Intrinsic ADP-ATP Exchange Activity Is a Novel Function of the Molecular Chaperone, Hsp70*

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Hsp70 is a multifunctional molecular chaperone whose interactions with protein substrates are regulated by ATP hydrolysis and ADP-ATP exchange. We show here that, in addition to ATPase activity, purified Hsp70 free from nucleoside-diphosphate (NDP) kinase exhibits intrinsic ADP-ATP exchange activity. The rate constants for ATP hydrolysis and ATP synthesis were in a similar range at the optimum pH of 7.5–8.5 in the presence of 5 mM ATP and 0.5 mM ADP. Hsp70 exhibited a considerably strict preference for ATP as a phosphate donor, and a biased substrate specificity, unlike NDP kinase; ADP, UDP, CDP > dTDP, dCDP > GDP, dGDP. During the reaction, Hsp70 formed an acid-labile auto-phosphorylated intermediate, and nucleoside diphosphate-dependent dephosphorylation of the latter then occurred. These properties of Hsp70 are not identical but similar to those of NDP kinase, but are not similar to those of adenylate kinase and ATP synthase.

The cytoplasmic 70-kDa heat shock protein (Hsp70) is thought to act as a “molecular chaperone” in protein folding by, at least, binding to nascent or misfolded segments of polypeptides, thereby regulating protein homeostasis (protein translocation, assembly, disassembly, and degradation) (1–3). Hsp70 binds tightly to ATP and ADP (4, 5) and exhibits very weak ATPase activity (6–8), which is able to separate conventional NDP kinase from Hsp70 (17). Hsp70 was eluted with approximately 0.1 M KCl and separated from contaminating proteins. Conventional NDP kinase, eluted with approximately 0.04 and 0.07 M KCl, was not detected among the contaminants in the commercial products of Hsp70 on that chromatography. The fractions of Hsp70 were concentrated and then subjected to HPLC on a double-linked TSK-Gel G3000 SW column (7.5 × 600 mm each) with 50 mM ammonium formate buffer, pH 5.5, 0.1 mM EDTA, and 0.5 mM dithiothreitol. The final preparation exhibited a purity of greater than 99% and was judged to be free from conventional NDP kinase on silver-stained SDS-PAGE.

**Experimental Procedures**

Materials—Hsp70 from bovine brain, nucleoside-diphosphate kinase (EC 2.7.4.6) from bovine liver, adenylate kinase (EC 2.7.4.3) from chicken muscle, various ribo- and deoxyribonucleoside tri-, di-, and monophosphates, AMP-PNP, and ATP-S were purchased from Sigma. [8-14C]ADP, [2-14C]CDP, [8-14C]ATP, and [γ-32P]ATP were obtained from NEN Life Science Products. The monoclonal antibody against the human nm23-H1 protein (human NDP kinase-A) was from Novoceastra Laboratories Ltd., UK. All other reagents were commercial products of the highest grade available.

Assaying of ATP Hydrolysis and ATP Synthesis—Almost all the data for Hsp70 ATPase reported were obtained by measurement of inorganic phosphate liberated from ATP, we found that Hsp70 exhibits the reverse activity, i.e. ADP-ATP exchange, and thus the amount of inorganic phosphate in the reaction is not reflected by the ATPase activity. In this regard, we analyzed the ATPase activity by measuring the conversion of [14C]ATP to [14C]ADP. The ATP synthesis activity was analyzed by measuring the conversion of [14C]ADP to [14C]ATP as described (18, 19). The reactions were carried out at 37 °C for 30 min in 100 mM Hepes-KOH buffer, pH 8, containing 5 mM ATP, 0.5 mM ADP, and 6 mM MgCl2 with 0–20 pmol of Hsp70 (monomeric form), and 0.05 μCi of [8-14C]ATP and 0.02 μCi of [8-14C]ADP for the assaying of ATPase and ATP synthesis activities, respectively, in a total volume of 10 μl. After incubation, the reaction mixtures were immediately spotted onto a polyethyleneimine-cellulose TLC plate (Macherey-Nagel). ADP and ATP were separated by ascending chromatography with 1 M formic acid containing 0.7 M LiCl, and the radioactivity in the resolved spots was quantitated with a Bio Imaging Analyzer BAS 1500 (Fuji Photo Film Co., Ltd., Japan).

Nucleotide Specificity as a Phosphate Acceptor of Hsp70—The nucleotide specificity of Hsp70 was examined by measuring the conversion of [14C]ATP to [14C]ADP. The ATP synthesis activity was examined by measuring the conversion of [14C]ADP to [14C]ATP as described (18, 19). The reactions were carried out at 37 °C for 30 min in 100 mM Hepes-KOH buffer, pH 8, containing 5 mM ATP, 0.5 mM ADP, and 6 mM MgCl2 with 0–20 pmol of Hsp70 (monomeric form), and 0.05 μCi of [8-14C]ATP and 0.02 μCi of [8-14C]ADP for the assaying of ATPase and ATP synthesis activities, respectively, in a total volume of 10 μl. After incubation, the reaction mixtures were immediately spotted onto a polyethyleneimine-cellulose TLC plate (Macherey-Nagel). ADP and ATP were separated by ascending chromatography with 1 M formic acid containing 0.7 M LiCl, and the radioactivity in the resolved spots was quantitated with a Bio Imaging Analyzer BAS 1500 (Fuji Photo Film Co., Ltd., Japan).

The abbreviations used are: NDP, nucleoside diphosphate; AMP-PNP, 5′-adenyl-γ-3-pyrophosphate; ATP-SH, adenosine 5′-O-(thio)triphosphate; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Mes, 4-morpholineethanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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enrichment of Hsp70 was analyzed (Fig. 2).

Detection of an Autophosphorylated Hsp70 Intermediate—Autophosphorylation and CDP-dependent dephosphorylation of Hsp70 were analyzed using 10 μg of Hsp70, 10 μCi of [γ-32P]ATP (600 Ci/mmol), 100 μM ATP, and 6 mM MgCl₂ in 100 mM Hepes-KOH buffer, pH 8, in the absence and presence of 5 mM CDP, respectively, in a total volume of 10 μl. After incubation at 37 °C for 2 h, both reactions were quenched by the further addition of 10 mM EDTA, and half of each sample was then treated with the traditional (pH 6.8) SDS sample buffer without boiling and subjected to 15% SDS-PAGE. After electrophoresis, the gel was dried without acid fixation and analyzed with an imaging analyzer. The remaining samples were analyzed as to the acid and base stability of the phosphorylated Hsp70 as described (20). These samples were then analyzed with an imaging analyzer.

RESULTS

Identification of ADP-ATP Exchange Activity of Hsp70—We found the purified Hsp70 free from NDP kinase exhibited ADP-ATP exchange activity, in addition to ATPase activity. As shown in Fig. 2A, Hsp70 exhibited weak ATPase activity in a dose-dependent manner, but no activity was observed for the control protein, BSA. Although ADP is a product inhibitor of common ATPase, a limited amount of ADP, i.e. concentrations between 0.1 and 1.0 mM, in the reaction mixture stimulated the ATPase activity of Hsp70 by 2–3.7 fold, as shown in Fig. 2A. Inhibition by ADP was then observed at concentrations in excess of 1 mM (data not shown). AMP had no effect on this activity.

The reverse reaction of ATP hydrolysis, i.e. ATP synthesis activity, of Hsp70 was analyzed (Fig. 2B). Surprisingly, Hsp70 catalyzed ATP synthesis at a slow but linear rate in the presence of substrate ADP but not in the presence of AMP. The ATP synthesis activity of Hsp70 gave saturation curves with ADP as a phosphate acceptor and with ATP as a phosphate donor. The reaction of ATP synthesis, as catalyzed by Hsp70, was entirely different from that of adenylate kinase, which catalyzes the reversible ATP-dependent synthesis of ADP from AMP, as shown in Fig. 3C. Furthermore, the reaction was different from that of conventional ATP synthase, which catalyzes ATP synthesis from ADP and P_ᵢ. When ATP was replaced with 5 mM P_ᵢ, Hsp70 failed to produce ATP from ADP (Fig. 2B). The conversion of ADP to ATP, i.e. ADP-ATP exchange, catalyzed by Hsp70 was similar to the reaction catalyzed by NDP kinase, which catalyzes the nucleoside triphosphate-dependent synthesis of ribo- and deoxyribonucleoside triphosphates from the corresponding diphosphates (21–23). No ATP synthesis activity was observed for the control protein, BSA (Fig. 2B).

From the kinetic results for ATP hydrolysis and for ATP synthesis, the K_m value of ATP for the ATP hydrolysis in the presence of 0.5 mM ADP and the K_m value of ADP for the ATP synthesis of Hsp70 were calculated to be 4.3 mM and 0.31 mM, respectively (data not shown). The specific activities of Hsp70 for ATP synthesis at pH 7.4, measured under the conditions in Fig. 2B, were 2.8–4.0 molecules of ATP min⁻¹ (Hsp70 monomer)⁻¹ with the five different purified Hsp70 preparations tested. The values are nearly the same as for the activity of ATP hydrolysis of Hsp70 in the presence of 0.5 mM ADP at pH 8. The K_m value of ATP and the specific activity for the ATPase.
activity of Hsp70 determined by measuring the conversion of $[^{14}C]$ATP to $[^{14}C]$ADP in our experiments were greatly different from the data previously reported (15, 24), which were determined by measuring the release of inorganic phosphate from ATP. Since Hsp70 exhibits both the activities of ATP hydrolysis and ATP synthesis, the kinetic constants for Hsp70 ATPase determined by measurement of inorganic phosphate in the reaction are not correct. Furthermore, the $K_m$ value of ATP for the ATPase activity of Hsp70 in our experiments is much higher than the values reported in the range of 0.7–1.4 $\mu$M (15, 24) and is in a range similar to that reported for NDP kinase (25), because the $K_m$ value was determined with 0.5 mM ADP in our experiments. Since both the $K_m$ values are in a range similar to the concentrations of ATP (~5 mM) and ADP (~0.5 mM) in the cytosol, changes in substrate availability would be expected to significantly affect the in vivo enzyme reaction.

The pH optima for Hsp70 ATPase and ATP synthesis were determined using 100 mM Mes buffer (pH 5.5–6), Hepes buffer (pH 7–8), Ches buffer (pH 9), and Chaps buffer (pH 10). Without ADP, the pH optimum for ATPase was found to be 7, consistent with a previous report (26). The optimum pH of the stimulated Hsp70 ATPase in the presence of 0.5 mM ADP, however, shifted to 7.5–9. The pH optimum for Hsp70 ATP synthesis was 7–9, which overlapped that of the stimulated ATPase. It is noteworthy that, at pH 7.5–8.5, the rate constants for ATP hydrolysis and ATP synthesis of Hsp70 were similar in the presence of 5 mM ATP and 0.5 mM ADP in the reaction mixture. The ATP synthesis activities were slightly higher below pH 7.5 and lower above pH 8.5 than the ATP hydrolysis activities.

**Hsp70 Functions as a NDP Kinase-like Enzyme—**NDP kinase is known to use any nucleoside triphosphate as a phosphate donor with similar efficiency. Hsp70, however, utilized ATP most efficiently, with the other ribo- and deoxyribonucleoside triphosphates being utilized at rates 16–33% of the rate of ATP utilization (data not shown). NDP kinase transfers the terminal phosphate of a nucleoside triphosphate to a nucleoside diphosphate. To determine whether this mechanism is the same for Hsp70, ATP analogs, such as ATP-$\gamma$S and AMP-PNP, were added together with ATP to the reaction mixture, and then the transfer of the $\gamma$-phosphate from ATP to ADP was analyzed. This transfer by both NDP kinase and Hsp70 was competitively inhibited by ~60–50% in the presence of equal concentrations of ATP-$\gamma$S and ATP and was completely inhibited by 5-fold excess concentrations of these analogs over those of ATP (data not shown).

Next we examined the specificity of each nucleotide as an acceptor of the transfer of the $[\gamma^{32}P]$phosphate of ATP, as catalyzed by Hsp70. As shown in Fig. 3, A and B, NDP kinase converted all ribo- and deoxyribonucleoside triphosphates to the corresponding nucleoside triphosphates with almost similar efficiency, consistent with previous reports (25, 27, 28). Hsp70, however, exhibited a biased substrate specificity, i.e. UDP, CDP > dTDP, dCDP > GDP, dGDP. Under these assay conditions, the conversion of ADP and dADP to ATP and dATP, respectively, could not be analyzed, because the newly formed products overlapped the phosphate donor, $[\gamma^{32}P]$ATP. Nevertheless, it was confirmed that the rates of conversion by Hsp70 of $[^{14}C]$ADP and $[^{14}C]$CDP to the corresponding nucleoside triphosphates exhibited almost similar efficiency (data not shown).

Since NDP kinase requires Mg$^{2+}$ or Mn$^{2+}$ for its activity, the effects of divalent cations on the ATP hydrolysis and ATP synthesis activities of Hsp70 were analyzed. Both activities of Hsp70 are almost equally stimulated by 6 mM Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ (data not shown).

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**Fig. 4. SDS-PAGE and immunoblot analyses of Hsp70 (A), autophosphorylation of Hsp70 in an alkali-stable and acid-labile manner, and CDP-dependent dephosphorylation (B and C).** A, silver-stained SDS-PAGE (4–20% gradient) of 1 $\mu$g of Hsp70 (lane 2) and immunoblot analysis of 1 $\mu$g of Hsp70 (lane 3) and NDP kinase (lane 4) using a 1:100 dilution of a monoclonal antibody raised against human nm23-H1 protein (NDP kinase-A), followed by ECL Western blotting detection reagent (Amersham). Lane 1, molecular weight markers. B, the autophosphorylation and CDP-dependent dephosphorylation of Hsp70 (10 $\mu$g) were analyzed as described under "Experimental Procedures." The autophosphorylation of Hsp70 in the absence (lane 1) and presence (lane 2) of 5 mM CDP for 2 h. Half of each sample was treated with the traditional (pH 6.8) SDS sample buffer without boiling, subjected to 15% SDS-PAGE, and then dried without acid fixation. To determine the stability of the autophosphorylated Hsp70 as a function of pH, phosphorylated Hsp70 was treated with basic (pH 8.5) (lane 3) SDS sample buffer without boiling and then electrophoresed, and the gel was dried without acid fixation. For acid stability, phosphorylated Hsp70 was boiled in the SDS sample buffer at pH 6.3 and then electrophoresed. The gel was fixed in 20% trichloroacetic acid, followed by Coomassie staining, destaining in methanol/acetic acid, and drying (lane 4). The samples were then analyzed with an imaging analyzer. C, the remaining halves of the samples in B with (lane 1) or without (lane 2) 5 mM CDP in the reaction mixture were then analyzed by TLC, followed by imaging analysis.

**Formation of an Autophosphorylated Hsp70 Intermediate and Its CDP-dependent Dephosphorylation—**We examined whether or not the intrinsic ADP-ATP exchange activity of Hsp70 is due to a contaminant, since its activity is similar to that of NDP kinase. This possibility is unlikely for the following reasons. Firstly, the commercially available Hsp70 was further purified by HPLC on a Mono Q anion-exchange and then a gel permeation column to remove possible contaminants with affinity for ATP, such as NDP kinase. The purified Hsp70 was >99% pure and was confirmed to be free from conventional NDP kinase. This was also confirmed by the fact that no immunoreactivity of the Hsp70 preparation against anti-NDP kinase-A antibodies was observed (Fig. 4A). Secondly, the nucleotide specificity for the activity of ATP synthesis by Hsp70 was different from that reported for NDP kinase. Thirdly, an autophosphorylated intermediate was observed in the protein band of Hsp70, as described below, as in the case of the autophosphorylated intermediate of conventional 16-kDa NDP kinase.

NDP kinase autophosphorylates the active site histidine of the intermediate in the process of the catalytic phosphate transfer reaction (21–23). Hsp70 gave a single autophosphorylated intermediate band corresponding to a protein with a molecular mass of 70 kDa, lane one of the nucleoside diphosphate, CDP, in the reaction mixture, which contained 10 $\mu$Ci of $[^{32}P]$ATP, 100 mM ATP, and 6 mM MgCl$_2$ (Fig. 4B, lane 1), and no formation of CTP was observed (Fig. 4C, lane 1). In the presence of 5 mM CDP in the reaction mixture, however, the radioactivity of the phosphorylated intermediate decreased significantly with the concomitant formation of $^{32}$P-labeled CTP from CDP (Fig. 4B, lane 2, and Fig. 4C, lane 2, respectively).
Furthermore, a significant decrease in the radioactivity of the autophosphorylated intermediate was also observed on the addition of 5 mM CDP to the reaction mixture after the first reaction of the formation of an autophosphorylated intermediate (data not shown). These results indicate that Hsp70 catalyzes the transfer of the γ-phosphate group from ATP to CDP, and that this transfer involves a phosphoenzymic intermediate. These properties are similar to those of the conventional NDP kinase reported. To characterize the phosphorylation of Hsp70, autophosphorylated Hsp70 was subjected to acid, neutral, and basic treatments as described (20, 22), which allow the evaluation of a high energy phosphate on a histidine residue in NDP kinase and histidine protein kinase. The phosphorylated intermediate of Hsp70 was stable as to alkaline treatment, but labile as to acid treatment, with a significant decrease in the autophosphorylation level, as compared with neutral treatment, as shown in Fig. 4B, lanes 1, 3, and 4, respectively. That phosphorylation at serine, threonine, and tyrosine is stable as to acid treatment suggests that the acid-labile and alkali-stable phosphorylated residue(s) in Hsp70 may be a basic amino acid residue, although the phosphorylated residue has not yet been identified.

**DISCUSSION**

When we analyzed the ATPase activity of Hsp70, we observed a discrepancy in the ATPase activity determined with two different methods; the ATPase activity determined as the conversion of [14C]ATP to [14C]ADP was higher than that determined as the release of P1 from ATP. In turn, we obtained Hsp70 which is free from contaminants with affinity to ATP, such as conventional NDP kinase, and measured the activities of ATP hydrolysis as well as ATP synthesis of Hsp70 and found that Hsp70 also exhibits the activity of ADP-ATP exchange.

A mechanism for Hsp70-protein/peptide interaction and ATP hydrolysis has been proposed (3, 14). The first step in the mechanism involves the binding of a substrate protein/peptide to an Hsp70-ADP complex, resulting in a conformational change which causes acceleration of ATP-ATP exchange in the presence of ATP (step 2). The binding of ATP causes a conformational change which triggers substrate release from the complex (step 3). Finally, ATP is hydrolyzed to ADP to afford an Hsp70-ADP complex that can then participate in a new cycle of binding (step 4). The mechanisms underlying ADP-ATP exchange (step 2) and ATP hydrolysis (step 4) are best understood for the bacterial Hsp70 homolog, DnaK, with its cofactors, DnaJ and GrpE, which accelerate the ATP-dependent cycle of substrate binding and release (3, 17, 29). Interaction with DnaJ accelerates the ATP-ATP exchange activity of Hsp70 and found that Hsp70 catalyzes the transfer of the γ-phosphate group from ATP to CDP, and that this transfer involves a phosphoenzymic intermediate.

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