Hsp110 Is a Nucleotide-activated Exchange Factor for Hsp70*

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Hsp110 proteins constitute a subfamily of the Hsp70 chaperones and are potent nucleotide exchange factors (NEFs) for canonical Hsp70s of the eukaryotic cytosol. Here, we show that the NEF activity of the yeast Hsp110 homologue Sse1 itself is controlled by nucleotide. Nucleotide binding results in formation of a stabilized conformation of Sse1 that is required for association with the yeast Hsp70 Ssa1. The interaction triggers release of bound ADP from Ssa1, but nucleotide persists bound to Sse1 in the complex. Surprisingly, removal of this nucleotide does not affect the integrity of the complex. Instead, rebinding to Sse1 in the complex. Our data demonstrate that in contrast to previously characterized NEFs for Hsp70 chaperones, the NEF activity of Sse1 requires nucleotide binding and let us propose a new model for Hsp110 function.

Members of the abundant Hsp70 class of chaperones play essential roles in diverse cellular processes, including the folding and assembly of newly synthesized proteins, the transport of proteins across membranes, the refolding of misfolded proteins, and the regulation of signal transduction proteins (1). Hsp70 chaperones transiently associate with peptide segments of protein substrates, thereby affecting their folding (2). The binding and release of substrates is driven by the ATPase cycle of the Hsp70. ATP binding to the N-terminal nucleotide-binding domain (NBD)6 induces conformational changes in the C-terminal substrate-binding domain that result in low affinity for substrates and high binding and release rates. ADP on the other hand results in higher affinity for substrates and low binding/release rates and thereby causes the trapping of substrates.

The basic chaperone cycle of Hsp70 proteins is modulated by cofactors that affect either ATP hydrolysis or exchange of ADP for ATP. The hydrolysis of ATP is stimulated by J-domain-containing co-chaperones that also deliver substrates and thereby facilitate substrate binding as well as provide specificity to the Hsp70s. Nucleotide exchange factors (NEFs) accelerate nucleotide release by associating with specific conformations of the Hsp70 NBD that exhibit low affinity for ADP and ATP. Rebinding of ATP and the concomitant dissociation of the NEF complete the exchange reaction.

Recently, it has become clear that several members of the Hsp70 superfamily function as NEFs for canonical Hsp70 chaperones. The first example of this functional diversification within the Hsp70 superfamily came from the finding that the yeast Grp170-class protein Lhs1 is a NEF for the endoplasmic reticulum resident Hsp70 chaperone Kar2 (3). In addition, the eukaryotic cytoplasm harbors a divergent branch of the Hsp70 superfamily with NEF activity. The Hsp110 proteins differ from other Hsp70 chaperones by an extended C-terminal domain, with an inserted acidic region between the terminal strands of the predicted β-sheet subdomain of the C-terminal domain as well as an elongated C terminus (4). A recent crystal structure of the yeast Hsp110 Sse1 lends further support to the close relationship between Hsp70 and Hsp110 (5). However, despite this similarity, Hsp110 proteins do not appear to be effective folding chaperones but rather act as NEFs for the cytosolic Hsp70 machinery (6, 7).

The yeast Saccharomyces cerevisiae harbors two highly homologous members of the Hsp110 family, the abundant Sse1 and the less expressed Sse2. Mutational analysis suggests that Sse1 and Sse2 have overlapping and essential activities; sse1 null mutant cells are slow growing, whereas sse2 null mutant cells exhibit no apparent phenotype and double null mutant cells are inviable (7, 8). Viability is restored by overexpression of Fes1, another NEF cognate to Hsp70 chaperones (7), strongly suggesting that the essential in vivo role of Sse1 and Sse2 is NEF activity.

The canonical Hsp70 chaperones in the cytoplasm of yeast fall into the two classes, Ssa (Ssa1–4) and Ssb (Ssb1–2). The Ssa chaperones are required for viability and are involved in housekeeping functions as well as stress-related protein folding processes, whereas the Ssb chaperones are dispensable for viability and appear to facilitate nascent polypeptide folding at the ribosome. Sse1 forms high affinity complexes with both Hsp70 classes, whereas Sse2 is restricted to the Ssa class (7, 9–11). The meaning of these stable complexes in the cell is unclear and has lately been the subject of controversy. One possibility is that the Hsp70 in these complexes are trapped in a conformation with low affinity for nucleotide and that they therefore represent a nucleotide-sensitive intermediate state in the nucleotide-exchange reaction. Indeed, co-immunoprecipitation and surface plasmon reso-
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nce spectroscopy (Biacore) experiments indicate that the presence of ATP drastically reduces the interaction between Ssa1 and Sse1 (7). Paradoxically, a set of experiments using gel shift assays to assess the interaction between Sse1 and a C-terminally truncated bovine Hsc70 indicates that ATP is required for formation of a heterodimeric complex (10). Furthermore, labeled ATP was found to persist in the complex and only very slowly undergo hydrolysis, leading the authors to postulate an unconventional and multi-step mechanism for the Sse1 NEF function (10, 12). These apparently conflicting observations call for a thorough analysis of how nucleotides influence the formation, integrity, and dissociation of the complex between Sse1 and its cognate Hsp70, Ssa1.

Here we show that nucleotide binding stabilizes the highly flexible Sse1 and in two steps induces a conformation that is able to associate with Ssa1 and catalyze nucleotide release. Nucleotide persists in the stable heterodimeric complex; nevertheless, removal of all bound nucleotide does not affect complex integrity nor does it appear to compromise the folding status of Sse1 in the complex. Excess nucleotide triggers dissociation of the complex by binding to Ssa1, via a mechanism similar to that of other characterized NEFs. Taken together, our data indicate that nucleotide is the key regulator of the NEF cycle of Sse1, driving both complex formation and dissociation.

EXPERIMENTAL PROCEDURES

Reagents—N\textsubscript{6}-(4-N\textsuperscript{-}methylanthraniloylaminobutyl)-8-aminooasenosine 5′-diphosphate (MABA-ADP) was purchased from TrilLink Technologies Inc., San Diego, CA. ATP, ADP, and AMP were obtained from Roche Applied Science. ADP was purified to remove contaminating ATP by anion exchange chromatography using a ResourceQ 1-mL column eluted with a 0.1–1 M NH\textsubscript{4}CO\textsubscript{3} gradient followed by lyophilization. D\textsubscript{2}O buffer for hydrogen-deuterium exchange (HX) experiment was prepared using 99.85% D\textsubscript{2}O (Euriso-top, Gif-sur-Yvette, France), lyophilized, and re-dissolved five times in fresh D\textsubscript{2}O volumes.

Plasmids—The expression vector pCA528 supports T7-promoted expression of proteins as His\textsubscript{6}-SUMO fusions and was constructed by ligation of a SMT3 promoter from yeast chromosomal DNA (primers CCTTGGCATATGGGTGTCGGTATGTCAAAATAGCAGCTTGAAC) and KpnI/XhoI-restricted StrepTactin-Sepharose (IBA, GmbH, Göttingen, Germany), and bound complexes were eluted by addition of 2.5 mM des-ribiotinylated Ssa1 carrying a C-terminal His\textsubscript{10} tag at its C terminus was expressed from pCA599 and purified similarly.

Nucleotide Removal—Proteins were made nucleotide-free by a protocol modified from Theysen et al. (15). Briefly, the protein was diazylated overnight against 25 mM Hesper-KOH, 50 mM KCl, 5 mM MgCl\textsubscript{2}, 2 mM dithiothreitol in the presence of calf intestine phosphatase (Roche Applied Science) at 4 °C. Calf intestine phosphatase was removed by ion exchange chromatography over a ResourceQ 6-ml column in the same buffer containing 5% (v/v) glycerol. Sse1 and Sse1\textsubscript{K69M} carrying a C-terminal Strep-Tag II (WSH-PQFEK) were puréed using Ni-IDA matrix. Eluted material was applied onto a final concentration of 250 mM imidazole was supplemented with His\textsubscript{6}-Ulpl protease, dialyzed overnight, and contaminants were removed by incubation with Ni-IDA matrix. The NBD of Ssa1 was purified similarly but expressed from pCA592. Sse1 was expressed and purified as described previously (14) or from pCA534 and purified in a similar manner as Ssa1.

Ssa1 and Ssa1\textsubscript{G198D} carrying a C-terminal His\textsubscript{10} tag (7) were expressed as described for the SUMO-fused Ssa1 with exclusion of isopropyl-1-thio-β-d-galactopyranoside. Sse1 and Sse1\textsubscript{K69M} carrying a C-terminal Strep-Tag II were expressed from the vector pCA568. To isolate stoichiometric complexes, cells from separate expression cultures were pooled, lysed, and purified using Ni-IDA matrix. Eluted material was applied onto StrepTactin-Sepharose (IBA, GmbH, Göttingen, Germany), and bound complexes were eluted by addition of 2.5 mM des-ribiotinylated doubly tagged Ssa1 carrying a Strep-Tag-II-SUMO at its N terminus and a His\textsubscript{6} tag at its C terminus was expressed from pCA599 and purified similarly.

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For expression of the NBD domain of Ssa1, pCA592 was constructed by PCR-amplifying a fragment of SSA1 (primers CCAAGGTCAATGAGCTTG, GCGGCCTCGAGTTAACCAAAATAGCAGCTTGAAC) and KpnI/Xhol-restricting the product and ligating it with similarly restricted pCA533. The expression vector pCA569 was constructed in multiple steps generating a pUHE21-2fd12 (13) derivative expressing Sse1 carrying a C-terminal Strep-Tag II (WSHPQFEK). The Sse1 K69M mutation was introduced into pCA569 as a BamHI/HindIII fragment obtained from pUHESSE1-K69M (14). pET20b-SSA1\textsubscript{G198D}-His\textsubscript{10} was obtained by site-directed mutagenesis of pET20b-SSA1-His\textsubscript{10} (7).

Protein Expression and Purification—Ssa1 with a Ulpl-cleavable N-terminal His\textsubscript{6}-SUMO tag was expressed from the vector pCA533 in BL21-SI/pCodonPlus cells (Invitrogen) by induction at 20 °C with 0.3 mM NaCl and 0.5 mM isopropyl-1-thio-β-d-galactopyranoside. The protein was purified using Ni-IDA matrix (Proton; Macherey-Nagel, Düren, Germany); eluted material containing 250 mM imidazole was supplemented with His\textsubscript{6}-Ulpl protease, dialyzed overnight, and contaminants were removed by incubation with Ni-IDA matrix. The NBD of Ssa1 was purified similarly but expressed from pCA592. Sse1 was expressed and purified as described previously (14) or from pCA534 and purified in a similar manner as Ssa1.

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Nucleotide Removal—Proteins were made nucleotide-free by a protocol modified from Theysen et al. (15). Briefly, the protein was diazylated overnight against 25 mM Hesper-KOH, 50 mM KCl, 5 mM MgCl\textsubscript{2}, 2 mM dithiothreitol in the presence of calf intestine phosphatase (Roche Applied Science) at 4 °C. Calf intestine phosphatase was removed by ion exchange chromatography using a ResourceQ 6-ml column in the same buffer containing 5% (v/v) glycerol. The nucleotide content (<3–5%) in the final protein fraction was assessed by analytical ion exchange chromatography (ResourceQ 1 ml in 25 mM Hesper-KOH, pH 6.5, 0–1.5 mM KCl).

Ssa1-Sse1 Association Assay—To assess complex formation, 2 μM nucleotide-free Sse1 was incubated for 10 min at 4 °C with ATP or purified ADP at concentrations ranging from 0.2 to 2 mM in 250 μL of LWB (40 mM Hesper-KOH, 150 mM KCl, 5 mM MgCl\textsubscript{2}, 5% (v/v) glycerol, 20 mM β-mercaptoethanol). Ssa1 carrying a N-terminal Strep-Tag-II-SUMO fusion was added to a final concentration of 2 μM and incubated for 10 min before 25 μL of StrepTactin-Sepharose suspension was added. After 20 min, unbound material was removed by washing and bound material was eluted by proteolytic cleavage of the SUMO tag by
incubation with Ulp1 protease. Eluted material was analyzed on 8% SDS-PAGE that was stained with Coomassie Brilliant Blue.

Ssa1-Sse1 Dissociation Assay—Ssa1-His6, Sse1-Strep-Tag-II complexes and mutant derivatives were immobilized onto Ni-NTA-agarose (Qiagen, Hilden, Germany) by incubation of 600 μl of 5 μM protein solutions in LWB with 75 μl of matrix suspension at 4°C for 60 min. Unbound protein was removed by washing, and beads were incubated with buffer with and without addition of 4 mM ATP or purified ADP for 30 min at 4°C. Released material was collected from the supernatant, and remaining bound material was eluted with 250 mM imidazole. Collected fractions were separated on 8% SDS-PAGE and stained with Coomassie Brilliant Blue.

Size Exclusion Chromatography—8 μM Sse1 was incubated alone or with 24 μM Ssa11–378 for 30 min at 30°C in the absence of nucleotide or in the presence of 10 mM ATP. The samples (100 μl) were analyzed on an analytical Superdex 200 column equilibrated in 25 mM Hepes-KOH, pH 7.4, 150 mM KCl, 5 mM MgCl2, 2 mM dithiothreitol, 0.05% (v/v) Tween 20. The fractions were analyzed on a silver-stained 12% SDS-PAGE gel.

Nucleotide Release Analysis—Nucleotide release was measured using the fluorescently labeled analogue of ADP, MABA-ADP (15), and the stopped flow instrumentation SX-18MV from Applied Photophysics (Surrey, UK). 0.5 μM Ssa1 was preincubated with 0.5 μM MABA-ADP in HKM buffer (25 mM Hepes-KOH, pH 7.6, 50 mM KCl, 5 mM MgCl2) for 30 min at 30°C. For determination of the basal dissociation rate, equal volumes of protein-MABA-ADP complex and HKM buffer, or HKM buffer containing excess unlabeled nucleotide (250 μM ATP, 250 μM or 2 μM purified ADP), were rapidly mixed in the stopped flow device and the decrease in fluorescent signal was monitored. Stimulation of the dissociation rate was assessed by mixing Ssa1-MABA-ADP with a solution of 2 μM nucleotide-free Sse1 or 2 μM Sse1 preincubated at 30°C with 250 μM unlabeled ATP, 250 μM or 2 μM purified ADP, respectively. The measurement time scales ranged from 0.1 to 10 s. The kinetic rate constants were obtained by fitting the data using the Applied Photophysics SpectraKinetic Work station v4.56-1.

Hydrogen-deuterium Exchange Experiments and Mass Spectrometry—HX experiments were performed in a manner similar to that described earlier (16, 17). To initiate amide proton-deuteron exchange, 100–200 pmol of purified proteins alone or supplemented with defined nucleotides were diluted 10-fold (Fig. 2) or 20-fold (Figs. 3 and 4) into D2O-based buffer (25 mM Hepes-KOH, pH 7.4, 50 mM KCl, 5 mM MgCl2). After incubation at 30°C for a fixed time period, the exchange reaction was stopped by addition of 1 volume of ice-cold quench buffer (0.4 M potassium phosphate buffer, pH 2.2). Quenched samples were immediately injected into the high performance liquid chromatography setup and analyzed on an electrospray ionization quadrupole time-of-flight mass spectrometer (QSTAR Pulsar; Applied Biosystems, Foster City, CA) as described previously (17, 18).

In the experiments of Figs. 2, A and D, 3B, and 4B, the respective proteins were preincubated for 5 min at 30°C with the appropriate concentrations of nucleotide before initiating amide hydrogen exchange by dilution into D2O buffer. For the data of Fig. 2C, excess nucleotide was removed by passage over a Zeba Desalt Spin Column (Pierce) before the HX experiment.

RESULTS

Nucleotides Are Required for Formation of an Ssa1-Sse1 Complex—To understand how Hsp110 regulates the chaperone cycle of Hsp70, we set out to characterize the complex that is formed between yeast Ssa1 and Sse1. We started by investigating the formation of the complex using purified components in a defined nucleotide state. Ssa1 was incubated with nucleotide-free Sse1 (see "Experimental Procedures") on StrepTactin matrix by means of a Strep-Tag-II-SUMO moiety fused to its N terminus. After washing, Ssa1 and bound Sse1 were eluted by proteolytic severing of the SUMO tag and analyzed on SDS-PAGE. In the absence of added nucleotide, Sse1 did not form any detectable complex with Ssa1 (Fig. 1A, lower panel, lane 2). In contrast, the presence of ADP (purified free from contaminating ATP, see "Experimental Procedures") in amounts ranging from equimolar to 1000-fold excess supported weak formation of the complex (Fig. 1A, lower panel, lanes 3–6) and addition of ATP supported the interaction even more robustly than ADP (lanes 7–9). In the assay, ADP and ATP appear to be most effective in promoting Ssa1-Sse1 association when added in an equimolar ratio or up to a 100-fold excess, whereas a 1000-fold excess resulted in significantly less detectable complex (compare lanes 7–9 with lane 10).

In an independent approach, we assessed the nucleotide requirement for complex formation using size exclusion chromatography applied on full-length Sse1 and the isolated NBD of Ssa1 (Ssa11–378). In the absence of added nucleotide, Sse1 and Ssa11–378 eluted in separate fractions according to their respective sizes when the mixture was analyzed on a size exclusion column (Fig. 1B, upper panel), indicating that no complex was formed. In contrast, addition of 10 mM ATP to the mixed proteins before chromatography resulted in significant complex formation as evidenced by co-elution of Ssa11–378 and Sse1 (Fig. 1B, lower panel). Note that excess ATP was separated from the protein during size exclusion chromatography, because the running buffer did not contain nucleotide. The NBD of Ssa1 is predicted to be sufficient for a bona fide Hsp70-NEF interaction (10, 19, 20) but cannot support a putative Hsp70-substrate interaction between Sse1 and Ssa1. Consequently, the experiment formally rules out the possibility that the complex studied is an Hsp70-substrate interaction. Similar experiments using full-length Ssa1 and Sse1 also revealed interactions of the proteins only when ATP was present in the sample (data not shown).

Sse1 Requires Nucleotide to Efficiently Catalyze Nucleotide Release from Ssa1—The nucleotide dependence for complex formation between Ssa1 and Sse1 enabled us to directly test whether the observed interaction correlates with catalyzed release of ADP from Ssa1. Preloaded complexes consisting of Ssa1 and the fluorescent nucleotide derivative MABA-ADP were rapidly mixed with Sse1, and dissociation of MABA-ADP was followed by decrease in its fluorescence signal in stopped flow measurements (7, 15). In the presence of a 4-fold excess of nucleotide-free Sse1 over Ssa1, only 6-fold acceleration of the basal release rate could be detected (Fig. 1C). Under identical
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A

B

C

FIGURE 1. Nucleotides Control the Hsp110-Hsp70 Complex

A, Sse1 binding to Ssa1 is more efficiently supported by ATP than ADP. SUMO-Ssa1 immobilized onto StrepTactin beads was incubated with Sse1 (2 μM) in the presence or absence of increasing concentrations (2, 20, and 200 μM and 2 mM) purified ADP or ATP. After washing, Ssa1 was eluted by proteolytic cleavage of the SUMO tag and the amount of co-isolated Sse1 was assessed by SDS-PAGE analysis. The upper panel shows a control experiment performed in the absence of SUMO-Ssa1. B, co-migration of the Ssa1 nucleotide-binding domain with Sse1 in size exclusion chromatography is observed only in the presence of nucleotide. 8 μM Sse1 was preincubated with 24 μM Ssa1(1-378) in the absence or the presence of 10 mM ATP, and samples were analyzed by size exclusion chromatography using a Superdex 200 column. C, the Sse1 nucleotide exchange factor activity is nucleotide-regulated. Dissociation rate of MABA-ADP from Ssa1 in the presence of nucleotide-free or nucleotide-bound Sse1. 2 μM Sse1 was preincubated with 0 or 250 μM ATP, 250 μM ADP, or 2 mM ADP before measurements of the stimulated MABA-ADP release rate with a stopped-flow instrument. The error bars indicate S.D.

NMCs promote the NEF activity of Sse1 and that ATP induces this activity far more potently than ADP.

ATP Binding Induces a Stabilization of Sse1—We next sought to explain why nucleotide binding to Sse1 is required to generate a functional NEF complex with cognate Hsp70s. We applied amide hydrogen exchange (HX) combined with mass spectrometry to analyze the effects of nucleotides on Sse1. With this technique, conformational changes and binding interfaces to partners can be resolved by monitoring changes in solvent accessibility of amide hydrogen positions in the protein (21). Briefly, purified nucleotide-free proteins were equilibrated with defined nucleotides and then diluted in D2O-based buffer solution for a fixed time period. The number of incorporated deuterons was determined by mass spectrometry after stopping the HX reaction. Nucleotide-free Sse1 exchanges as much as 62% of all available amide hydrogens in the 2-min reaction (410 deuterons incorporated, Fig. 2A), which indicates a very dynamic and loosely folded conformation of the molecule. This unusual behavior is in contrast to that of canonical Hsp70 chaperones, which exchange much more slowly. For comparison, 42 and 37% of all available amide hydrogen positions exchange within a 2-min reaction in Ssa1 and DnaK, respectively (17).

When increasing concentrations of ATP were added to Sse1, we observed a gradual decrease in the number of deuterons incorporated in the 2-min exchange reaction down to ~59% of exchangeable positions (391 deuterons, Fig. 2A, panels 1–6), indicating stabilization of the molecule. In addition, ATP promoted the emergence of a second distinct species of Sse1 that exchanges much more slowly than the nucleotide-free protein (272 deuterons incorporated, i.e. 41% of available amide hydrogens that exchanged within 2 min; Fig. 2A, spectra 3–8). These observations suggest that Sse1 undergoes a two-step ATP-driven compacting reaction (Fig. 2B). According to this model, nucleotide-free Sse1 predominantly adopts a highly flexible conformation (Fig. 2B, I) whereas it exists mostly in a stabilized state when bound to nucleotide (Fig. 2B, II). This form can in turn adopt a fully compacted and nucleotide-bound structure (Fig. 2B, III). ATP is efficient at inducing stabilization and conversion to the compact form of Sse1, whereas ADP at 2 mM induces full stabilization (Conformation II) but does not yield any detectable compact form (Fig. 1A, lane 9, III). However, inclusion of phosphate in the ADP condition resulted in detection of the compact form (data not shown), suggesting that ADP-Sse1 is maintained in a strongly biased equilibrium between conformations II and III. To make sure that our HX experiments were performed under saturating ATP and ADP conditions, we determined the affinities that Sse1 exhibits toward these nucleotides. The $K_d$ for ADP is 23 ± 9 μM and for ATP 2.1 ± 0.6 μM as determined by MABA-ADP competition conditions, a 4-fold excess of Sse1 preincubated with 250 μM ADP resulted in 20-fold acceleration of the basal release rate. In contrast, when incubated with 250 μM ATP Sse1 accelerated the basal release rate 85-fold. Increasing the ADP concentra-

\[ J. \text{Fiaux and B. Bukau, unpublished observations.} \]
Sse1 was preincubated with Ssa1 in the absence of nucleotide, increases after 2-min HX reactions were determined. When titration (data not shown) (15). Thus, in contrast to ATP, ADP only very inefficiently supports conversion of Sse1 to the compact configuration III even under nucleotide saturation conditions (Fig. 1A, compare lanes 3–6 with lane 9).

We used HX experiments to characterize the complex formed between Ssa1 and Sse1 (Fig. 2C). Nucleotide-free Sse1 was incubated with ADP or ATP and a 2-fold excess of Ssa1. Unbound nucleotide was removed by quickly passing the solution over a size exclusion chromatography column, and mass increases after 2-min HX reactions were determined. When Sse1 was preincubated with Ssa1 in the absence of nucleotide, whereas ADP only poorly induces the same active conformation and mainly maintains Sse1 in an inactive conformation (Fig. 2B, II).

**Nucleotide Is Not Required for the Integrity of the Ssa1-Sse1 Complex**—Others have observed that ATP persists in the Ssa1-Sse1 complex (227 deuterons) when compared with nucleotide-free (410 deuterons) or ATP-bound (272 deuterons) Sse1 (compare Fig. 2A, lanes 3–8 with Fig. 2C, lane 5). This indicates that formation of a complex between Ssa1 and Sse1 results in significant amide protection at the complex interface or further stabilization of the ATP-bound Sse1 through binding to Ssa1. Preincubation of Sse1 with Ssa1 in the presence of 250 μM ADP weakly supported formation of an Ssa1-Sse1 complex (Fig. 2C, lane 3). Increasing the ADP concentration to 10 mM resulted only in a modest increase of formed complex (Fig. 2C, compare lanes 3 and 4), consistent with the interpretation that 250 μM ADP already almost fully saturates Sse1.

Attempts to detect an Ssa1-Sse1 complex in the presence of excess nucleotide resulted in less amide protection (264 deuterons incorporated in 2 min of HX) compared with the above situation (Fig. 2D). The lower extent of protection likely represents dynamic association and dissociation of the Ssa1-Sse1 complex. Indeed, excess nucleotide (re-)binding to Ssa1 triggers complex dissociation (see below and “Discussion”). This dynamic behavior is also apparent in Fig. 1A, lane 10.

Taken together, these results suggest that ATP efficiently induces a compact conformation of Sse1 (Fig. 2B, III) that can interact with Ssa1 to mediate nucleotide exchange,
Sse1, respectively, and purified the complexes free from unbound individual components and excess nucleotide using serial affinity chromatography. The ADP and ATP contents in the purified complex exhibited great variation between purifications (data not shown). However, of the total amount co-purified nucleotide, consistently ADP was more abundant than ATP and the total level of nucleotide was substoichiometric to the amount of Ssa1-Sse1 complex, suggesting that nucleotide is not strictly required for complex integrity. To rigorously test whether removal of nucleotide destabilizes the Ssa1-Sse1 complex, we treated a nucleotide-containing complex (40% ADP, 10% ATP) with phosphatase and verified the complete removal of nucleotide (nucleotide not detectable, <3% stoichiometric ratio). We incubated the nucleotide-containing and the nucleotide-free protein samples with Ni-NTA matrix and assessed the amount of Sse1 that remained in complex with Ssa1 (Fig. 3A). The isolated complexes were stoichiometric even when all nucleotide had been removed, indicating that bound nucleotide is not strictly required for complex integrity.

To investigate whether nucleotide-free and nucleotide-bound Ssa1-Sse1 complexes exhibited any differences with regard to their conformations, we determined the HX properties for the two types of complexes. Sse1 exhibited identical exchange kinetics whether nucleotide was present in the complex or removed by phosphatase treatment (Fig. 3B). Hence, removal of all bound nucleotide does not affect complex integrity or compromise the folding status of Sse1 once bound in the protein complex.

Nucleotide Binding to Ssa1 Induces Dissociation of the Ssa1-Sse1 Complex—According to the general understanding of NEF action, Sse1 preferentially binds to the NBD of Ssa1 when the latter attains a conformation with low affinity for nucleotides. Consequently, the re-binding of nucleotide to Ssa1 should induce complex dissociation by favoring nucleotide-bound NBD conformations that are unable to associate with Sse1. To directly test whether excess nucleotide binds to Ssa1 and induces complex dissociation, we immobilized stable stoichiometric complexes of Ssa1-His$_{10}$ and Sse1-Strep tag II on Ni-NTA matrix and incubated the complexes with 4 mM ADP or ATP. Buffer alone did not result in any detectable release of Sse1 from Ssa1 whereas ADP, and more potently ATP, stimulated dissociation (Fig. 4A, lanes 1–6). In contrast, when we introduced nucleotide binding deficiency through a G198D mutation in Ssa1 (22), nucleotide did not trigger any detectable dissociation of Ssa1 from a complex (Fig. 4A, lanes 13–18), indicating that binding of nucleotide to Ssa1 is required for the release event. In addition, nucleotide-induced dissociation between Ssa1 and Sse1 is apparent in Figs. 1A and 2D. The

**FIGURE 3.** Nucleotide is not required for the maintenance of the Ssa1-Sse1 complex. A, nucleotide removal does not compromise the interaction between Sse1 and Ssa1. The Ssa1-His$_{10}$-Sse1 complex was purified in the presence of residual-bound ADP or made nucleotide-free by calf intestine phosphatase (CIP) treatment. The samples (load) were incubated with Ni-IDA matrix, the matrix was washed (unbound), and the bound material eluted with imidazole (eluted). The amount of Sse1 that co-isolated with Ssa1 (lanes 1–6) was assessed from SDS-PAGE analysis. B, nucleotide removal affects the properties of free Sse1 in HX experiments, but not of Ssa1-bound Sse1. Mass increase through deuteron incorporation is shown for Sse1-Strep-Tag II alone or in its complex with Ssa1-His$_{10}$ as a function of the HX reaction time. The measurements were performed at 30 °C in the presence of residual ADP in the complex (open symbols) and after complete removal of nucleotide by calf intestine phosphatase treatment (filled symbols). For comparison, Sse1-Strep-Tag II was incubated with ATP (+) or measured in its nucleotide-free state after calf intestine phosphatase treatment (○). The inset shows the same data plotted with a logarithmic time scale.

**FIGURE 4.** Dissociation of the Ssa1-Sse1 complex is triggered by nucleotide binding to Ssa1. A, purified complexes of Ssa1-His$_{10}$ with Sse1-Strep-Tag II, Ssa1-His$_{10}$ with Sse1$_{K69M}$-Strep-Tag II, and Ssa1$_{G198D}$-His$_{10}$ with Sse1-Strep-Tag II were immobilized onto Ni-NTA beads, washed, and incubated with buffer alone or buffer supplemented with excess purified ADP or ATP. The remaining bound protein (bound) was eluted with imidazole and analyzed together with the supernatants (released). B, complex dissociation by ATP addition assessed by HX. Mass increase through deuteron incorporation is shown for Sse1-Strep-Tag II alone (○) or in its complex with Ssa1-His$_{10}$ (●) as a function of the HX reaction time. The measurements were performed at 30 °C with nucleotide-free proteins (open symbols) and after addition of an excess ATP (filled symbols).
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Our model for Sse1-driven nucleotide exchange follows the principle that characterize NEF function. Accordingly, nucleotide exchange is the result of catalyzed nucleotide release, which in turn relies on the transient stabilization of the NBD in a conformation that exhibits low affinity for nucleotides. However, in contrast to other known NEFs, we find that Sse1 itself requires nucleotide for full activity. Without ATP or ADP present, Sse1 exhibits only very low basal NEF activity (Fig. 1C) and does not form readily detectable complexes with Ssa1 (Figs. 1 and 2). ATP-bound Sse1 forms complexes with Ssa1 and potently catalyzes ADP release from Ssa1. Also, ADP induces formation of a complex with Ssa1 and accelerates the basal NEF activity of Sse1, but not to the same extent as ATP. This difference in potency between ADP and ATP cannot be explained by the affinity difference Sse1 exhibits for ADP (23 μM) and ATP (2.1 μM), because even a saturating concentration of ADP does not induce full activity of Sse1 (Fig. 1C). Instead, our HX experiments are consistent with the interpretation that Sse1 undergoes a two-step compacting reaction resulting in an active conformation (Fig. 2A). Accordingly, Sse1 is in a fast equilibrium between its nucleotide-free form (Conformation I) and its nucleotide-bound but inactive form (Conformation II). ATP very efficiently triggers the critical conformational change into a third active conformation (Conformation III), whereas ADP even at concentrations that allow full occupancy of conformation II (2 mM ADP) does so only very inefficiently. Likely, the γ-phosphate of ATP plays a critical role in triggering the conversion into conformation III, and in support of this notion, addition of phosphate to the HX ADP condition resulted in detectable formation of conformation III.

Because ATP addition results in the simultaneous compaction of Sse1, the formation of Ssa1-Sse1 complexes, and the appearance of NEF activity, we surmise that ATP binding induces the functional Sse1 conformation, which locks Ssa1 in a state with poor affinity for ADP or ATP. Transient stabilization of this Ssa1 conformation results in catalyzed nucleotide release.

The critical role that ATP plays to obtain a compacted conformation of Sse1 is supported by previous studies that have documented protection of Sse1 from proteolysis upon addition of ATP (7, 10) and the observation that Sse1 only could be successfully crystallized in the presence of ATP but not in its nucleotide-free or ADP-bound form (5). We consider the possibility that the reported crystal structure of Sse1-ATP represents the active NEF conformation (Conformation III) of Sse1. No indications of the two-step compacting reaction of Sse1 can be derived from this structure.

In a recent study, Morano and coworkers (10) have considered how nucleotide affects formation of a complex between Sse1 and a bovine Hsp70 mutant. The authors document that...
20–25% of Sse1 forms a complex with the Hsc70 in the absence of added nucleotide whereas addition of ATP, and to a lesser extent addition of ADP, results in more robust complex formation. The observed enhancing effect of ATP on complex formation is consistent with our findings.

The stoichiometric complexes of Ssa1 and Sse1 that we purified contained variable sub-stoichiometric amounts of ADP and ATP. Morano and coworkers (10, 12) have shown that nucleotide is bound to Sse1 in the complex with Hsp70 and proposed a model in which the loss of bound ATP by release or hydrolysis might regulate dissociation of the complex. Our data are not consistent with this model. First, our purified stoichiometric complexes of Ssa1 and Sse1 contain only sub-stoichiometric amounts of ADP and ATP relative to the complex. In addition, complete enzymatic removal of nucleotides by phosphatase treatment does not impair complex integrity. Finally, the hydrolysis-deficient Sse1K69M mutant forms a stable complex with Ssa1 that exhibits dissociation properties indistinguishable from that of wild type protein. Furthermore, Sse1K69M fully complements sse1, sse2 double mutations in vivo, indicating that the ATP hydrolysis is not required for NEF activity in the cell (7, 24). Thus, ATP bound to Sse1 in complex with Ssa1 does not play a central role in regulating the dissociation of the complex. Similarly, we note that the ATPase activity of Sse1 remains without functional assignment.

We find that Ssa1-Sse1 complexes are dissociated by the mere presence of excess ADP and ATP. This observation is consistent with the behavior of other NEF-Hsp70 interactions (25, 26) and can be explained by Sse1 exhibiting the highest affinity for a Ssa1 conformation with low affinity for nucleotide. Association of excess ADP or ATP with Ssa1 would favor alternative conformations that exhibit higher affinity for nucleotide but lower affinity for Sse1. As a consequence, nucleotides trigger the dissociation of Sse1 by binding to Ssa1.

The nucleotide-triggered conformational changes that induce Sse1 NEF activity represent a potential mechanism by which the cell could regulate its Hsp70 NEF activity in response to varying cellular demand. Formally, a drop in the cytoplasmic ATP level below 2 μM is sufficient to down-regulate Sse1 activity. So far, in yeast cells the most severe nutrient starvation conditions have been reported to result in a 100-fold drop of ATP levels to 10–50 μM. Hence, this condition alone is not sufficient to inactivate Sse1.

At present, it is unclear whether Hsp110 proteins other than Sse1 exhibit nucleotide dependence for interaction with their cognate Hsp70 chaperones. For example, the mammalian Hsp105α has been demonstrated to catalyze nucleotide release from Hsp70 (6) but nucleotide dependence for this function has not been tested directly. However, upon complex formation with Hsp70 the ATPase activity of Hsp105α is activated (27), suggesting a stabilization of the NBD in line with the behavior we observe for Sse1.

A more detailed understanding of the mechanism by which Hsp110s mediate nucleotide release from Hsp70 is urgently needed. It appears that more than an individual domain of Sse1 is directly or indirectly involved in the catalytic activity, because the NBD domain in isolation as well as C-terminal segments are not sufficient for activity (6, 24). This raises the intriguing possibility that multiple domains of Hsp110 contact the NBD of Hsp70s, perhaps resulting in a novel mechanism for the opening of the nucleotide-binding site.

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