SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Protein Mutagenesis

Im7 L18AL19AL37AH40W ($Im7_{A3W}$) and Im7 L18AL19AL37AW75F ($Im7_{A3W75F}$) were derived from Im7 L18AL19AL37A ($Im7_{A3}$) (Pashley et al., 2012) by site directed mutagenesis using the QuikChange II kit (Agilent) with the primers: 5′-ctggatgctcagtaatttttacaaaccattcgagtgccacatctaacacatca-3′ and 5′-tgatgtgttagatgtggcactcgaatggtttgtaaaaattactgagcatccag-3′ for $Im7_{A3W}$, and 5'-cgaagggattgtcaaggaaattaaagaatttcgagctgctaacggt-3' for $Im7_{A3W75F}$.

Generation of the α-S1-casein Peptide (amino acids 133–193)

Bovine casein is a naturally disordered protein with little secondary structure that is abundant in bovine milk (Koczan et al., 1991). Because of its disorder and exposed hydrophobic surfaces, casein has been previously used as a model chaperone substrate (Creamer et al., 1981; Lin et al., 1995; Nam and Walsh, 2003; Quan et al., 2011). However, commercially available bovine casein (Sigma-Aldrich) is a mixture of a least three proteins: α-S1-casein, α-S2-casein, and β-casein, containing possibly multiple chaperone binding sites. To simplify the analysis of complex formation we identified the binding sites for Spy on casein in order to derive a peptide that could be used to investigate client binding and release. For that, we used a similar approach as was used to identify a Spy-binding Im7 peptide (Quan et al., 2014). Commercially available bovine α-casein (Sigma-Aldrich) was digested with trypsin followed by affinity chromatography using a Strep-Tactin Sepharose column (IBA Life Sciences) that had been previously saturated with N-terminally Strep-tagged Spy. After washing the column, bound casein peptides were eluted together with tagged Spy by competition with 2.5 mM desthiobiotin. Co-eluted peptides were identified by mass spectrometry (Quan et al., 2014). For this study, we chose the most abundant casein peptide identified using this approach, which consisted of amino acids 133 through 193 of α-S1-casein (termed $casein_{133\text{-}193}$).

Protein Expression and Purification

Spy_{WT}, Spy variants H96L and Q100L, and $Im7_{A3}$ were expressed and purified as described previously (Stull et al., 2016). $Im7_{A3W}$ and $Im7_{A3W75F}$ were expressed and purified as described for $Im7_{A3}$. $Casein_{133\text{-}193}$ was expressed with an N-terminal His_6-SUMO-tag in *E. coli* BL21 (DE3) cells using a pET28b vector. Cells were harvested by centrifugation, resuspended in lysis buffer (40 mM Tris,10 mM sodium phosphate pH 8, 400 mM sodium chloride, 10% glycerol), and lysed using a French press. Cell lysate was spun down twice at 36,000*g* for 30 min at 4 °C. Supernatant was supplemented with 10 mM imidazole and passed through a 5 mL HisTrap column (GE Healthcare) using an ÄKTA PURE (GE Healthcare). The protein bound to the column was washed with 1 column volume of lysis buffer, then

washed with 3 column volumes of lysis buffer containing 10 mM imidazole and 6 M guanidine hydrochloride. The guanidine hydrochloride concentration was gradually reduced to 0 M by passing 15 column volumes lysis buffer through the column. The column was then washed with 6 column volumes of lysis buffer containing 100 mM imidazole, and the protein was eluted with 2 column volumes of lysis buffer containing 500 mM imidazole. Fractions containing protein were pooled and 500 μg of the SUMO protease (ULP1)(Li and Hochstrasser, 1999) was added to cleave off the His-SUMO tag, followed by dialysis overnight against buffer B (50 mM Tris pH 8, 300 mM sodium chloride, 5% glycerol). The following day, the protein was passed over a 5 mL HisTrap column (GE Healthcare) to remove the tag and the ULP1. The flow-through was then concentrated with an Amicon centrifugal filter (Millipore) and loaded onto a Superdex 75 HiLoad 16/600 column (GE Healthcare), pre-equilibrated with 40 mM HEPES (pH 7.5), 150 mM sodium chloride, 5% glycerol. Fractions containing the casein$_{133\text{-}193}$ peptide were then pooled and run over 0.5 mL Ni-NTA resin (Qiagen) in buffer B for final polishing. The clean peptide was pooled, concentrated, and frozen in liquid nitrogen.

α-lactabumin was purchased from Sigma and carboxymethylated to obtain a fully unfolded and soluble version of α-lactabumin. The carboxymethylation was done as described previously (Schechter et al., 1973).

Equilibrium Fluorescence

Dissociation constants of Spy-client complexes in standard buffer (40 mM Hepes [pH 7.5], 100 mM sodium chloride) were determined either by titrating 50 μM Im7$_{A3}$ with increasing concentrations of Spy$_{WT}$ or by titrating 250 nM Im7$_{A3W}$ with increasing concentrations of Spy$_{WT}$, Spy$_{H96L}$, or Spy$_{Q100L}$ at 22°C. Dissociation constants of Spy-client complexes were also determined at different salt concentrations by titrating 62.5 nM, 125 nM, 500 nM, or 1.5 μM of Im7$_{A3W}$ with increasing concentrations of Spy$_{WT}$ in 40 mM Hepes (pH 7.5) containing 25 mM, 50 mM, 200 mM, or 300 mM sodium chloride, respectively. Binding was monitored using the tryptophan fluorescence of Im7$_{A3W}$. The fluorophore was excited at 296 nm, and the fluorescence change upon complex formation was monitored using a QuantaMaster 400 (Photon Technology International). The fluorescence at 340 nm was then plotted as a function of Spy dimer concentration. The resulting binding isotherms were fitted either with a square hyperbola function [1] (for Im7$_{A3W}$ titrated with Spy$_{WT}$ in the presence of 100, 200, or 300 mM sodium chloride) or with a quadratic equation [2] (for Im7$_{A3}$ and Im7$_{A3W}$ titrated with Spy$_{WT}$, Spy$_{H96L}$, or Spy$_{Q100L}$ in the presence of 25, 50, or 100 mM sodium chloride) to obtain the K_d.

$$F = \frac{F_{max} * L}{K_d + L} + C \qquad [1]$$

where F is the recorded fluorescence signal, F_{max} is the maximum fluorescence reached upon saturation of the complex, L is the concentration of free Spy in solution, K_d is the dissociation constant, and C is a parameter for the offset.

$$F = \frac{F_0 * (C + L_0 + K_d - \sqrt{(C + L_0 + K_d)^2 - 4 * C * L_0})}{2 + I} \quad [2]$$

where F is the recorded fluorescence signal, F_0 is a fluorescence correction factor, C is the concentration of Im7, L_0 is the concentration of total Spy titrated to the reaction, K_d is the dissociation constant, and I is a parameter for the y-intercept.

Fluorescence Anisotropy

Tryptophan fluorescence anisotropy was used to determine the stoichiometry of the complexes. 50 μM $Im7_{A3}$ or 10 μM $Im7_{A3W}$ were titrated with increasing concentrations of Spy in 40 mM Hepes (pH 7.5), 100 mM sodium chloride at 22°C. The client tryptophan was excited at 296 nm, and the fluorescence emission was recorded at 340 nm using a Cary Eclipse spectrofluorometer (Agilent Technologies). Fluorescence anisotropy was calculated as described previously (Tapley et al., 2010). The resulting fluorescence anisotropy values were plotted as a function of Spy dimer concentration and fitted with a quadratic equation [2] to obtain the complex stoichiometry.

Cirular Dichroism (CD) Spectroscopy

The secondary structure content of $Im7_{A3}$ and $Im7_{A3W}$ was determined by CD spectrocopy using a Jasco J-810 CD spectropolarimeter (Jasco, Analytical Instrument). Prior to the experiment, both proteins were dialyzed against 40 mM potassium phosphate (pH 7.5), diluted to 20 μM, and loaded into a 1 mm pathlength cuvette. Spectra were collected from 195 nm to 260 nm at 22°C. In total, 6 scans of each protein were averaged. The CD spectra of both $Im7_{A3}$ and $Im7_{A3W}$ are similar to spectra reported earlier for $Im7_{A3}$ (Pashley et al., 2012), indicating that both proteins are unstructured and the H40W mutation does not substantially affect the secondary structure content.

Analytical Ultracentrifugation

Sedimentation velocity experiments for $Im7_{A3}$, $Im7_{A3W}$, and $Im7_{A3W75F}$ in the presence or absence of Spy were performed using a Beckman Proteome Lab XL-I analytical ultracentrifuge (Beckman Coulter) to determine complex stoichiometry. All proteins were first dialyzed against 40 mM Hepes, 100 mM sodium chloride (pH 7.5) prior to performing the experiments. To determine the complex stoichiometry of $Im7_{A3}$ and $Im7_{A3W75F}$ with Spy, 40 μM of Spy dimer was mixed with increasing concentrations of either $Im7_{A3}$ or $Im7_{A3W75F}$. For $Im7_{A3W}$, 30 μM of Spy dimer was mixed with increasing concentrations of $Im7_{A3W}$. Samples were loaded into cells containing standard sector shaped two-channel Epon centerpieces with 1.2

cm path-length (Beckman Coulter) and equilibrated to 22°C in the centrifuge for at least 1 hr prior to sedimentation. All samples were spun at 42,000 rpm in a Beckman AN-50 Ti rotor, and the sedimentation of the protein was monitored continuously by absorbance at 280 nm. Data analysis was conducted with SEDFIT (version 14.1) using the continuous c(s) distribution model (Schuck, 2000). The confidence level for the ME (maximum entropy) regularization was set to 0.95. Buffer density and viscosity were calculated using SEDNTERP (http://sednterp.unh.edu). The sedimentation distribution plots, c(s) as a function of s, obtained by SEDFIT were integrated to determine the relative distribution of complexed and freely sedimenting client protein. The integrated surface area was then plotted as a function of Im7 or Spy concentration to obtain binding isotherms. The stoichiometry was calculated by fitting the binding isotherms with a quadratic equation [2].

Stopped-Flow Fluorescence for Casein$_{133\text{-}193}$ and carboxymethylated α-Lactalbumin

As for Im7, the transient kinetics of Spy-client complex formation were recorded using a SF-2004 stopped-flow spectrofluorimeter (KinTek) by monitoring the change in tryptophan fluorescence of the casein$_{133\text{-}193}$ and carboxymethylated α-lactalbumin upon addition of Spy$_{WT}$. Casein$_{133\text{-}193}$ or carboxymethylated α-lactalbumin were mixed with increasing concentrations of Spy$_{WT}$ at a flow rate of 8 ml s^{-1}. The tryptophan was excited at 296 nm and fluorescence emission was recorded using a 340 ± 10 nm bandpass filter. Monochromator slits were set to 4 nm each. All experiments were carried out at 22°C in 40 mM Hepes (pH 7.5) and different amounts of sodium chloride to investigate the salt dependence of the binding reaction. The final concentration after mixing of casein$_{133\text{-}193}$ or carboxymethylated α-lactalbumin and Spy was chosen such that a pseudo-first-order approximation could be used for the data analysis and so that the observed rate constants did not exceed the limits of the instrument (the dead time was determined to be 1.3 ms). Casein$_{133\text{-}193}$: 250 nM casein$_{133\text{-}193}$ at 100 mM to 1000 mM sodium chloride; carboxymethylated α-lactalbumin: 125 nM carboxymethylated α-lactalbumin at 100 mM sodium chloride, 500 nM carboxymethylated α-lactalbumin at 150 mM sodium chloride, and 2 μM carboxymethylated α-lactalbumin at 200 mM and 250 mM sodium chloride.

As for Im7$_{A3W}$, an increase in tryptophan fluorescence upon binding of Spy to casein$_{133\text{-}193}$ or carboxymethylated α-lactalbumin was detected (Figure S4). For both casein$_{133\text{-}193}$ and carboxymethylated α-lactalbumin, two phases were observed upon mixing with Spy. Therefore, the transients were fit to a double exponential function to derive observed rate constants (k_{obs}). The binding rate constant (k_{on}) was determined the same way as for Im7$_{A3W}$ or Im7$_{WT}$ (see Experimental Procedures in the main text).

Table S1. K_d Values and Complex Stoichiometries[a]. Related to Figure 1, 2, 3, 4, 5, 6.

Im7 Variant	Spy Variant	Experimental Conditions[b]	Stoichiometry (Spy dimer:Im7)[c]	$K_{d,\,equ}$ (μM)[d]	$K_{d,\,kin}$ (μM)[e]	$K_{d,\,ITC}$ (μM)[f]	k_{on} (x 10^6 M^{-1} s^{-1})	$k_{off,\,intercept}$ (s^{-1})[g]	$k_{off,\,competition}$ (s^{-1})[h]	ΔH^i (kcal mol^{-1})	ΔS^j (cal mol^{-1} K^{-1})
$Im7_{A3}$	Spy_{WT}	T = 22˚C I = 0.12 M	1.2±0.1	10.4±5.8	n.d.	5.3±0.2	n.d.	n.d.	n.d.	7.11±0.13	48.23±0.45
		T = 22˚C I = 0.045 M	1.1±0.1	0.048±0.018	0.009±0.001	0.021±0.010	1190±37	18.0±16.0	10.5±0.2	4.53±0.10	49.75±0.72
		T = 22˚C I = 0.07 M	1.1±0.1	0.07±0.02	0.08±0.01	0.09±0.02	518±20	70.1±19.7	41.2±1.5	5.49±0.04	50.43±0.03
	Spy_{WT}	T = 22˚C I = 0.12 M	1.2±0.1	1.4 ±0.2	1.1±0.1	0.7±0.1	121±4	134±8	134±3 26.3±4.4[l]	6.12±0.04	48.97±0.51
		T = 22˚C I = 0.22 M	1.1±0.1	10±1	13±5	6.9±0.4	18.4±7.4	215±8	234±13 19.5±4.4[m]	7.17±0.06	47.90±0.50
$Im7_{A3W}$		T = 22˚C I = 0.32 M	1.1±0.2	36±4	66±5	34±1	4.7+0.2	298±7	310±17 27.6±5.4[n]	7.62±0.08	46.27±0.07
	Spy_{H96L}	T = 22˚C I = 0.12 M	1.2±0.1	0.27±0.03	0.16±0.01	0.16±0.01	166±5	45.6±9.5	27.0±1.1	8.58±0.09	60.23±0.15
	Spy_{Q100L}	T = 22˚C I = 0.12 M	1.2±0.1	0.07±0.02	0.007±0.001	0.062±0.034	147±6	14.8±19.9	1.0±0.1	2.77±0.24	43.90±1.4
	Spy_{WT}	T = 4˚C I = 0.045 M	1.1±0.1	n.d.	0.019±0.002	0.011±0.010	515±46	8.2±23.4	9.8±0.2 0.6±0.1[o]	11.43±0.55	74.83±0.90
$Im7_{WT}$	Spy_{WT}	T = 4˚C I = 0.045 M	0.7±01[p] 1.0±0.1[q]	0.05±0.06[r]	n.d.	4.4±0.9	2622±178[s]	n.d.	131.1±7.4 6.4±1.5	-5.67±0.25	4.07±0.54

[a]All values are averages of at least 3 independent experiments. Fitting errors of each experiment are propagated to yield an average standard error. n.d. = not determined.

[b]In this study, we varied the temperature (T) as well as the ionic strength (I) of the buffer to investigate the mechanism of client binding and release of the molecular chaperone Spy.

[c]Stoichiometry as determined by isothermal titration calorimetry (ITC).

[d, e, f]The K_d of Spy client interaction was determined either by equilibrium fluorescence titration (d), kinetically (e), or calorimetrically using isothermal titration calorimetry (f) (Figures S1, S5, and S6). The K_d for the Spy $Im7_{A3}$ complex could not be determined accurately by fluorescence due to the low signal change associated with binding (e) The kinetic K_d was calculated as a ratio of k_{off}/k_{on}, using the k_{off} determined by binding competition experiments.

[g, h]The release rate constant k_{off} is derived either from the y-intercept of the linear fit of the observed binding rate constants or from binding competition experiments (see Experimental Procedures and Figures 3, S4 and S5). As a competitor we used $Im7_{A3}$ where we replaced the endogenous W75 by an F.

[i, j, k]Thermodynamic parameters of Spy-client complex formation as determined by ITC.

[l, m, n, o]We observed a second phase when the Spy_{WT}-$Im7_{A3W}$ complex was mixed with the competitor $Im7_{A3W75F}$ to obtain a release rate constant. However, only at an ionic strength ≥ 0.12 M at 22˚C and 0.045 M at 4˚C was the contribution in amplitude > 5%, allowing k_{off} to be determined (see Figures S4A and S8D).

[p]Stoichiometry as determined by ITC.

[q, r]Stoichiometry and K_d derived from a fit of the initial fluorescence change during the burst phase of binding transients of $Im7_{WT}$ with Spy_{WT} recorded via stopped flow and therefore depict the stoichiometry and affinity of the natively folded state of Im7 to Spy (Figure S8). Note that this K_d is much lower than the observed K_d determined by ITC, presumably because the observed ITC K_d includes partial unfolding of $Im7_{WT}$ due to binding to Spy.

[s]Binding rate constant of the natively folded Im7 to Spy was back-calculated using the K_d (r) and k_{off} determined by fluorescence stopped flow.

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