S100A1 Is a Novel Molecular Chaperone and a Member of the Hsp70/Hsp90 Multichaperone Complex*

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Although calmodulin is known to be a component of the Hsp70/Hsp90 multichaperone complex, the functional role of the protein remains uncertain. In this study, we have identified S100A1, but not calmodulin or other S100 proteins, as a potent molecular chaperone and a new member of the multichaperone complex. Glutathione S-transferase pull-down assays and co-immunoprecipitation experiments indicated the formation of stable complexes between S100A1 and Hsp90, Hsp70, FKBP52, and CyP40 both in vitro and in mammalian cells. S100A1 potently protected citrate synthase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, and rhodanese from heat-induced aggregation and suppressed the aggregation of chemically denatured rhodanese and citrate synthase during the refolding pathway. In addition, S100A1 suppressed the heat-induced inactivation of citrate synthase activity, similar to that for Hsp90 and p23. The chaperone activity of S100A1 was antagonized by calmodulin antagonists, such as fluphenazine and prenylamine, that is, indeed an intrinsic function of the protein. The overexpression of S100A1 in COS-7 cells protected transiently expressed firefly luciferase and Escherichia coli beta-galactosidase from inactivation during heat shock. The results demonstrate a novel physiological function for S100A1 and bring us closer to a comprehensive understanding of the molecular mechanisms of the Hsp70/Hsp90 multichaperone complex.

Heat shock proteins (Hsp)1 90 and 70 are abundant molecular chaperone proteins under physiological conditions (1). Hsp90 alone can act to prevent protein aggregation and promote refolding in vitro, but in vivo it operates as part of the multichaperone machinery in the cytosol, which includes Hsp70, peptidyl-prolyl isomerases, and other cochaperone proteins (2). Several Hsp90-associated cochaperones contain multiple copies of the tetra-tricopeptide repeat motif that mediates protein-protein interactions in diverse cellular pathways (3). The tetra-tricopeptide repeat domains of peptidyl-prolyl isomerases such as FKBP52, CyP40, and phosphoprotein phosphatase 5 mediate the binding of these proteins to Hsp90 (4–6). Unfolded polypeptide substrates associate first with the Hsp70/Hsp40 system and are then bound by Hsp90 via Hop that interacts with both Hsp70 and Hsp90, comprising an intermediate chaperone complex (7, 8). Further remodeling results in the mature chaperone complex consisted of Hsp90, p23, and FKBP52 or CyP40 (5). Similar to Hsp90 and Hsp70, cochaperone proteins such as FKBP52, CyP40, and p23, but not Hop, have also been found to display chaperone activity (9, 10). These proteins prevent the thermal aggregation of citrate synthase (CS) or refold a denatured beta-galactosidase in vitro (9, 10).

Ca2+ signal plays a pivotal role in regulating various cellular responses including cell metabolism, cytoskeletal dynamics, cell cycle, gene expression, neurotransmission, and intracellular signal transduction processes (11, 12). The signal-induced change in the intracellular free Ca2+ concentration has been portrayed as a switch through a class of Ca2+-binding proteins (13). Over the years, many Ca2+-binding proteins have been identified, reflecting the biological importance of Ca2+ and its regulative function in the cell. Among such proteins, it is well known that calmodulin (CaM) is involved in many aspects of the Ca2+ regulating system in various cell types (14). Although several reports indicate that CaM binds to Hsp90 (15), Hsp70 (16), FKBP52 (17), and CyP40 (18) in a Ca2+-dependent or -independent manner, the regulating role of CaM remains unclear. The overlapping specificity of CaM and S100 proteins for the interaction with target proteins has been reported (19). It has also been known that CaM and S100 proteins can have differential effects on a common target protein (20, 21). These reports prompted us to investigate the functional role of S100 proteins in the multichaperone complex. To establish whether CaM or S100 proteins exhibited properties of molecular chaperones, we examined their interaction with the components of the chaperone complex and activities in the refolding of substrate proteins in vivo and in vitro. In the present report, we have identified S100A1 to be a potent molecular chaperone and a new member of the multichaperone complex.

EXPERIMENTAL PROCEDURES

Materials—Fluphenazine (4-[3-[3-(trifluoromethyl)-10H-phenothiazin-10-ylpropyl]-1-piperazin ethanol) and prenylamine (N-(1-methyl-2-phenethyl)-gamma-phenylbenzenepropanamine) were purchased from Wako. Q-Sepharose, phenyl-Sepharose, glutathione-Sepharose, benzamidine-Sepharose, Protein-G-Sepharose, and CaM-Sepharose were purchased from Amersham Biosciences. CS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Roche Diagnostics.

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‡ Materials—Fluphenazine (4-[3-[3-(trifluoromethyl)-10H-phenothiazin-10-ylpropyl]-1-piperazineethanol) and prenylamine (N-(1-methyl-2-phenethyl)-gamma-phenylbenzenepropanamine) were purchased from Wako. Q-Sepharose, phenyl-Sepharose, glutathione-Sepharose, benzamidine-Sepharose, Protein-G-Sepharose, and CaM-Sepharose were purchased from Amersham Biosciences. CS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Roche Diagnostics.

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anti-rhodanese and aldolase from Sigma. Anti-Hsp90 monoclonal antibody (AC88) and anti-Hsp40 polyclonal antibody were purchased from StressGen Biotech. Anti-Hsp70 polyclonal antibody, anti-FKBPs2 polyclonal antibody, and anti-CyP40 (C-terminal) polyclonal antibody were purchased from Medical and Biological Laboratories (Nagoya, Japan), Santa Cruz Biotechnology, and BIOROLL Research Labs, respectively. Anti-S100A1 polyclonal antibodies were purchased from QED Bioscience and Dako Cytomation. Anti-luciferase antibody and anti-β-galactosidase antibody were purchased from Promega and Roche Diagnostics, respectively. Anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies were purchased form Amersham Biosciences. Anti-ti-sheep and anti-goat peroxidase-conjugated secondary antibodies were purchased from Southern Biotechnology and Chemicon International, respectively. The γ-galactosidase-expressing plasmid pGL3-Promoter vector from Promega, Bovine brain Hsp90, Hsp70, and CaM were purified as described previously (22–24). All other reagents were at least analytical grade.

Expression and Purification of S100 Proteins, Glutathione S-transferase (GST)-truncated Hsp90, GST-FKBPs2, Wild-type FKBPs2, GST-CyP40, and Wild-type CyP40—S100A1 was expressed and purified as described previously (24). The bacterial expression plasmids for the production of other S100 proteins were prepared by PCR using bovine brain and the human lung cDNA library (Clontech) as templates and specific primers. PCR products were subcloned into pCT11a vector (Novagen). S100 proteins were expressed in BL21 (DE3) and purified by Q-Sepharose followed by phenyl-Sepharose column chromatography (25). Truncated Hsp90 (237–628) and truncated Hsp70 (1–382) were prepared by PCR using human Hsp90a and Hsp70 cDNAs (kindly provided by Dr. Kazunori Yokoyama, Department of Biological Systems, Gene Engineering Division, RIKEN Tsukuba Institute Biosource Center) as templates and specific primers. Human FKBPs2 and bovine CyP40 were prepared by PCR using human testis and bovine brain cDNA library (Clontech) as templates and specific primers. PCR products were subcloned into pGEX4T-1 vector (Amer sham Biosciences). GST fusion proteins were expressed in BL21 and purified according to the manufacturer’s instructions. In some cases, GSTs were removed from fusion proteins by thrombin digestion. The GST-FKBPs2 or GST-CyP40 containing fractions were dialyzed against 20 mM Tris-HCl, pH 8.0. Then the GST fusion proteins were incubated with 50 units of thrombin/mg of protein at 37°C for 3 h. To separate the FKBPs2 and CyP40 proteins from free GST, the protein samples were loaded again onto a glutathione-Sepharose column (2 ml bed volume). The flow-through fraction containing recombinant FKBPs2 or CyP40 was collected. Finally, to remove residual thrombin, the flow-through fractions were applied to a benzamidine-Sepharose column (2 ml bed volume) and eluted with the same buffer. As judged from SDS-PAGE, this method yielded the FKBPs2 and CyP40 proteins with greater than 95% purity.

In Vivo Protein Binding Studies—Ca²⁺-binding protein (S100 proteins or CaM: 25 μg each) and GST fusion protein (25 μg each) were mixed in a binding buffer (20 mM Tris-HCl, pH 7.5, and 0.1 mM KCl). The reaction mixtures in a total volume of 150 μl were incubated with 50 μl of glutathione-Sepharose beads for 60 min at 25°C. After pelleting by microcentrifugation, the gels were subjected to replicate washes (4 × 1 ml), boiled in SDS sample buffer, and examined for protein retention by SDS-PAGE.

Estimation of equilibrium dissociation constant (Kd) was performed by incubating a fixed concentration of GST-truncated Hsp90 (7.18 μM), GST-truncated Hsp70 (1.99 μM), GST-FKBPs2 (1.08 μM), GST-CyP40 (1.26 μM) beads or, alternatively, GST beads alone (3.2 μM) with increasing concentrations of purified recombinant S100A1 (0–100 nM) in 150 μl of a buffer containing 20 mM Tris-HCl, pH 7.6, 0.1 mM KCl, and 0.1% Triton X-100 with 1 mM CaCl₂ or 1 mM EGTA. The beads were washed 4 times with the buffer and then eluted and used for gel electrophoresis. Coomassie-stained gels were scanned by an image analyzer (LAS-100plus; Fuji Film), and arbitrary densitometric values for S100 proteins were subtracted from the initial amount added to the samples. Kd was calculated by Scatchard plot analysis of bound and free fractions.

Immunoprecipitation and Western Blotting—Antibody resin was prepared by incubating anti-Hsp90, anti-Hsp70, anti-FKBPs2, or anti-CyP40 antibody (1 μg each) with a slurry of Protein G-Sepharose (20 μl) in phosphate-buffered saline for 1 h at 25°C. The antibody-conjugated resins were washed three times in an incubation buffer (20 mM Tris-HCl, pH 7.6, and 0.1 mM KCl with 1 mM CaCl₂ or 1 mM EGTA). The resins (20 μl) were suspended in 60 μl of the incubation buffer and incubated for 1 h at 25°C with purified Hsp90, purified Hsp70, recombinant FKBPs2, and recombinant CyP40 proteins (2 μg each). After washing with the incubation buffer, the resin (20 μl) was individually incubated in 60 μl of the incubation buffer containing 20 μg of recombinant S100A1 for 3 h at 25°C and washed three times with the incubation buffer. The immunoprecipitates were analyzed by Western blotting with antibodies specific for S100A1, Hsp90, Hsp70, FKBPs2, or CyP40. Binding of anti-sheep and anti-rabbit, anti-rabbit, or anti-goat peroxidase-conjugated secondary antibodies for Western blotting was made visible by enhanced chemiluminescence (ECL, PerkinElmer Life Sciences) and immunoprecipitates were visualized using Western blotting with antibodies specific for S100A1, Hsp90, Hsp70, FKBPs2, CyP40, or CaM (a gift from Dr. Yassuo Watanabe, Kagawa University Faculty of Medicine). Where noted, the brain extracts (100 μl) were immunoprecipitated with 1 μg of the anti-S100A1 antibody and Protein G-Sepharose as described above. The immunoprecipitates were analyzed by Western blotting with the respective antibodies as indicated.

Surface Plasmon Resonance (SPR)—BIAcore 2000, CM5 chip, N-ethyl-N-((3-dimethylaminopropyl)carbodiimide, N-hydroxysuccinimide, and ethanolamine–HCl (Biacore, Inc.) were used for amine coupling of S100A1 to the dextran surface of the CM5 chip. Recombinant S100A1 (10 μg) was immobilized on the CM5 in 10 mM NaCl, and 0.05% Tween 20 with 1 mM CaCl₂ or 1 mM EGTA were washed at a flow rate of 20 μl/min. Purified Hsp90, purified Hsp70, recombinant FKBPs2, and recombinant CyP40 were injected at various concentrations (50, 100, 200, 400, and 800 nM). S100A1-coupled sensor chip was regenerated between protein injections with a brief (60-s) wash with 2 mM NaCl followed with 0.5% SDS, 5% Nonidet P-40 until the response unit base line returned to its pre-injection level. Response curves were prepared for fitting by subtraction of the signal generated simultaneously on the control flow cell. Biacore sensorgram curves were evaluated by BIAevaluation 3.0 (Biacore) with a numerical integration algorithm.

Thermal-induced Aggregation of CS, Aldolase, GAPDH, and Rhodanese—The effects of S100 proteins and CaM on the thermal aggregation of mitochondrial CS at 43°C were monitored as described previously (26). To monitor thermal aggregation, the CS concentration was used 0.19 μM in 40 mM Heps, pH 7.4, in the presence or absence of S100 proteins and CaM. Light scattering of CS was monitored over 20 min at an optical density of 500 nm in an Amershamb Sciences Ultraspex 3000 UV-visible spectrophotometer equipped with a temperature control unit using semi-microcuvettes (1 ml) with a path length of 10 mm.

The thermal-induced aggregation of aldolase, GAPDH, and rhodanese was performed according to the established protocols (27). Protein solutions (3 μl each) with or without S100A1 (5 μM) were mixed at room temperature and then heated at 65°C for 5 min in a thermal cycler. The light scattering of the solution at 488 or 360 nm was measured in a spectrophotometer (model UV-1600; Shimadzu).

Thermal Inactivation and Reactivation of CS—CS (1.9 μM) was incubated at 43°C in the presence of S100A1 or bovine serum albumin. Through incubation, aliquots were withdrawn at intervals of 2–10 min, and the enzyme activity was determined as described (26). To initiate reactivation of CS, 100 μM oxaloacetic acid (OAA) (in 50 mM Tris-HCl, pH 8.0, and 2 mM EDTA) was diluted 1:100 into the incubation mixture, to a final concentration of 1 mM. After the reactivation reaction was incubated at 43°C (Fig. 7B) or the temperature was lowered to 25°C (Fig. 7C).

Rhodanese and CS Aggregation Assays—The ability of CaM and S100 proteins to inhibit rhodanese and CS aggregation was assayed essentially as described previously (28, 29). CaM and S100 proteins...
were equilibrated for 5 min at 25 °C in 95 µl of 50 mM Hepes, pH 7.0, and 100 mM NaCl. After adding 5 µl of 37.5 µM rhodanese or 35.8 µM CS in 6 µl guanidine-HCl and 50 mM Hepes, pH 7.0, the rate of the aggregation of these proteins was determined by measuring the increase in absorbance at 320 nm. Aggregation was essentially completed after 15 min. Effects of fluphenazine and prenylamine on the chaperone activity of S100A1 were measured by the CS aggregation assay as described above.

Calcium/Calmodulin-dependent Protein Kinase II (CaM KII) Assay—Rat CaM KII holoenzyme was purified from rat forebrain as described previously (30). CaM KII was incubated with 50 mM Hepes, pH 7.5, 10 mM Mg(Ac)₂, 1 mM dithiothreitol, 40 µM syntide-2, 2 mM CaCl₂, and 1 µM CaM in the presence or absence of fluphenazine and prenylamine. The reaction was initiated by the addition of 100 µM [γ-32P]ATP (6500 cpm/pmol) (Amersham Biosciences) and terminated by spotting aliquots (15 µl) onto phosphocellulose paper (Whatman, P-81) followed by washing with 75 mM phosphoric acid. Phosphate incorporation into syntide-2 was quantitated by liquid scintillation counting of the filters. CaM KII activity was measured for 5 min at 30 °C under the linear condition.

Cloning of pME18S-S100A1, Cell Culture, Transfection, Heat Shock, and Cell Lysis—The S100A1-expressing plasmid pME18S-S100A1 was prepared by PCR using pET11a-S100A1 as a template (24). The PCR product was digested with EcoRI and SpeI and subcloned into the EcoRI site of pME18S (Promega) with 2.5 kbp M silent change Syntide-2 (Promega) with 2.5 µg of the luciferase-expressing plasmid pG53-Promoter (Promega) with 2.5 µg of either empty vector or pME18S-S100A1. Transient transfections were performed by using LipofectAMINE 2000 according to the instructions of the manufacturer (Invitrogen). In co-transfection experiments, the total amount of plasmid DNA was kept constant by the addition of pME18S. After 2 days of transfection, the dishes were washed with paraflin and heat shocked at the appropriate temperatures in an incubator. Immediately after heat treatment, the cells were washed with ice-cold phosphate-buffered saline and lysed in 200 µl of 25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane N,N,N,N-tetraacetic acid, 10% glycerol, and 1% Triton X-100.

β-Galactosidase and Luciferase Assays—β-Galactosidase assays were performed in triplicate samples using the β-Galactosidase Reporter Gene Assay, chemiluminescent kit (Roche Diagnostics). β-Galactosidase activity was measured for 20 s with Luminoskan (Labsystems) with a 100:2.5-µl ratio mixture of the β-galactosidase assay reagents and cell lysate. Luciferase assays were performed in triplicate samples using the luciferase assay kit (Picogene L77.5 Luminescence kit; Toyo Inki Co., Japan). The intensity of light emission from the assay was determined for 20 s with Luminoskan with a 100:50-µl ratio mixture of the luciferase assay reagents and cell lysate.

RESULTS

S100A1 but Not CaM Interacts with Hsp90, Hsp70, FKBP52, and CyP40 in Vitro—Previous studies have provided evidence that CaM-binding motifs are localized in: the middle domain of Hsp90 (25), N-terminal half of Hsp70 (26), C-terminal domains of FKBP52 (27), and C-terminal domains of FKBP52 (28), which are involved in the chaperone and cochaperone proteins with GST. Selective adsorption of the GST fusion proteins onto glutathione-Sepharose allowed the resin to be used in affinity chromatography to screen for proteins, which would target the chaperone and cochaperone functions. We performed in vitro binding assays using GST-truncated Hsp90, GST-truncated Hsp70, GST-FKBP52, and GST-CyP40 fusion proteins to confirm the interactions between CaM and the chaperone and cochaperone proteins described in previous reports (15–18) and exclude the possibility that these interactions were indirect or nonspecific. We also analyzed binding of S100 proteins to the chaperone and cochaperone proteins, because the binding properties of CaM and S100 proteins often overlap (19). Surprisingly, S100A1, but not CaM or other S100 proteins, exclusively bound to GST-truncated Hsp90, GST-truncated Hsp70, GST-FKBP52, and GST-CyP40 in the absence of Ca²⁺ (Fig. 1, B–E). Some other S100 proteins, such as S100B, S100A2, S100A4, and S100A6 were also bound weakly to GST-truncated Hsp90 (Fig. 1B). Previous biochemical studies have identified many potential S100 target enzymes such as glycogen phosphorylase (21), twitchin kinase (20), aldolase (22), Ndr kinase (21), phosphoglucomutase (23), and GAPDH (24), some exhibiting Ca²⁺-dependent whereas others Ca²⁺-independent modulation.

Next, we examined the effects of Ca²⁺ on the binding of S100A1 to the chaperone and cochaperone proteins. Neither Ca²⁺ nor EGTA influenced the affinity of S100A1 for Hsp70, FKBP52, and CyP40 (Fig. 2A, 2C–2E). Although S100A1 tightly bound to GST-truncated Hsp90 in the presence of Ca²⁺, S100A1 binding to the protein was only slightly reduced in the presence of EGTA (Fig. 2A, 2D). Although, CaM did not interact with GST-truncated Hsp90, GST-truncated Hsp70, GST-FKBP52, and GST-CyP40 in this assay, wild-type Hsp90, Hsp70, FKBP52, and CyP40 bound weakly to CaM-Sepharose in the presence or absence of Ca²⁺ (data not shown). All Ca²⁺-binding proteins studied did not interact with GST either in the presence or absence of Ca²⁺ (data not shown). These experiments demonstrate that S100A1 exclusively interacts with the chaperone and cochaperone proteins in a Ca²⁺-independent fashion.

We next sought to quantify the binding affinity between S100A1 and the chaperone and cochaperone proteins in vitro using the GST pull-down method followed by Scatchard analysis of binding data. GST-truncated Hsp90, GST-truncated Hsp70, GST-FKBP52, or GST-CyP40 were incubated with a logarithmic range of concentrations of recombinant S100A1 protein. Bound protein amounts were measured by SDS-PAGE followed by densitometric analysis. In Fig. 2B, I, the Scatchard analysis of S100A1 binding to GST-truncated Hsp90, in the presence of Ca²⁺, indicates two linear functions that give evidence of a minimum of two different S100A1 binding sites. One binding phenomenon. Higher affinity to bind S100A1 with a low dissociation constant (KD = 810 nM) than the other, which has a KD of 10.5 µM. In the presence of 1 mM EGTA, the Scatchard plot demonstrates the presence of a high affinity site (KD = 3.47 µM) and a low affinity site (KD = 56.8 µM) (Fig. 2B, 2). The Ca²⁺-dependent conformational change of S100A1 influenced its affinity to bind to GST-truncated Hsp90.

To determine both the number and affinity of S100A1 binding to GST-truncated Hsp70, GST-FKBP52, and GST-CyP40, Scatchard analysis was also performed. In all cases, the best fit of the data indicated a minimum of two classes of binding sites. As shown in Fig. 2B, 3–5, the KD values for S100A1 interaction with GST-Hsp70 (KD = 45.4 nM), FKBP52 (KD = 385 nM), and CyP40 (KD = 991 nM) are all in the submicromolar range indicating that the high affinity binding of S100A1 to the chaperone and cochaperone proteins is a fairly general phenomenon.

For further confirmation of the GST pull-down results, full-length proteins such as purified Hsp90, purified Hsp70, recombinant FKBP52, and recombinant CyP40 were used for co-immunoprecipitation experiments. Antibodies specific for Hsp90, Hsp70, FKBP52, and CyP40 were coupled to Protein G-Sepharose and then incubated with recombinant S100A1. When the resulting immunoprecipitates were subjected to Western blot analysis with anti-S100A1 antibody, S100A1 of expected size was detected (Fig. 3, A–E). In a reciprocal experi-
iment, Hsp90, Hsp70, FKBP52, or CyP40 were incubated with S100A1-coupled Protein G-Sepharose and then immunoprecipitates were analyzed by Western blotting with antibodies specific for Hsp90, Hsp70, FKBP52, and CyP40. As shown in Fig. 3, F–J, all the proteins were co-immunoprecipitated with S100A1. Taking the above results together as depicted in Figs. 1 and 2, we revealed that S100A1 interacted with Hsp90, Hsp70, FKBP52, and CyP40 (data not shown). Binding of Hsp90, however, was partially a Ca$^{2+}$-dependent process, which was in agreement with the GST pull-down experiments. The binding curves of Hsp90, Hsp70, FKBP52, and CyP40 (data not shown). Binding of Hsp90, however, was partially a Ca$^{2+}$-dependent process, which was in agreement with the GST pull-down experiments. The binding curves of Hsp90, Hsp70, FKBP52, and CyP40 (data not shown). Binding of Hsp90, however, was partially a Ca$^{2+}$-dependent process, which was in agreement with the GST pull-down experiments. The binding curves of Hsp90, Hsp70, FKBP52, and CyP40, respectively. Protein molecular weight markers (Bio-Rad) are shown on the right side.

**Biosensor Analyses**—To study the real-time binding kinetics of full-length Hsp90, Hsp70, FKBP52, and CyP40 to S100A1, recombinant S100A1 was immobilized on a biosensor chip surface and the protein complex formation was analyzed by SPR. Binding was analyzed in the presence or absence of Ca$^{2+}$, which gave identical results in the binding of Hsp70, FKBP52, and CyP40 (data not shown). Binding of Hsp90, however, was partially a Ca$^{2+}$-dependent process, which was in agreement with the GST pull-down experiments. The binding curves of Hsp90, Hsp70, FKBP52, and CyP40 were fit to the heterogeneous ligand binding model. The equation describing the heterogeneous ligand binding model involves two independent binding sites on S100A1 (analyte + ligand 1 ↔ analyte-ligand 1; analyte + ligand 2 ↔ analyte-ligand 2). When the binding curves of Hsp90, Hsp70, FKBP52, and CyP40 were fit to the “two-site” model, the “goodness of fit” was indicated by $\chi^2$ of <1.0. All other models had $\chi^2 > 1.0$, indicating higher non-random deviation from the fitted curve. Apparent rate constants of Hsp90 (in the presence of Ca$^{2+}$) obtained from this model were $k_{a1} = 4.15 \times 10^4$ M$^{-1}$ s$^{-1}$, $k_{d1} = 1.05 \times 10^{-2}$ s$^{-1}$, and $k_{a2} = 8.02 \times 10^4$ M$^{-1}$ s$^{-1}$, $k_{d2} = 4.31 \times 10^{-3}$ s$^{-1}$ (Fig. 4A). In the presence of EGTA, apparent rate constants of Hsp90 were $k_{a1} = 1.21 \times 10^5$ M$^{-1}$ s$^{-1}$, $k_{d1} = 3.68 \times 10^{-3}$ s$^{-1}$, and $k_{a2} = 7.46 \times 10^3$ s$^{-1}$, $k_{d2} = 3.65 \times 10^{-3}$ s$^{-1}$ (Fig. 4B). We observed that chelating of Ca$^{2+}$ caused decreases in $k_{a1}$ and $k_{d2}$ (dissociation) and increases in $k_{a2}$ and $k_{d2}$ (association). Fig. 4, C–E, shows the kinetics of the interaction between S100A1 with Hsp70, FKBP52, and CyP40. Dissociation of Hsp70, FKBP52, and CyP40 from S100A1 occurs very slowly, which results in a relatively large overall binding affinity (Table I).

**S100A1 Interacts with Hsp90, Hsp70, FKBP52, and CyP40 in Vivo**—We performed co-immunoprecipitation experiments to demonstrate that in vitro S100A1-chaperone and -cochaperone protein associations also occur under physiological conditions in mammalian cells. As a typical example of such association, immunoprecipitates of bovine brain cellular lysates using an anti-CyP40 antibody were found to contain, in addition to CyP40 itself, a specific 10-kDa band that was identified by an anti-S100A1 antibody (Fig. 5A, lane 4). In contrast, the specific 17-kDa band was not recognized by an anti-CaM antibody (Fig. 5A, lane 6). Both CyP40 and S100A1 were not detected after immunoprecipitation with rabbit preimmune serum or other nonspecific antibodies (data not shown). These experiments indicate the formation of stable complexes between S100A1 and CyP40 under physiological conditions in mammalian cells, although the possibility that the complexes were formed after lysis cannot be excluded.

A physical interaction between S100A1 and Hsp90, Hsp70, or FKBP52 was also demonstrated by the observation that S100A1 co-immunoprecipitates with the anti-Hsp90 antibody (AC88) (Fig. 5, B and C), anti-Hsp70 antibody (Fig. 5D), or anti-FKBP52 antibody (Fig. 5E) from bovine brain extracts. As shown in Fig. 5, F–J, immune adsorption of S100A1 from bovine brain extracts was accompanied by co-adsorption of Hsp90, Hsp70, FKBP52, and CyP40, again suggesting that S100A1 is bound to the chaperone and cochaperone proteins in mammalian cells.

**S100A1 Protects CS, Aldolase, GAPDH, and Rhodanese from Heat-induced Aggregation**—One of the characteristic features of molecular chaperones is their ability to suppress the aggre-
Novel Chaperone Function of S100A1

A 2.5-fold molar excess amount of S100A1 (0.475 μM) suppressed the heat-induced (43 °C) aggregation of CS and a 6.5-fold molar excess of S100A1 (1.235 μM) inhibited the aggregation completely (Fig. 6A). When a fixed large amount (2.6 μM) of Ca²⁺-binding protein was used, S100B and S100A2 suppressed the aggregation completely, whereas CaM and S100A4 suppressed the aggregation partially (Fig. 6B). Other S100 proteins had no effect on heat-induced CS aggregation. In
FIG. 3. S100A1 interaction with Hsp90, Hsp70, FKBP52, and CyP40 in vitro. Co-immunoprecipitation experiments were performed as described under “Experimental Procedures.” Hsp90-coupled Sepharose was incubated with S100A1 in the presence of 1 mM CaCl₂ (A) or 1 mM EGTA (B). The pellets were analyzed by Western blotting with antibodies specific for either S100A1 or Hsp90. Hsp70-coupled Sepharose (C),
the light scattering analysis, none of the S100 proteins or CaM alone caused any optical changes.

To demonstrate that the chaperone action of S100A1 has general substrate specificity, we verified that the protein was able to inhibit heat-induced aggregation of aldolase, GAPDH, and rhodanese. In this case, heating 3/4H9262 aldolase, GAPDH, or rhodanese at 65°C caused extensive protein aggregation in less than 5 min, shown as an increase in absorbance at 488 or 532 nm.

FKBP50-coupled Sepharose (D), or CyP40-coupled Sepharose (E) were incubated with S100A1 in the presence of 1 mM EGTA. Immunoprecipitates were analyzed by Western blotting with antibodies specific for either Hsp90 or S100A1. S100A1-coupled Sepharose was incubated with Hsp90 (H), FKBP52 (I), or CyP40 (J) in the presence of 1 mM EGTA. Immunoprecipitates were analyzed by Western blotting with antibodies specific for S100A1 (H–J), Hsp70 (H), FKBP52 (I), and CyP40 (J). Lane 1, unbound fraction; lane 2, bound fraction; IP, immunoprecipitation; WB, Western blotting; H-chain and L-chain, heavy chain and light chain of antibody.

**TABLE I**

| Analyte | Ca2⁺ | kᵢ | k₋ᵢ | Kᵢ1 | k₋₁ | k₋₂ | K₋₂ |
|---------|------|----|-----|-----|-----|-----|-----|
| Hsp90   | +    | 4.15 × 10⁶ | 1.05 × 10⁻² | 2.53 × 10⁻⁹ | 8.02 × 10⁴ | 4.31 × 10⁻³ | 5.37 × 10⁻⁷ |
| Hsp90   | −    | 1.21 × 10⁵ | 3.68 × 10⁻³ | 3.04 × 10⁻⁸ | 7.46 × 10⁵ | 4.38 × 10⁻³ | 4.89 × 10⁻⁷ |
| Hsp70   | −    | 2.68 × 10³ | 8.33 × 10⁻⁶ | 3.11 × 10⁻⁸ | 3.73 × 10⁴ | 8.93 × 10⁻⁸ | 2.39 × 10⁻⁷ |
| FKBP52  | −    | 3.45 × 10³ | 6.15 × 10⁻⁶ | 1.78 × 10⁻⁸ | 1.68 × 10⁴ | 2.04 × 10⁻⁸ | 1.21 × 10⁻⁷ |
| CyP40   | −    | 3.83 × 10⁴ | 3.31 × 10⁻⁴ | 8.64 × 10⁻⁹ | 4.04 × 10⁴ | 7.62 × 10⁻⁴ | 1.89 × 10⁻⁷ |

The rate constants were calculated by BIAevaluation 3.0 software from the traces in Fig. 4. The affinity constants, Kᵢ₁ and K₋₂ values, were calculated from the equations Kᵢ₁ = kᵢ₁/k₋₁ and K₋₂ = k₋₂/k₋₁, respectively. Other experimental details are described under “Experimental Procedures.”

**FIG. 4.** SPR sensorgrams of the binding of purified Hsp90, purified Hsp70, recombinant FKBP52, and recombinant CyP40 to immobilized S100A1. Hsp90 was injected over 2600 response units (RU) of immobilized S100A1 at various concentrations (50, 100, 200, 400, and 800 nM) in 1 mM CaCl₂ (A) or 1 mM EGTA (B). Hsp70 (C), FKBP52 (D), or CyP40 (E) was injected over immobilized S100A1 at various concentrations (50, 100, 200, 400, and 800 nM) in 1 mM EGTA. Sensorgrams have been corrected to the electrostatic binding of proteins to the carboxymethyl-dextran matrix using “mock-coupled” sensor chips.
360 nm. S100A1 did not aggregate when heated at 65 °C for up to 24 h (data not shown). When co-incubated with any of the proteins subjected to heat, 5 µM S100A1 potently suppressed protein aggregation to less than 50% (Fig. 6, C–E).

**FIG. 5.** S100A1 interaction with Hsp90, Hsp70, FKBP52, and CyP40 in vivo. **A**, bovine brain extracts were immunoprecipitated with anti-CyP40 antibody. Lane 1, authentic CyP40; lane 3, authentic S100A1; lane 5, authentic CaM; lanes 2, 4, and 6, immunoprecipitate with anti-CyP40 antibody. Lanes 1 and 2, Western blots with anti-CyP40 antibody; lanes 3 and 4, Western blots with anti-S100A1 antibody; lanes 5 and 6, Western blots with anti-CaM antibody. **B** and **C**, brain extracts were immunoprecipitated with an anti-Hsp90 antibody in the presence of 1 mM CaCl₂ (**B**) or 1 mM EGTA (**C**). The immunoprecipitates were analyzed by Western blotting with anti-Hsp90 antibody and anti-S100A1 antibody. **D** and **E**, brain extracts were immunoprecipitated with an anti-Hsp70 antibody (D) or anti-FKBP52 antibody (E) in the presence of 1 mM EGTA. The immunoprecipitates were analyzed by Western blotting with antibodies specific for Hsp70 (D), FKBP52 (E), and S100A1 (D and E). **F** and **G**, brain extracts were immunoprecipitated with an anti-S100A1 antibody in the presence of 1 mM CaCl₂ (**F**) or 1 mM EGTA (**G**). The immunoprecipitates were analyzed by Western blotting with anti-S100A1 antibody and anti-Hsp90 antibody. **H**–**J**, brain extracts were immunoprecipitated with an anti-S100A1 antibody in the presence of 1 mM EGTA. The immunoprecipitates were analyzed by Western blotting with anti-Hsp70 antibody (**H**), anti-FKBP52 antibody (**I**), or anti-CyP40 antibody (**J**). IP, immunoprecipitation; WB, Western blotting; H-chain and L-chain, heavy chain and light chain of antibody.
Novel Chaperone Function of S100A1

FIG. 6. Inhibition of the thermal aggregation of CS, aldolase, GAPDH, and rhodanese by S100A1. A, the effects of S100A1 on the thermal aggregation of CS at 43 °C were monitored as described (26). Curves 1–4 represent S100A1 concentrations of 0, 0.475, 0.95, and 1.235 μM, respectively. B, the effects of S100 proteins and CaM on the thermal aggregation of CS. Concentrations of S100 proteins and CaM used were 2.6 μM. Curve 2 is a control. Curves 1 and 3–11 represent the addition of S100A13, S100A6, S100A12, S100A10, S100A11, CaM, S100A4, S100A1, S100A2, and S100B, respectively. C–E, the effects of S100A1 on the thermal aggregation of aldolase (C), GAPDH (D), and rhodanese (E). The enzymes (3 μM each) were incubated at 65 °C for 5 min in the presence or absence of S100A1 (5 μM). Light scattering because of thermal aggregation was measured as absorbance at 488 or 360 nm in a spectrophotometer. Results represent the mean and S.D. of triplicate determinations.

information. As shown in Fig. 7A, CS rapidly lost its activity on incubation in the absence of S100A1 at 43 °C. However, S100A1 suppressed the heat-induced inactivation of CS in a concentration-dependent manner, similar to that for Hsp90 (26). When CS and S100A1 at a molar ratio of 1:10 were co-incubated for 30 min, the enzyme retained nearly 40% activity (Fig. 7A). In control experiments at 37 °C, co-incubation with S100A1 did not influence the specific activity of the enzyme (data not shown). When 2 mg/ml bovine serum albumin or IgG were used instead of S100A1, protection of the enzyme activity was not observed (data not shown). These results suggest that S100A1 recognizes and binds CS that unfolds during thermal stress, and protects it from irreversible aggregation and loss of enzyme activity.

Following the observations by Jakob et al. (26), we performed reactivation experiments of thermally unfolding CS with OAA, a substrate of CS, in the presence or absence of S100A1. After 6 min incubation of CS at 43 °C in the absence of S100A1, when ~25% of activity remained, OAA was added (Fig. 7B). After addition of OAA, the activity of CS increased by 35% and the net enzyme activity reached ~60% of the initial activity (Fig. 7B). However, in the presence of S100A1, the reactivation yield increased only by 5% and the net enzyme activity reached only 55%.

To try to reactivate CS, we used a temperature shift to 25 °C after 10 min incubation of CS (at 43 °C) to achieve nearly complete inactivation (35). In the absence of S100A1, after 10 min incubation of CS at 43 °C for 10 min, only 10% of the enzyme activity was left (Fig. 7C). After addition of OAA and a temperature shift to 25 °C, more than 50% of the initial activity of CS was regained and reached 65% of the initial activity. However, in the presence of S100A1, the reactivation yield increased only slightly (5% of the initial activity) and the net enzyme activity reached only about 45% (Fig. 7C).

S100A1 Suppresses Aggregation of Denatured Substrate during the Refolding Pathway—Similar to the thermal aggregation of CS, distinctive chaperones show different effects on the refolding pathway of “chemically denatured,” that is, guanidine HCl-denatured substrate proteins (29). In the present study, we investigated the interaction between S100A1 and the chemically denatured substrate protein, rhodanese. When the protein in 6 M guanidine HCl solution was allowed to refold spontaneously by diluting 20-fold in 50 mM Hepes, pH 7.0, a rapid aggregation took place. The aggregation was found to be suppressed significantly when the diluting buffer contained an increasing concentration of S100A1. With a stoichiometric amount (1.9 μM) of S100A1, ~90% aggregation was suppressed as compared with control (rhodanese alone) (Fig. 8A). CaM and other S100 proteins had essentially no effect (Fig. 8B).

Next, we examined the inhibitory effects of S100A1 on the aggregation of chemically denatured CS. As shown in Fig. 8C, S100A1 inhibited CS aggregation in a concentration-dependent manner. Whereas S100A2 had a weak inhibitory activity, CaM
FIG. 8. Suppression by S100A1 of the aggregation of chemically denatured rhodanese and CS during the refolding pathway. A, effects of S100A1 on the aggregation of chemically denatured rhodanese during the refolding pathway. Aggregation was essentially complete after 15 min. Curves 1–5 represent S100A1 concentrations of 0, 0.475, 0.95, 1.9, and 9.5 μM, respectively. B, effects of Ca2+-binding proteins on the aggregation of denatured rhodanese during the refolding pathway. Concentrations of S100 proteins and CaM were 2 μM each. Curve 9 is a control. Curves 1–8 and 10–12 represent the addition of S100A13, CaM, S100A6, S100A11, S100A2, S100A8, S100B, S100A10, S100A12, S100A4, and S100A1, respectively.

Curves 1–4 represent S100A1 concentrations of 0, 4.75, 9.5, and 19 μM, respectively. D, effects of Ca2+-binding proteins on the aggregation of denatured CS during the refolding pathway. Curves 1–4 represent S100A1 concentrations of 0, 4.75, 9.5, and 19 μM, respectively. E, effects of Ca2+ on the S100A1 chaperone activity using the rhodanese aggregation assay. The concentration of S100A1 was 4.75 μM. Curves 1–4 represent the addition of 1 mM Ca2+, 1 mM EGTA, 1 mM EGTA, and 4.75 μM S100A1, and 1 mM Ca2+ and 4.75 μM S100A1, respectively.

and other S100 proteins had no effect on CS aggregation (Fig. 8D). These results indicate that S100A1 recognizes and binds conformers of rhodanese and CS during the refolding pathway, thereby preventing their aggregation.

Because Ca2+ had no effect on the binding of S100A1 to the chaperone and cochaperone proteins, we examined the effects of Ca2+ on the chaperone activity of S100A1 by rhodanese aggregation assay. Neither Ca2+ nor EGTA influenced the chaperone activity of S100A1 (Fig. 8E).

Effects of CaM Antagonists on the Chaperone Activity of S100A1—Phenothiazines and diphenylalkylamines were originally identified as CaM antagonists (36), which bind to CaM in a Ca2+-dependent manner and antagonize the interaction of CaM with its target proteins; however, some CaM antagonists also bind S100 proteins, including S100A1, and thus would be expected to antagonize S100 proteins (21). To investigate the possible antagonistic activity of two CaM antagonists against S100A1, effects of fluphenazine (a representative of the phenothiazines) and prenylamine (a representative of the diphenylalkylamines) on the chaperone activity of S100A1 by the CS aggregation assay were examined. As shown in Fig. 9, A and B, the chaperone activity of S100A1 (5.4 μM) was inhibited by both fluphenazine and prenylamine with concentrations at 50% inhibition (IC50) values of 25–50 μM. The spontaneous aggregation of CS was not affected in the presence of fluphenazine and prenylamine, also both drugs did not cause any optical change in the light scattering assay. We also examined the effects of these drugs on CaM KII activity using 1 μM CaM to confirm their antagonistic action against CaM (Fig. 9C). As expected, both fluphenazine and prenylamine inhibited the enzyme activity with IC50 values of 19 and 10.6 μM, respectively. These results suggest that the chaperone activity of S100A1 is antagonized effectively by CaM antagonists and is indeed an intrinsic function of the protein.

Thermal Denaturation of β-Galactosidase and Luciferase in Vivo—We examined the role of S100A1 in protection against heat-induced protein denaturation in mammalian cells. As a model system, Escherichia coli β-galactosidase or firefly luciferase were expressed in mammalian cells and the effect of expression of S100A1 on the thermostability of the enzymes was examined. Previous reports indicated that these foreign reporter proteins are inactivated upon heat treatment of the mammalian cells (37). In addition, no induction of Hsp90, Hsp70, and Hsp40 could be observed in COS-7 cells by the overexpression of S100A1 (Fig. 10, A and B). COS-7 cells were transiently transfected with the pMH-LacZ6 with or without pME18S-S100A1 and expression of the S100A1 and β-galactosidase was evaluated by Western blotting (Fig. 10, C and D). Neither low grade (β-galactosidase:S100A1 = 1:5) nor high grade expression (β-galactosidase:S100A1 = 1:10) of S100A1 affected β-galactosidase expression. In the absence of S100A1 expression, β-galactosidase activity was slightly but significantly decreased to ~94% (Fig. 10C, lane 2) or ~92% (Fig. 10D, lane 2) of the initial activity after a heat shock at 45 °C for 2 h. The protective effect of S100A1 on heat inactivation of β-galactosidase was seen after heat shock. Both low grade and high grade expression of S100A1 enhanced enzyme activity slightly (Fig. 10, C, lane 4, and D, lane 4, ~103 and 106%, respectively).

To further confirm the chaperone activity of S100A1 in intact cells, COS-7 cells were transiently transfected with a luciferase expressing vector pGL3-Promoter and with pME18S-S100A1 or a control vector pME18S. As shown in Fig. 10E, upper panel, the levels of luciferase were not influenced by co-expression of S100A1. The cells were heated at 42 °C, and luciferase activity
in the lysate was followed as a function of time (Fig. 10). After heat shock at 42°C for 1 h, luciferase activity was slightly decreased (−87% of the initial activity) in the control cells, whereas S100A1 enhances luciferase activity slightly (−115% of the initial activity). After 2 h, luciferase activity was protected by co-expression of S100A1 against heat inactivation (−96% of the initial activity), whereas in the absence of exogenous S100A1, luciferase activity was lost to 23% of the initial activity.

**DISCUSSION**

Identification of the S100 target proteins and characterization of their mode of interaction with S100 proteins are essential to understand the cellular function of the S100-dependent signaling pathway. Although it has been reported previously that CaM, a prototypical Ca²⁺ sensor, binds to Hsp90 (15), Hsp70 (16), FKBP52 (17), and CyP40 (18), the regulatory function of CaM has been a conundrum. To establish whether CaM or S100 proteins exhibit properties of molecular chaperones, we examined their interaction with the components of the chaperone complex. We used a glutathione-Sepharose affinity chromatography-based method with GST fusion proteins containing truncated Hsp90 (237–628), truncated Hsp70 (1–382), wild-type FKBP52, and wild-type CyP40 to identify chaperone- or cochaperone-binding proteins among 10 S100 proteins and CaM. This method allowed us to identify S100A1, but not CaM or other S100 proteins, as a new member of the multichaperone complex.

In co-immunoprecipitation studies using bovine brain extracts, we found that S100A1 associated with native Hsp90, Hsp70, FKBP52, and CyP40. This observation was further supported by a similar co-immunoprecipitation experiment employing the pure protein mixtures of S100A1 and the chaperone and cochaperone proteins. Quantitative GST pull-down experiments indicated a biphasic interaction of S100A1 and Hsp90, Hsp70, FKBP52, and CyP40, with a minimum of two equilibrium binding constants. There are several explanations for this observation, for example, two S100A1 binding sites in Hsp90, Hsp70, FKBP52, and CyP40 or two binding sites of Hsp90, Hsp70, FKBP52, and CyP40 in S100A1. The future task would be to determine the S100A1 interacting domains of Hsp90, Hsp70, FKBP52, and CyP40. Also, the binding domains of Hsp90, Hsp70, FKBP52, and CyP40 in S100A1 should be determined. TheKD of S100A1 for the chaperone and cochaperone proteins, ranging from 4.54 × 10⁻⁸ M to 3.47 × 10⁻⁶ M, is similar to those of chaperone/cochaperone interactions such as Hop/Hsp90 (K₈ = 3.5 × 10⁻⁶ M, Ref. 38), Hop/Hsp70 (K₈ = 1.21 × 10⁻⁸ M, Ref. 38), FKBP51/Hsp90 (K₈ = 3.7 × 10⁻⁸ M, Ref. 38), FKBP52/Hsp90 (K₈ = 2.4 × 10⁻⁵ M, Ref. 38), Sti1/Ssa1 (K₈ = 7.5 × 10⁻⁶ M, Ref. 39), BAG1/Hsc70 (K₈ = 0.5 × 10⁻⁶ M, Ref. 40), auxilin/Hsc70 (K₈ = 0.6 × 10⁻⁶ M, Ref. 41), DnaK/DnaJ (K₈ = 5.4 × 10⁻⁷ M, Ref. 42), and CaM/Hsp90 (K₈ = 1.8 × 10⁻⁶ M, Ref. 43). The interaction of S100A1 with native Hsp90, Hsp70, FKBP52, or CyP40 was further characterized with SPR spectroscopy. Consistent with the GST pull-down experiments, SPR experiments showed a biphasic interaction suggesting a minimum of two independent binding sites on S100A1. However, K₈ values obtained from SPR experiments are 10²–10³ fold lower than the K₈ values obtained from the GST pull-down assays. The difference in the apparent K₈ values determined by the two methods is likely to result from the usage of different types of assay. For example, the immobilization of proteins to a solid phase support can theoretically affect rate constants by 10-fold compared with solution measurements (44). In view of these problems, we consider this assay simply as a semi-quantitative tool to monitor interaction.

An overlap specificity of different S100 proteins and CaM for interacting target proteins, peptides, and drugs is often observed (20, 21, 45). It has also been reported that CaM and S100 proteins can have different effects on the same target protein. For example, both CaM and S100A1 bind to twitchin kinase, but only S100A1 activates the enzyme. In the present study, Hsp90, Hsp70, FKBP52, and CyP40 weakly bound to CaM-Sepharose in the presence and absence of Ca²⁺ (data not shown) similar to that reported previously (17, 18), although nonspecific interactions cannot be excluded.

S100 proteins (−20 known so far) show a very divergent pattern of cell- and tissue-specific expression consistent with their pleiotropic intracellular functions (19, 46). About 40 target proteins are presently known to interact with S100 proteins (19, 46). Although much of the research has focused on the Ca²⁺-bound form, intracellular Ca²⁺-binding proteins such as CaM and S100 proteins can exist primarily in the two states,
the other being effectively Ca\(^{2+}\) free (47). Either state can interact with a different or overlapping sets of target proteins (48, 49). However, in a few cases, some S100 proteins bind to their target proteins \textit{in vitro} in a Ca\(^{2+}\)-independent manner (50), suggesting the possibility that they use different mechanisms for the regulation of their target proteins. The identification of the Ca\(^{2+}\)-free form of S100A1 as a component of the Hsp70/Hsp90 multichaperone complex and the Ca\(^{2+}\)-independence of the S100A1 chaperone activity provide an experimental system for future studies of S100A1/target protein interactions.

The chaperone ability of S100A1 and other Ca\(^{2+}\)-binding proteins was examined by two different assays: one involving thermal aggregation of CS, and the other monitoring the aggregation of rhodanese or CS following denaturation with 6 M guanidine HCl. Both methods are excellent tools with which to determine the ability of chaperone proteins to interact with unfolding proteins and suppress their aggregation (28, 29). Results of the thermal aggregation assay showed that S100A1 potently protects CS from heat-induced aggregation. This behavior is very similar to that of some of the well studied molecular chaperones such as GroEL, Hsp90, Hsp70, and small Hsps (29). Whereas large amount of S100B and S100A2 suppressed the aggregation, other S100 proteins and CaM had little or no effect on the aggregation assay (Fig. 6B). S100A1 also protected aldolase, GAPDH, and rhodanese from heat-induced aggregation (Fig. 6, C–E). Thus, S100A1 appears to have the ability to protect many different proteins from heat-induced aggregation. As in the case of the thermal aggregation of CS, different chaperone proteins may have different effects on the refolding of chemically denatured substrate proteins (29). For example, stoichiometric amounts of GroEL or excess amounts of Hsp90 completely suppressed aggregation of substrate proteins; whereas small Hsps had no effect on the aggregation of chemically denatured substrate proteins (35).

In the present study, dilution of chemically denatured rhodanese and CS into S100A1 (0.475–19 μM) maintained these proteins in a soluble state. In contrast to the situation in the presence of S100A1, aggregation of denatured rhodanese and CS occurred in the presence of other S100 proteins or CaM (Fig. 8, B and D). These findings are similar to the data reported for the aggregation suppression of chemically denatured CS during refolding by Hsp90 and GroEL (50) and are in contrast to the results for small Hsps (35).

Hsp90 and p23 have been shown to slow the inactivation process of the enzyme CS, which suggests that these proteins interact transiently with the unfolding enzyme (9, 26). In contrast, Hsp70, GroEL, and small Hsps, although able to suppress heat-induced CS aggregation effectively, appear to have no effect on the inactivation process (35). S100A1 suppressed the heat-induced inactivation of CS in a concentration-dependent manner, similar to that of Hsp90 and p23 (9). These results may suggest that S100A1 recognizes and binds to CS that unfolds during thermal stress, and protects it from irreversible aggregation and loss of enzyme activity.
Addition of OAA, a substrate of CS, to thermally inactivating CS leads to the reactivation of a certain amount of folding intermediates (26). In contrast to the situation with small Hsp90s, where unfolding intermediates of CS are trapped in a folding competent state and then are reactivated by OAA (29), S100A1 binds to the intermediates in a much more stable manner and inhibits the reactivation by OAA.

CaM antagonists also bind to several S100 proteins, including S100A1 and would be expected to antagonize the function of these proteins as well (20, 21). In the present experiments, the chaperone activity of S100A1 was effectively antagonized by CaM antagonists, such as fluphenazine and prenylamine, suggesting being an intrinsic function of the protein. Because relatively high molar ratios of S100A1:fluphenazine and S100A1:prenylamine were 1:5–1:9, at their respective IC50 values. However, molar ratios of S100A1:fluphenazine and S100A1:prenylamine were 1:19 and CaM:prenylamine = 1:10.

Consistent with the in vitro experiments described above, we found that transient overexpression of S100A1 in COS-7 cells indeed protected the enzymatic activities of E. coli β-galactosidase and firefly luciferase during heat stress. These studies may suggest that the main chaperone function of S100A1 is to protect the client proteins against irreversible damage during heat shock. Conclusively, we propose that S100A1 represents a potent molecular chaperone and a new cochaperone protein of Hsp70/Hsp90 multichaperone complex.

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S100A1 Is a Novel Molecular Chaperone and a Member of the Hsp70/Hsp90 Multichaperone Complex
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