide segments themselves had little or no affinity for ClpA and did not compete on their own (data not shown). Interestingly, competitive binding required the presence of the SsrA sequence at the C terminus of the extended peptides. The peptide BBA-SsrA was a good inhibitor of DNS-SsrA binding, whereas the peptide SsrA-BBA, which has the same peptide segments in the opposite orientation, was a much poorer inhibitor.

ClpA recognizes disparate peptide motifs found near the ends of other protein substrates. To determine if different motifs can bind simultaneously to one ClpA hexamer, we performed direct competition experiments with DNS-SsrA and peptides bearing N-terminal sequences of known ClpAP substrates. We used an 18-amino acid peptide corresponding to the N-terminal sequence of HemA and another 18-residue peptide corresponding to the N-terminal sequence of RepA (see Table III for $K'_{2}$ values). Both peptides were good inhibitors of DNS-SsrA binding to ClpA. Strong competition was also found when the full-length RepA protein was used. We also tested another peptide, referred to as Cleptide (36), corresponding to the 10 residues surrounding the ClpP auto-processing site, which was previously found to be a good substrate for ClpP and to bind to ClpA, affecting ATPase activity and competing for casein binding (37). Cleptide also competed with DNS-SsrA for binding to ClpA with $K'_{2} = 3.7 \times 10^{6} \text{ M}^{-1}$ in agreement with $K'_{2} = 6.6 \times 10^{6} \text{ M}^{-1}$ for ClpA binding of N-terminal dansylated Cleptide.

Fluorescence anisotropy measurements were made to confirm that binding of the competitor is accompanied by dissociation of the DNS-SsrA and not simply a change in environment of the fluorescent adduct (Fig. 3B). The anisotropy decreased in parallel with the decrease in fluorescence intensity upon addition of competitor. When competitor was in excess, the anisotropy of DNS-SsrA fluorescence approached that observed for the peptide in buffer, indicating that competitive binding resulted in total displacement of DNS-SsrA from the ClpA$_6$ binding site. The order of addition of DNS-SsrA and peptide competitors did not influence final fluorescence intensities or anisotropies.

### Table II

| Competitor          | $\Delta F$ for DNS-SsrA in presence of ClpA$_6$ | 2 $\mu$M | 6 $\mu$M | %  |
|---------------------|-----------------------------------------------|---------|---------|----|
| AANDENYLAA (SsrA)   | 67                                            | 39      |        |    |
| AANDENYLDA (SsrA)   | 94                                            | 76      |        |    |
| AANDENYDAA (SsrA)   | 97                                            | 84      |        |    |
| DANDENYLA (SsrA)    | 74                                            | 42      |        |    |
| LADENYLAA (SsrA)    | 62                                            | 36      |        |    |

* SsrA, 11-amino acid SsrA motif; HemA-N18aa, 18 N-terminal-amino acid motif of the HemA protein; RepA-N18aa, 18 N-terminal-amino acid motif of the RepA protein; $\beta$-SsrA, SsrA tag conjugated to the 18-amino acid model peptide forming three strands of $\beta$ structure; $\alpha$-SsrA, SsrA tag conjugated to the 16-amino acid motif forming a regular $\alpha$-helix. HemA (50) and RepA (51) are proteins specifically degraded by ClpAP; sequences of the N-terminal 18 amino acids of each are provided under "Experimental Procedures."

### Table III

| Competitor   | $[C_{total}]_{0}$ | $[C_{free}]_{0}$ | $K'_{2}$ |
|--------------|------------------|-----------------|----------|
| SsrA         | 2.25             | 2               | 0.88     |
| $\alpha$-SsrA| 0.79             | 0.54            | 3.2      |
| HemA-N18aa   | 0.32             | 0.07            | 25       |
| $\gamma$-SsrA| 0.3              | 0.05            | 35       |
| RepA-N18aa   | 0.3              | 0.05            | 35       |

Fluorescence binding to ClpA and ClpX Deletion Mutations—The N-terminal domains of ClpA and ClpX either directly or indirectly through adaptor proteins play a role in substrate recognition and binding (38–41). To test the effects of the ClpA and ClpX N-domains on SsrA binding, DNS-SsrA titrations were performed with deletion mutants, ClpA-A153 and ClpXΔN, which lack their respective N-terminal domains (29). ClpA-A153 and ClpXΔN form stable hexamers in the presence of nucleotide. Titrations of the deletion mutants in the presence of 1 mM ATP$_\gamma$S showed a binding stoichiometry of 1:1 peptide to protein hexamer, indicating that the ATPase domains of ClpA and ClpX are sufficient for specific peptide binding (Table IV). The DNS-SsrA association constants for both ClpA-A153 and ClpXΔN were lower than those obtained with their respective full-length proteins. The weaker binding could reflect some structural perturbation in the ATPase domains resulting from the absence of the N-domain. Interestingly, the DNS-SsrA fluorescent enhancement upon binding to ClpA$_6$ was 1.4-fold greater than that observed with ClpX$_6$, indicating additional differences in the extended binding pocket for peptide motifs in the two chaperones.

We also examined peptide binding to another deletion mutant of ClpA (ClpA-N438), which lacks the C-terminal ATPase domain, NBD2, one of two tandem AAA domains in this protein. In the ClpAP complex, the NBD2 domain contacts ClpP while the NBD1 domain is at the outer end of the complex (29, 39). In separate studies, we have found that ClpA-N438 oligomerizes in the presence of ATP$_\gamma$S as judged by the increase in mobility on a Superdex 200 gel filtration column and by the ladder of six bands that appear on SDS gels following cross-linking with glutaraldehyde (data not shown). ClpA-N438 does not interact with ClpP (29). ClpA-N438 bound DNS-SsrA in the presence of ATP$_\gamma$S, although the binding affinity was 10-fold lower than that observed for binding to intact ClpA$_6$ (Table IV).
**DISCUSSION**

This study has shown that ClpA and ClpX directly bind short peptides with sequences corresponding to motifs used for targeting proteins to these chaperones in vivo. Tight binding of peptides requires the nucleotide-bound assembled form of the chaperone, as is the case for recognition of intact protein substrates in vivo and in vitro (43). For ClpA, we tested the binding of specific variants of SsrA in which key residues shown to be important for binding and degradation of SsrA-tagged proteins were altered, and the results showed an excellent correlation between binding of the mutant peptide and the ability of proteins bearing the mutant tag to be degraded. Thus, the binding of peptides under these in vitro conditions reflects biologically important binding reactions, which correlate with interactions involving the substrate recognition sites on the ClpA and ClpX chaperones.

Binding of small peptides with sequence motifs recognized by ClpA and ClpX occurs with high affinity and at a stoichiometry of just one peptide per hexamer. Binding stoichiometry was determined by ITC and by end point titrations using fluorescent analogs of peptide substrates. ClpA and ClpX are symmetric particles composed of six identical subunits arranged in a ring. A preliminary report of the 1:1 stoichiometry of SsrA peptide binding to ClpA hexamer was made in 2002 (44); others (20) have calculated that ClpX also binds an SsrA-peptide at a stoichiometry of 1:1, although no definitive peptide binding studies for ClpX or ClpA have been previously reported. Restricting the number of peptides that can bind simultaneously to a 6-fold symmetric complex requires a geometry or mechanism of binding that limits either the number of potential binding sites available at a given time or their relative affinities. Limiting initial substrate binding stoichiometry by Clp/Hsp100 chaperones would seem to be advantageous and correlate with sequential processing of protein substrates. Biochemical experiments and direct visualization (15, 16, 25, 26) have shown that substrates are initially immobilized near the axial channel of a Clp/Hsp100 chaperone by binding of specific peptide sequence motifs located at the N or C termini of the substrate to chaperone sites, as yet unidentified, accessible from the distal ring surface. The substrate is then unfolded and translocated through axial channels of the chaperone and its functional partners, such as ClpP, into active centers, where they are processed (7, 8, 45, 46). Constraints imposed by the sizes of the axial channels and internal chambers of the complex would appear to limit processing and translocation to one protein at a time, and therefore, a binding mechanism that ensured engagement of only one protein substrate molecule at a time would avoid aggregation of partially unfolded substrates and prevent clogging of substrate engagement sites along the translocation pathway.

The thermodynamic properties of SsrA binding reactions provide some indication of conformational changes that affect the binding interactions. SsrA, DNS-SsrA, and the longer α-SsrA peptide all bind to ClpA hexamer with high affinity (ΔG, −9.5 ± 0.2 kcal mol⁻¹ at pH 7.5, 28 °C). For SsrA binding, the reaction is enthalpically controlled at 10 °C but entropically controlled at 28 °C; ΔH and −TΔS contribute equally to ΔG at 14 °C (Fig. 1E). A small positive ΔCp value of +0.74 kcal K⁻¹ mol⁻¹ has been determined after correcting for buffer proton effects. The small positive value of ΔCp suggests that any ordering of the ClpA hexamer on binding peptide substrates to produce a decrease in solvent accessible hydrophobic surfaces is offset by solvent rearrangement and/or burial of polar surfaces. The switch from a net uptake of 2 protons at 10 °C to a net release of 1 proton at 28 °C during SsrA binding to ClpA suggests that a conformational change in ClpA occurs between these temperatures that affects the pKₐ of one or more protein groups.

The locations of the binding sites in ClpA and ClpX are still under investigation. The binding sites lie within the nucleotide binding domains (NBDs) of both proteins as evident from the ability of deletion mutants lacking their N-terminal domains to bind peptides with high affinity. The minor decreases in affinity caused by the absence of N-terminal domains observed probably reflect structural perturbation in the nucleotide binding domains. In ClpA, the peptide binding site appears to reside in NBD1, because a mutant lacking NBD2 (ClpA-N438) also binds peptides with high affinity. This result suggests that a previously proposed substrate binding site in NBD2, referred to as the sensor and substrate discrimination site (42), is probably not the initial binding site for substrate motifs. Slightly weaker binding of peptides to ClpA-N438 than to intact ClpA and the 20% lower fluorescence intensity of the bound DNS-SsrA suggest that this peptide is more exposed to solvent. The increased solvent exposure could reflect either a structural change in NBD1 caused by the absence of NBD2 in the mutant or could indicate that the binding site lies near the interface between the two domains resulting in more complete burial of the ligand in intact ClpA. So far, cross-linking of peptide substrates to ClpX and NBD1 of ClpA (data not shown) has indicated that at least a portion of the peptide binding sites lies within the α/β subdomain, which also contains the structural components of the axial channel (47). More precise location of the peptide binding site is needed to help distinguish binding mechanisms and to help explain substrate discrimination in the Clp/Hsp100 chaperones.

Comparing the fluorescence change of DNS-SsrA bound to ClpA in the presence of ATP or ATPγS also suggests more solvent exposure of the bound peptide when ATP hydrolysis can occur. We could not detect a large difference in binding affinity between the ATP- and ATPγS-bound states of ClpA. However, nucleotide hydrolysis might increase on rates and off rates, thereby having little overall effect on binding affinity. One source of uncertainty in this conclusion, however, is that titrations with ATP were carried out at 6 °C, at which temperature ATP hydrolysis is slow. In addition, it is possible that the
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induces an allosteric change in the binding sites for other peptides. For instance, binding of each peptide occurs at different sites but the different peptides also contain divergent motifs. The different peptides also peptides containing divergent motifs were able to compete with DNS-SsrA for binding to ClpA. The different peptides also appeared to bind with a stoichiometry of 1 peptide per hexamer of ClpA. Thus, a single site or overlapping sites on the ClpA hexamer apparently recognize different sequence motifs. Alternatively, binding of each peptide occurs at different sites but induces an allosteric change in the binding sites for other peptides, which either alters their accessibility or their intrinsic affinity for their motifs. A third possibility is that peptide binding sites on ClpA are malleable or deformable and undergo an induced fit to accommodate specific motifs. Any of these mechanisms would suffice to limit binding to one peptide or protein substrate at a time and would lead to competition between substrates under physiological conditions in which the chaperones were limiting. Further details of the binding mechanism and stoichiometry are currently being studied and should lead to a more complete understanding of substrate recognition and selection in vitro.

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