Hsp70 molecular chaperones are ATPases that bind to hydrophobic regions of proteins and guide their folding, assembly, and translocation across membranes. The ability of purified Hsp70s to uncoat clathrin-coated vesicles or to stimulate the post-translational translocation of precursor proteins into the endoplasmic reticulum, mitochondria, and the nucleus was previously shown not to be sensitive to the sulfhydryl-modifying reagent N-ethylmaleimide (NEM). During purification of factors required for protein folding in the cytosol, we found that the ATP-agarose binding activity of the yeast Hsp70 Ssa1p in postribosomal supernatants was inhibited by NEM. We also found that completely removing nucleotides from purified Ssa1p rendered its ATP-agarose binding activity, ATPase activity, and post-translational translocation-stimulating activity sensitive to NEM. We modified nucleotide-free Ssa1p with [14C]NEM and then digested it with proteases. Purification and sequencing of the radiolabeled proteolytic fragments revealed that each of Ssa1p's three cysteine residues (Cys-15, Cys-264, and Cys-303) was modified with [14C]NEM. ADP protected each from modification. We also found that yeast postribosomal supernatants very efficiently released the conformation of Ssa1p or interfere with its ability to bind nucleotides. Together the results demonstrate that Ssa1p is an NEM-sensitive factor in cytosolic extracts from yeast that stimulates post-translational translocation of proteins into organelles.

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Addendum: We recently showed that Ssa1p and the DnaJ homolog Ydj1p form a protein folding machinery of the yeast cytosol (4). We also found that yeast postribosomal supernatants very efficiently stimulated luciferase refolding and that this activity was completely inhibited by NEM.2 While attempting to fractionate the postribosomal supernatant on ATP-agarose columns into two fractions with different NEM sensitivities, we found that Ssa1p binding to this resin was abolished by treating the postribosomal supernatant with NEM. This raised the possibility that Hsp70s are sensitive to NEM under certain conditions. We report here that removing nucleotides from purified Ssa1p renders its ATP-agarose binding, ATPase, and the post-translational translocation-stimulating activities susceptible to inactivation by NEM. ADP protects Ssa1p from inactivation. Furthermore, NEM reacts with all three cysteine residues of Ssa1p, and ADP protects each from modification.

2 E. J. Levy and W. J. Chirico, unpublished observations.
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Experimental Procedures

buffers—Buffers used were buffer KM (25 mM HEPES, pH 7.4, 50 mM KC1, 2 mM dithiothreitol (DTT), 3 mM MgCl2), Buffer K10% (25 mM HEPES, pH 7.4, 50 mM KC1, 10% (w/v) trichloroacetic acid, 2 × ATPase reaction buffer (60 mM HEPES, pH 7.4, 50 mM KC1, 22 mM MgCl2, 0.2 mM ATP, 1 μM of (α-32P)ATP (30 Ci/mmol, Du Pont NEN), and Buffer A (20 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT).

Postribosomal Supernatants and Nucleotide-free Ssa1p—Ssa1p was purified from postribosomal supernatants (7) of S. cerevisiae MW141 (27) using DEA-cellulose, ATP-agarose, and Mono Q chromatography as described previously (4). Nucleotides were removed from purified Ssa1p by centrifuging it through a 1 ml Sephadex G50 column (28) equilibrated in Buffer K10% containing 0.1 mM EDTA and then dialyzing it against the same buffer for 48 h. The nature of the bound nucleotide and its loss from Ssa1p during dialysis was monitored at 260 nm using reversed-phase high performance liquid chromatography (HPLC) as described previously (29, 30). Unless stated otherwise, protein concentrations were determined using the method of Bradford (31), and bovine γ-globulin was used as the standard (Bio-Rad).

Electrophoresis—Proteins were separated on 12% SDS-PAGE gels and stained with Coomassie Blue as described previously (25). The following molecular mass standards were purchased from Sigma: phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (35 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa).

Amino Acid Analysis—Ssa1p eluted from a Mono Q column was centrifuged sequentially through two Sephadex G50 columns (28) equilibrated with HPLC grade water. Six aliquots of Ssa1p were individually digested in 6 M HCl, 1% (v/v) phenol for 1 h at 105 °C using the gas-phase method previously described (32). Amino acids were derivatized, and phenylthiocarbamyl-derivatives were analyzed using a model 420A derivatizer/analyzer system from Applied Biosystems (Foster City, CA) in the Protein Sequencing Center of SUNY-Health Science Center at Brooklyn. Amino acid analysis indicated that the method of Bradford (31) overestimated the concentration of Ssa1p by 3.7 ± 0.2-fold.

NEM Inhibition of ATP-agarose Binding Activity—Four 210 μl aliquots of postribosomal supernatant (10 mg/ml of protein in Buffer KM) were treated separately with 1) 10.5 μl of water, 2) 10.5 μl of water, 3) 21 μl of 131 mM inactivated NEM, or 4) 10.5 μl of 262.5 mM NEM, and the resulting mixtures were incubated at 25 °C. (Inactivated NEM was prepared by incubating equal volumes of 262.5 mM NEM and 825 mM β-mercaptoethanol together for 10 min at 4 °C.) After 30 min, samples were filtered through 3 μM filters, and equal volumes of 100 mM DTT-β-mercaptoethanol, nothing, and 10.5 μl of 825 mM β-mercaptoethanol. These mixtures were incubated at 4 °C for 10 min. An aliquot (200 μl) of each sample was then applied to an ATP-agarose column (300 μl) equilibrated with Buffer KM at 4 °C. After 1 h, each column was washed with Buffer KM (3 × 500 μl). Bound proteins were eluted with Buffer KM containing 1 mM MgATP (3 × 500 μl). Proteins in equivalent amounts of each sample applied or eluted from the columns (% of total volume of each) were separated on 12% SDS-PAGE gels as described above.

Four aliquots (63 μl) of nucleotide-free Ssa1p (1.13 mg/ml of protein in Buffer KM) were treated separately with 1) 7 μl of water, 2) 7 μl of water, 3) 21 μl of 62.5 mM of inactivated NEM, or 4) 7 μl of 125 mM NEM, and the resulting mixtures were incubated at 25 °C. After 30 min, samples were filtered through 5 μM filters, and equal volumes of 100 mM iodoacetamide at room temperature were added to each sample. After 1 h, each column was washed with Buffer KM (3 × 8 M urea, 0.4 M NaCl, 100 mM KC1, 10% (v/v) trichloroacetic acid) and then placed the mixture at room temperature in a hood. After the penicillin evaporated, the amount of radioactivity was measured using scintillation counting, and the concentration of [14C]NEM was adjusted to 10 mM (based on the specific radioactivity provided by the manufacturer) with 0.6 × Buffer K10%.

Incorporation of [14C]NEM into Nucleotide-free Ssa1p—Nucleotide-free Ssa1p (33 μl) was preincubated with or without MgADP (666 μM) in Buffer K10% at 4 °C. After 30 min, 45 μl of each mixture was treated with 15 μl of either 10 μM [14C]NEM or 10 μM inactivated [14C]NEM. (Inactivated [14C]NEM was prepared by incubating final concentrations of 10 mM [14C]NEM and 20 mM DTT together at 4 °C for 10 min.) At various times, an aliquot (2.5 μl) was removed and incubated with an equal volume of 10 mM DTT in Buffer K10% at 4 °C for 10 min to inactivate residual NEM. ATPase activity was measured by adding 5 μ1 of [32P]ATP reaction buffer to each sample (5 μl) and incubating the resulting mixtures at 30 °C as described previously (4). Aliquots (1 μl) were removed from each reaction at 0 and 40 min and immediately spotted on thin-layer chromatography plates (polyethyleneimine-cellulose, E. Merck). The plates were developed using 1.1 M LiCl. Radiolabeled nucleotides were visualized, and the percentage of hydrolysis of ATP was quantified (4) using a PhosphorImager.

Preparation of [14C]NEM—A 10 μM stock solution of [14C]NEM was prepared immediately before use by mixing 50 μl of [14C]NEM in pentane (40 μC/mm, Du Pont NEN) with 6.25 μl of 0.6 × Buffer K10% and then placing the mixture at room temperature in a hood. After the pentane evaporated, the amount of radioactivity was measured using scintillation counting, and the concentration of [14C]NEM was adjusted to 10 mM (based on the specific radioactivity provided by the manufacturer) with 0.6 × Buffer K10%.

trypsin digestion—Nucleotide-free Ssa1p (36 μl) was preincubated with or without MgADP (500 μM) in 20 μl of Buffer K10% at 4 °C. After 30 min, 10 μl of 7.5 mM [14C]NEM was added, and the resulting mixtures were incubated at 30 °C. After 1 h, residual [14C]NEM was inactivated by incubating the mixtures with 10 μl of 20 mM DTT for 10 min. The volume of each [14C]NEM-labeled Ssa1p sample was immediately raised to 100 μl with 50 mM ammonium acetate, and then the resulting mixtures were centrifuged through 1 ml Sephadex G50 columns (28) equilibrated with 50 mM ammonium acetate. After lyophilizing the samples, each was dissolved in 50 μl of 8 M urea, 0.4 M sodium phosphate, pH 6.8. Each sample then received 0.2 μl of 1 M DTT, and the resulting mixtures were incubated at 50 °C (34). After 15 min, the mixtures were incubated with 5 μl of 100 mM iodoacetamide at room temperature for 15 min (34). The mixtures were diluted with 140 μl of water (50 μl of each sample) and then each was incubated with 9 μl of 0.2 mg/ml of sequencing grade trypsin (Boehringer Mannheim) at 37 °C for 16 h. Use of an acidic buffer system (35), such as the sodium phosphate-based system used above, prevented the complete loss of [14C]NEM from Ssa1p that was observed in preliminary experiments using an ammonium bicarbonate-based system (data not shown) recommended for trypsin digestions of other proteins (34).
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Purification of Radiolabeled Proteolytic Fragments—Proteolytic digests were injected directly onto a C18 reversed-phase column (220 × 4.6 mm, Brownlee RP Spheri-5). Fragments of Ssa1p were eluted using a linear gradient from 100% solvent A (0.06% trifluoroacetic acid) to 100% solvent B (0.06% trifluoroacetic acid, 80% acetonitrile) in 90 min at a flow rate of 0.5 ml/min. The 400 solvent delivery system, model 491 injector, and 783A programmable absorbance detector were from Applied Biosystems. The integrator was from Spectra-Physics. Radioactivity in aliquots (50 μl) of fractions (500 μl) from the reversed-phase column was detected using liquid scintillation counting.

The tryptic fragments of Ssa1p containing [14C]NEM separated as four peaks (I–IV) on the C18 reversed-phase column (see “Results”). Peak fractions were pooled separately and lyophilized, and then each was dissolved in 50 μl of 2 mM urea, 100 mM sodium phosphate, pH 6.8. Each sample was then injected separately onto a Superdex Peptide HR 10/30 gel filtration column (Pharmacia Biotech Inc.) equilibrated with the same buffer at a flow rate of 0.5 ml/min. Radioactivity in aliquots (25 μl) of fractions (250 μl) from the Superdex column was detected using liquid scintillation counting. Fractions that contained [14C]NEM-labeled polypeptides were pooled separately and then injected onto Sep-Pak C18 cartridges (Waters Corp.). The cartridges were extensively washed with 0.1% trifluoroacetic acid, 95% acetonitrile and then concentrated using a SpeedVac (Savant).

Peak IV from C18 reversed-phase chromatography of tryptic fragments of [14C]NEM-labeled Ssa1p contained two radiolabeled fragments (Peaks IVa and IVb). Only peak IVa was recovered from the Sep-Pak column. The fragment corresponding to peak IVb was isolated by first lyophilizing an aliquot (148 μl) of the pooled fractions containing peak IV from C18 reversed-phase chromatography of the tryptic fragments. The lyophilized fragments were dissolved in 25 μl of 8 M urea, 0.4 M sodium phosphate, pH 6.8. The sample was diluted with 175 μl of water and then incubated with 0.03 μg of sequencing grade chymotrypsin (Boehringer Mannheim) at 37 °C for 16 h. The chymotrypsin digestion was injected directly onto the C18-reversed phase column, and the fragments were eluted as described above for the tryptic fragments. Fractions containing [14C]NEM-labeled fragments were pooled separately and then concentrated using a SpeedVac.

Protein Sequencing—Purified proteolytic fragments (4–30 pmol each) were sequenced using an Applied Biosystems Procise model 494 sequencer in the Protein Sequencing Center of SUNY-Health Science Center at Brooklyn. [14C]NEM-labeled residues were identified either by measuring the radioactivity in the eluant from each sequencing cycle or by comparison with the retention time of an NEM-modified cysteine standard.

RESULTS

We recently showed that Ssa1p and Ydj1p form a yeast cytosolic protein folding machinery capable of refolding denatured luciferase to its native form (4). We also showed that postribosomal supernatants from yeast cytosol efficiently refold denatured luciferase and that the refolding activity is completely inhibited by NEM.2 Neither purified Ssa1p’s ATPase activity nor Ydj1p’s ability to stimulate it was inhibited by NEM.2 These results suggested that the refolding machinery of the yeast cytosol is composed of Ssa1p, Ydj1p, and an NEM-sensitive factor. We attempted to fractionate the NEM-treated postribosomal supernatant into two fractions using ATP-agarose chromatography (Fig. 1A). The postribosomal supernatants were prepared from S. cerevisiae MW141, a strain in which Ssa1p is overproduced using a plasmid and in which the genomic copies of SSA1, SSA2, and SSA4 are disrupted (27). We hoped that the bound fraction would contain Ssa1p, which was previously shown to bind to ATP-agarose, and that the flow-through fraction would contain an NEM-sensitive activity required for luciferase refolding. As expected, Ssa1p in postribosomal supernatants bound to ATP-agarose and was eluted with ATP (Fig. 1A, lanes 1–3). Surprisingly, Ssa1p in postribosomal supernatants treated with NEM did not bind to ATP-agarose (Fig. 1A, lanes 10–12). The position of Ssa1p was confirmed using Western blots probed with anti-yeast Hsp70 antiserum (data not shown). Control experiments using postribosomal supernatants that were incubated with either inactivated NEM (Fig. 1A, lanes 7–9) or with β-mercaptoethanol alone (Fig. 1A, lanes 4–6) indicated that the above effects were NEM-specific. These results suggested that NEM either directly inhibits the ATP-agarose binding activity of Ssa1p or inhibits an NEM-sensitive factor in postribosomal supernatants that is required for Ssa1p to bind to ATP-agarose.

To determine whether the loss of ATP-agarose binding activity of Ssa1p in postribosomal supernatants is a direct effect of NEM modifying Ssa1p and not another factor, we repeated the above experiment with purified Ssa1p (Fig. 1B). Because purified Ssa1p contained tightly bound nucleotides that interfered with its ATP-agarose binding activity, we removed the nucleotides by exhaustively dialyzing Ssa1p against EDTA-containing buffers before treating it with NEM. Purified, nucleotide-free Ssa1p efficiently bound to ATP-agarose (Fig. 1B, lanes 1–3). In contrast, nucleotide-free Ssa1p treated with NEM did not bind to ATP-agarose (Fig. 1B, lanes 10–12). Incubating nucleotide-free Ssa1p with either inactivated NEM (Fig. 1B, lanes 7–9) or with β-mercaptoethanol alone (Fig. 1B, lanes 4–6) did not inhibit the ATP-agarose binding activity of Ssa1p. These results indicated that NEM directly inhibits the ATP-agarose binding activity of Ssa1p.
The results described above indicate that NEM treatment of Ssa1p prevents it from binding to ATP-agarose and from stimulating post-translational translocation. These results also suggest that NEM modification of Ssa1p interferes with its ability to bind and catalyze the hydrolysis of ATP. To determine whether Ssa1p’s ATPase activity is NEM-sensitive, we measured the time course of inhibition of Ssa1p’s ATPase activity by NEM (Fig. 3A). After 60 min of NEM treatment, Ssa1p’s ATPase activity was abolished (Fig. 3A, line –ADP). In the presence of ADP, NEM did not inhibit Ssa1p’s ATPase activity (Fig. 3A, line +ADP). ATP also protected Ssa1p’s ATPase activity from inhibition by NEM, but less effectively than ADP (data not shown). The results indicated that NEM inhibits the ATPase activity of Ssa1p and further supports the idea that NEM treatment of Ssa1p interferes with its ability to bind nucleotides.

To determine whether NEM disrupts Ssa1p function by modifying one or more sites, we determined how many mol of [14C]NEM are incorporated into Ssa1p (Fig. 3B). In the absence of ADP, 3.7 mol of [14C]NEM were incorporated into Ssa1p in 2 h (Fig. 3B, line –ADP). However, in the presence of ADP, only 0.7 mol were incorporated (Fig. 3B, line +ADP). Together, these results suggested that ADP protects three sites on Ssa1p from NEM modification. Modification of at least one site results in loss of activity.
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FIG. 4. Separation of tryptic fragments of [14C]NEM-labeled Ssa1p. Nucleotide-free Ssa1p (33 μM) was treated with 2.5 mM of [14C]NEM in the presence or absence of 500 μM MgADP. The samples were then digested with trypsin and separated on a C18 reversed-phase column as described under “Experimental Procedures.” The column was washed for 4 min with 0.06% trifluoroacetic acid and then for 90 min with a linear gradient to 80% acetonitrile, 0.06% trifluoroacetic acid. Fractions were collected at 215 nm (without ADP) or scintillation counting (with ADP). The radioactivity represents the total amount in each fraction. The numbers (I–IV) indicate the positions of the four major peaks of radioactivity generated from the tryptic digest of Ssa1p that was labeled with [14C]NEM in the absence of nucleotides.

Table I
Sequences of proteolytic fragments of [14C]NEM-labeled Ssa1p

| Peak  | Sequence | Location in Ssa1p | [14C]NEM-residue |
|-------|----------|------------------|-----------------|
| I     | TACERAK  | 262–268          | Cys-264         |
| II    | LRTACERAK| 260–268          | Cys-284         |
| III   | FEECLDLFR| 299–308          | Cys-303         |
| IVa   | ARFECLDLFR| 297–308        | Cys-303         |
| IVb   | SCVAHF   | 14–19            | Cys-15          |

To identify the NEM-reactive sites in nucleotide-free Ssa1p, we radiolabeled it with [14C]NEM in the presence or absence of ADP, proteolytically digested the samples, and then purified and sequenced the radiolabeled fragments. Nucleotide-free Ssa1p that was treated with [14C]NEM was digested with trypsin into an array of fragments that were separated on a reversed-phase column (Fig. 4A). Ssa1p that was protected by ADP during the NEM treatment was digested into a similar array of fragments (data not shown). In contrast to the patterns of fragments detected at 215 nm, the patterns of radiolabeled fragments obtained from samples treated with [14C]NEM in the presence or absence of ADP were not identical. In the absence of ADP, four major radiolabeled peaks (I–IV) were detected (Fig. 4B). A time course of trypsin digestion revealed that the amount of radioactivity in peaks I and III increased as that in peaks II and IV decreased (data not shown). This suggested that the radiolabeled fragments in peaks I and III can be generated from those in peaks II and IV. The distribution of radiolabeled peaks did not change after 12 h (data not shown). The amount of radioactivity recovered from reversed-phase chromatography of four separate experiments was 104 ± 11%. Of the 3.6 ± 0.2 mol of NEM incorporated per mol of Ssa1p, the radioactivity in peaks I and II together contributed 1.1 ± 0.1 mol, and that in peaks III and IV together contributed 2.1 ± 0.3 mol. The remaining radioactivity was distributed among at least 12 minor peaks. In the presence of ADP, the amount of radioactivity eluting at positions corresponding to peaks I–IV was negligible (Fig. 4B). These results suggested that 3 mol of NEM are distributed among at least four tryptic fragments of Ssa1p and that ADP protects each of these sites from NEM modification.

Fractions corresponding to peaks I–IV were pooled separately, and the enriched fragments were further purified individually on a gel filtration column. The radioactivity in peaks I–III migrated as single species on the gel filtration column, indicating that each contained only one radiolabeled fragment (data not shown). In contrast, peak IV separated into two peaks (IVa and IVb) on the gel filtration column (data not shown), indicating that two fragments coeluted from reversed-phase chromatography of the tryptic fragments. Together these results indicated that the 3 mol of NEM incorporated into Ssa1p were distributed among five tryptic fragments. After purifying radiolabeled peptides in peaks I, II, III, and IVa on the gel filtration column, they were sequenced (Table I). Peak IVb contained the largest of the five radiolabeled tryptic fragments. It had a propensity to adhere to microcentrifuge tubes and pipette tips, especially those that were siliconized, and could not be isolated. To generate a smaller and more soluble version of the radiolabeled fragment in peak IVb, the polypeptides in peak IV (Fig. 4B) were digested with chymotrypsin, and the resulting fragments were separated on the reversed-phase column (data not shown). Two peaks of radioactivity eluted from the column, and the radiolabeled fragments were sequenced (Table I, IVa and IVb). The sequence of the five radiolabeled fragments (Table I) and the change in their distribution during the time course of trypsin digestion indicated that peaks I and III were generated from peaks II and IVa, respectively. The sequence of the fragments revealed that each of the three cysteine residues of Ssa1p (Cys-15, Cys-264, and Cys-303) was modified by NEM. Furthermore, each of the cysteine residues was protected by ADP.

DISCUSSION

We showed above that the ATP-agarose binding, ATPase, and post-translational translocation activities of nucleotide-free Ssa1p are inactivated by NEM. We also showed that 3 mol of NEM are specifically incorporated into nucleotide-free Ssa1p. ADP protects Ssa1p from NEM modification and inactivation. Amino acid sequence analysis of radiolabeled proteo-
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TABLE II
Comparison of Ssa1p's NRSs to sequences of other Hsp70s and actin

Cys-15, Cys-264, and Cys-303 of Ssa1p are the NEM-reactive centers of NRS1, NRS2, and NRS3, respectively. Their positions are indicated by the asterisks. The sequences of the Ssa protein family were obtained from GenBank™, and the other sequences were obtained from SWISS-PROT. The accession numbers follow the names of the species.

| Source of Hsp70 or actin | NRS1 | NRS2 | NRS3 |
|-------------------------|------|------|------|
| Cytosol                 |      |      |      |
| Ssa1p (yeast, X12926)   | 4AVG| 265L| 297A|
| Ssa2p (yeast, X12927)   | DGL| LTACE| FEEL|
| Ssa3p (yeast, M97225)   | GID| 268 | CMDL|
| Ssa4p (yeast, J05637)   | LTYS| 266 | LPFR|
| Ssb1p (yeast, P11484)   | 8AIG| 269 | LGIF|
| Ssb2p (yeast, P40150)   | DGL| 289 | LRTA|
| Hsc70 (cow, P19120)     | GID| 286 | LRTA|
| Hsc70 (human, P11142)   | LTYS| 287 | LRTA|
| Hsc70 (petunia, P09149) | DGL| 288 | LRTA|
| Hsp70 (cow, P34933)     | 8AIG| 289 | LRTA|
| Hsp70 (human, P08107)   | DGL| 290 | LRTA|
| Actin (yeast, P02579)   | 7AVL| 291 | LRTA|
| Endoplasmic reticulum   |      |      |      |
| Kar2p (yeast, P16474)   | 7AVG| 292 | LRTA|
| BiP (human, P11021)     | DGL| 293 | LRTA|
| BiP4 (tobacco, Q03684)  | LTYS| 294 | LRTA|
| Mitochondria            |      |      |      |
| Sac1 (yeast, P12398)    | 3AVG| 295 | LRTA|
| GRP75 (human, P58646)   | DGL| 296 | LRTA|
| Hsp1 (pea, P37900)      | LTYS| 297 | LRTA|
| Chloroplast             |      |      |      |
| Hsp70 (pea, Q02028)     | 7AVG| 298 | LRTA|
| DnaK (red algae, P03723) | DGL| 299 | LRTA|
| Bacteria                |      |      |      |
| DnaK (E. coli, P04475)  | 3IG| 290 | LRTA|
| DnaK (M. mazei, P27094) | LGI| 291 | LRTA|

Lytic fragments revealed that all three cysteine residues (Cys-15, Cys-264, and Cys-303) of Ssa1p are modified. These results demonstrate for the first time that an Hsp70 molecular chaperone can be inhibited by NEM. Furthermore, they suggest that Ssa1p is an NEM-sensitive factor in yeast cytosolic extracts that stimulates post-translational translocation into the endoplasmic reticulum.

The inhibition of an Hsp70 by NEM is surprising because several groups have reported that Hsp70s are not sensitive to NEM (42). Our finding that Ssa1p is an NEM-sensitive factor in yeast cytosolic extracts is consistent with the results of several groups. In yeast cytosolic extracts, Ssa1p may become less sensitive to NEM as they are purified, because they become separated from putative polypeptide substrates or nucleotide exchange factors that promote greater accessibility of NEM to reactive cysteine residues. Our finding that Ssa1p is inhibited by NEM suggests that it is an NEM-sensitive factor in crude cytosolic extracts that stimulates protein folding (3), post-translational translocation of proteins into the endoplasmic reticulum (7), mitochondria (8), and nucleus (10). We cannot rule out the possibility that NEM-sensitive factors, distinct from Hsp70s, also contribute to these activities. For example, the activity of the mitochondrial import stimulation factor, which is composed of two different polypeptides, is sensitive to NEM (42).

To aid the comparison of reactive cysteine residues among Hsp70s, we have designated the proteolytic fragments of Ssa1p in which they are found as NRSs. Cys-15, Cys-264, and Cys-303 are the reactive centers of NRS1, NRS2, and NRS3, respectively. To further aid comparisons, we defined NRS1 as the tryptic fragment that was likely digested by chymotrypsin into the smaller fragment that we purified and sequenced. A comparison of the NRSs of Ssa1p with similar sequences found among a selection of Hsp70s and yeast actin is shown in Table II. Of the three sites, NRS1 is the most conserved. Among the selection of Hsp70s, 13 of its 20 amino acid residues either are identical or represent conserved substitutions. Based on the
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The three-dimensional structure of the ATPase core of Hsc70 (43), some of the amino acid residues of NRS1 are predicted to interact with ATP and its ligands. For example, Asp-10 interacts with one of the two potassium ions and two water molecules in the ATP binding pocket of Hsc70 (44). Interestingly, the cysteine residue of NRS1 (Cys-15 in Ssa1p) is the most conserved of the three residues. However, it is not invariant. Rare exceptions include the mitochondrial Hsp70 from yeast and the Hsp70s from chloroplasts. NRS2 contains nine amino acid residues that are also highly conserved. Among the Hsp70s chosen for comparison, five of the nine residues are invariant or represent conserved substitutions. In contrast to NRS1, the cysteine residue of NRS2 is less conserved. It is found in most Hsp70s of the cytosol but is lacking in those found in other subcellular compartments. Among family members of the S. cerevisiae cytosol, Ssa3p, Ssa4p, Ssb1, and Ssb2 lack a cysteine residue in NRS2. NRS3 is least conserved of the three sites. Only 6 of its 12 amino acid residues are invariant or conserved among the Hsp70s selected. However, NRS3 is identical in the Ssa protein family. The cysteine residue of NRS3 is also found in some of the other Hsp70s in the cytosol and chloroplasts but not in the endoplasmic reticulum. DnaK and mitochondrial Hsp70s show less identity in this region to other Hsp70s. Interestingly, cysteine is often replaced by alanine or valine in NRS1 and NRS2 regardless of the subcellular location of the Hsp70, while it is replaced by asparagine in NRS3 of Hsp70s of the cytosol and endoplasmic reticulum but by valine or threonine in DnaK and Hsp70s of mitochondria. The lack of absolute conservation of cysteine residues in the three NRSs suggests they are not essential for the function of Hsp70s. If most Hsp70s are sensitive to NEM, then the highly conserved nature of the cysteine residue of NRS1 suggests that its modification will play an important role in the inhibition. Changing Ssa1p's Cys-15 to an arginine, serine, or glycine residue does not affect its ability to bind to ATP-agarose (45). DnaK has only one cysteine residue, which is located in NRS1, and its nucleotide-free form is NEM-sensitive. This supports the idea that NRS1 plays an important role in the NEM sensitivity of Hsp70s. Hsp70s have different numbers of cysteine residues in their nucleotide and polypeptide binding domains. The importance of these cysteine residues to the structure and function of Hsp70s remains to be determined.

Although actin and the ATPase core of Hsp70s share little sequence identity, their three-dimensional structures are very similar (43, 46, 47). We searched actin sequences for NRSs and found sequence homology between the N terminus of actin and NRS1 as expected for a site that contributes to ATP binding (Table II). For example, 10 of the 20 residues of NRS1 of yeast actin are identical or conserved to those of Ssa1p. Interestingly, examination of a recent compilation of 122 actin sequences reveals that 69 actins contain a cysteine residue at position 17 (48). At position 18 is an invariant lysine residue. In the ADP form of actin, Lys-18 interacts with oxygens of the α- and β-phosphates (46). In Hsc70, K+ at site one interacts with the α- and β-phosphates, and Cys-17 occupies the equivalent position of Lys-18 in actin (44). Whether NEM can modify Cys-17 found in many actins and whether this modification would impair their function has not been reported. Like Hsp70s, actins bind nucleotides very tightly (Kd = 0.07 nM; Ref. 48). Based on our studies of Ssa1p, the NEM sensitivity of Cys-17 in a nucleotide-free form of some actins seems possible.

The positions of the amino acid residues in the three-dimensional structure of the ADP + P₄ form of bovine Hsc70 (43, 44) that correspond to Cys-15, Cys-264, and Cys-303 of Ssa1p are shown in Fig. 5. Because the conformations of nucleotide-free, ADP, and ATP forms of Ssa1p are different from each other, the positions of the cysteine residues in each form may be different. Cys-15 (Cys-17 in Hsc70) is located in subdomain IA in a β-strand at the base of the ATP binding pocket near the potassium ion located at site 1. Cys-264 (Cys-267 in Hsc70) is located in subdomain IIB in an α-helix that lines one side of the ATP binding cleft. Cys-303 (Asn-306) is also located in subdomain IIB, but in an α-helix on the outer surface of the protein. The positions of these residues suggest mechanisms for NEM inhibition of Ssa1p. NEM bound to Cys-15 (Cys-17 in Hsc70) may sterically interfere with the binding of the potassium ion at site 1, disrupt the interactions among the nucleotide, metal ions, and neighboring amino acid residues, or adversely alter Ssa1p's conformation. Potassium ions are essential for Hsp70 activity (17, 49) and ATP-dependent conformational changes (30). NEM modification of Cys-264 (Cys-267 in Hsc70) may inhibit Ssa1p activity either by disrupting the conformation of Ssa1p or by sterically blocking access of ATP to the bottom of the cleft. The distance of Cys-303 (Asn-306 in Hsc70) from the ATP binding pocket suggests that NEM modification of this site may disrupt the conformation of Ssa1p rather than directly interfering with nucleotide binding. Recently, we have shown that NEM dramatically alters the conformation of Ssa1p. This supports the idea that NEM-dependent conformational changes of Ssa1p may contribute to its inactivation. Although

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3 Q. Liu and W. J. Chirico, unpublished observations.

4 W. J. Chirico, unpublished observations.
all three cysteine residues of Ssa1p react with NEM, the contribution of each modified residue to the total inhibition and the mechanism of inhibition remains to be determined.

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