ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 11, pp. 1222-1229. © Pleiades Publishing, Inc., 2006.
Published in Russian in Biokhimiya, 2006, Vol. 71, No. 11, pp. 1505-1513.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM06-149, August 6, 2006.

ATPase Domain of Hsp70 Exhibits Intrinsic ATP–ADP Exchange Activity

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Received June 6, 2006
Revision received June 20, 2006

Abstract—The chaperone activity of Hsp70 in protein folding and its conformational switching are regulated through the hydrolysis of ATP and the ATP–ADP exchange cycle. It was reported that, in the presence of physiological concentrations of ATP (~5 mM) and ADP (~0.5 mM), Hsp70 catalyzes ATP–ADP exchange through transfer of γ-phosphate between ATP and ADP, via an autophosphorylated intermediate, whereas it only catalyzes the hydrolysis of ATP in the absence of ADP. To clarify the functional domain of the ATP–ADP exchange activity of Hsp70, we isolated the 44-kD ATPase domain of Hsp70 after limited proteolysis with α-chymotrypsin (EC 3.4.21.1). The possibility of ATP–ADP exchange activity of a contaminating nucleoside diphosphate kinase (EC 2.7.4.6) was monitored throughout the experiments. The purified 44-kD ATPase domain exhibited intrinsic ATP–ADP exchange by catalyzing the transfer of γ-phosphate between ATP and ADP with acid-stable autophosphorylation at Thr204.

DOI: 10.1134/S0006297906110071

Key words: ATP–ADP exchange, ATPase domain, autophosphorylation, Hsp70

The 70-kD heat shock proteins (Hsp70s) function as molecular chaperones in eukaryotes, being involved in various cellular functions, such as protein synthesis [1], folding [2, 3], translocation [4, 5], degradation [6], and modulation of protein expression [7]. Hsp70s have a highly conserved N-terminal ATPase domain (about 64% residue identity among eukaryotic Hsp70s) with a molecular mass of approximately 44-kD, and a C-terminal domain that can be further divided into a 15-kD substrate-binding domain and a 10-kD C-terminal subdomain of unknown function [8]. The chaperone activity of Hsp70 coupled with repeated cycles of binding and release of peptide substrates is regulated by ATP binding and ATP–ADP exchange coordinated through its conformational changes [8-11]. Co-chaperones such as Hdj1, Hip, and Bag1 accelerate the ATP-dependent cycle of substrate binding and release by switching between ATP- and ADP-binding states [12-14]. It has been reported that, in presence of physiological concentrations of ATP (~5 mM) and ADP (~0.5 mM), Hsp70 by itself catalyzes ATP–ADP exchange without cofactors through the transfer of γ-phosphate between ATP and ADP, whereas in absence of ADP, it only catalyzes the hydrolysis of ATP, as reported [15, 16]. During the ATP–ADP exchange reaction, Hsp70 forms an acid-labile autophosphorylated intermediate, and nucleoside diphosphate-dependent dephosphorylation of the latter then occurs in a similar manner to in the case of nucleoside diphosphate (NDP) kinase (EC 2.7.4.6). In addition, acid-stable autophosphorylation at conserved Thr204 and abolition of this autophosphorylation and the ATPase activity of a Thr204Ala mutant have been reported [17]. Although Hsp70 and NDP kinase both exhibit ATP–ADP exchange activity, obvious differences in enzymatic properties between these molecules have been detected. The nucleotide specificity of NDP kinase is nonspecific with respect to phosphate donor and acceptor nucleotides, whereas Hsp70 greatly prefers ATP as a phosphate donor, and ADP, UDP, and CDP as phosphate acceptors [15,

Abbreviations: DnaK) *Escherichia coli* heat shock protein 70; DTT) dithiothreitol; Hsc70) heat shock cognate 70; Hsp70) 70-kD heat shock protein; NDP) nucleoside diphosphate; P_i) inorganic phosphate; PMSF) phenylmethylsulfonyl fluoride; TLCK) tosyllysine chloromethyl ketone.

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16]. The turnover rate of approximately 1000 sec^{-1} for the ATP hydrolysis of NDP kinase [18] is much higher than that in the case of Hsp70, and the difference in the activity may reflect the efficiency of autophosphorylation by [γ-^{32}P]ATP of 16-kD NDP kinase and 70-kD Hsp70 with high and low efficiency, respectively, in the process of the ATP–ADP exchange reaction. To identify the functional domain of the ATP–ADP exchange reaction of Hsp70, we purified the N-terminal 44-kD fragment of bovine Hsp70 after extensive digestion with α-chymotrypsin (EC 3.4.21.1) and then characterized the enzyme activity. Since it has been reported that a weak, though measurable, physical interaction exists between DnaK or Hsc70 and NDP kinase [19, 20], which may mask the intrinsic enzymatic property of Hsp70, we carefully examined the possibility of NDP kinase contamination in our experiments. The purified N-terminal 44-kD ATPase domain exhibited ATP–ADP exchange activity in the presence of physiological concentrations of ATP and ADP in the reaction mixture, but only ATPase activity in the absence of ADP, and showed acid-stable autophosphorylation at Thr204, indicating that the 44-kD ATPase domain is the functional domain of the ATP–ADP exchange reaction, other than ATPase activity of Hsp70.

MATERIALS AND METHODS

Materials. Bovine brain Hsp70, bovine liver NDP kinase, tosyllysine chloromethyl ketone (TLCK)-treated α-chymotrypsin, and various nucleotides were purchased from Sigma (USA). All Hsp70 used in these experiments was further purified to homogeneity by HPLC on a Mono Q anion-exchange column as reported [15, 16]. [8-^{14}C]ADP, [8-^{14}C]ATP, and [γ-^{32}P]ATP were obtained from DuPont-New England Nuclear (USA). All other reagents were commercial products of the highest grade available.

Assaying of ATP hydrolysis and ATP synthesis activities. ATP hydrolysis and ATP synthesis activities were analyzed by measuring the conversion of [^{14}C]ATP to [^{14}C]ADP, and [^{14}C]ADP to [^{14}C]ATP, respectively, as reported [15, 16]. The reaction was carried out in 10 µl of 100 mM Hepes-KOH, pH 7.5, containing 5 mM ATP, 0.5 mM ADP, 6 mM $MgCl_2$, 0-40 pmol 44-kD fragment protein or recombinant Hsp70s, and 0.05 µCi of [8-^{14}C]ATP and 0.02 µCi of [8-^{14}C]ADP for the analysis of ATP hydrolysis and ATP synthesis, respectively. The reaction mixture was incubated at 37°C and the reaction was stopped at 1 h by the addition of 2 µl of 10 mM EDTA. Separation of ADP and ATP was achieved by thin layer chromatography on polyethylenimine cellulose sheets in 1 M formic acid and 0.7 M LiCl for 1 h. The radioactivity in the resolved spots was quantified with a Bio Imaging Analyzer BSA 1500 (Fuji Photo Film Co., Ltd., Japan).

Site-directed mutagenesis of Hsp70 and purification of mutant Hsp70s from *Escherichia coli*. Mutagenesis of human Hsp70 was performed according to the instructions for a QuikChange™ site-directed mutagenesis kit from Stratagene (USA). The sense oligonucleotides used for mutagenesis were as follows: 5′-TTTGATGCCGCACGACTTATTGGA-3′ for K71A, and 5′-CTGGGCGGGGGCGCCTTCGACGTGTC-3′ for T204A, the altered codons being underlined. The presence of the expected mutations without any artifact was confirmed by DNA sequencing. The recombinant genes encoding wild-type Hsp70 and mutants of it were cloned into the pET21 His-tagged plasmid (Qiagen, USA) and then overproduced in *E. coli* BL21(DE3). We found that the cells had to be grown at 28-30°C to obtain fully active proteins. The recombinant wild type and mutant human Hsp70s were purified on a HisTrap™ Ni^{2+}-agarose column (Pharmacia, USA), and then on a Mono Q anion-exchange column eluted with a narrow salt gradient of 0-0.2 M KCl in buffer A (25 mM Tris-HCl, pH 7.2, containing 1 mM EDTA and 1 mM dithiothreitol (DTT)). These materials were further purified on a gel permeation TSK-Gel G3000SW column (7.5 × 600 mm) in buffer A. Each purified recombinant protein was >99% pure, as judged on silver-stained SDS-PAGE.

Proteolytic digestion of Hsp70 and NDP kinase with α-chymotrypsin and separation of degradation peptides. Bovine brain Hsp70 (4 µg) or bovine liver NDP kinase (6 µg) in 10 µl of digestion buffer (20 mM Hepes-KOH, pH 7.5, containing 4.5 mM magnesium acetate, 75 mM KCl, and 0.8 mM DTT) was digested with 2 µg TLCK-treated α-chymotrypsin for 0-120 min at 37°C. The digestion was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). The reaction mixtures were then analyzed by SDS-PAGE, followed by Coomassie brilliant blue staining. For large-scale digestion and separation of peptides, bovine brain Hsp70 (100 µg) was digested with 50 µg TLCK-treated α-chymotrypsin in 200 µl of digestion buffer for 2.5 h, as described above. After termination of the reaction, samples were dialyzed against buffer A and then loaded onto a Mono Q HPLC column at the flow rate of 1 ml/min. The resulting peptides were eluted with a 0-0.2 M KCl gradient in buffer A, and then the separated fragments were dialyzed against buffer A. Protein concentrations were determined by Bradford's method using a Bio-Rad (USA) protein assay kit with BSA as the standard. Proteolytic digestion of an equimolar mixture of bovine brain Hsp70 (100 µg) and liver NDP kinase (23 µg) with α-chymotrypsin was also performed, as described above.

Autophosphorylation and identification of the phosphorylated residue. The purified bovine brain Hsp70 (7 µg) and 44-kD fragment (4.4 µg) were incubated at 37°C for 2 h with 10 µCi of [γ-^{32}P]ATP, 100 µM ATP, and 6 mM $MgCl_2$ in 100 mM Hepes-KOH, pH 8.0, in a total volume of 10 µl. After incubation, the reaction mixtures

were quenched by the further addition of 1 μl of 10 mM EDTA and then subjected to 10-20% gradient SDS-PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue under acidic conditions for 2 h and dried, and then the phosphorylated peptide was detected by autoradiography. For isolation of the phosphorylated peptide fragment, the autophosphorylated 44-kD fragment (20 μg) was dissolved in 100 μl of 70% formic acid containing 1% CNBr, and then the cleavage reaction was carried out in the dark at room temperature for 24 h. The reaction mixture was then diluted with 900 μl of H_2O, lyophilized to remove CNBr, dissolved in a small amount of H_2O, and then subjected to SDS-PAGE. The peptide fragments separated on SDS-PAGE were transferred to a Problot™ membrane (USA), stained with Coomassie brilliant blue, and then subjected to autoradiography. The N-terminal amino acid sequence of each fragment peptide was determined with an Applied Biosystems 492 protein sequencer (Applied Biosystems, USA) as described previously [21]. To determine the phosphorylated residue, the phosphorylated 44-kD fragment (44 μg) was reduced, S-pyridylethylated, and then digested with lysyl endopeptidase overnight as described previously [22]. The resulting peptides were separated by reversed phase HPLC on a Cosmosil 5C4-300 column (4.6 × 150 mm) (Nakarai Chemical, Japan), elution being performed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The N-terminal 22 amino acid sequence of the phosphorylated peptide was determined with an Applied Biosystems 492 protein sequencer, and the radioactivity of the Edman degradation product at each cycle was monitored.

RESULTS

The N-terminal 44-kD fragment is the ATP–ADP exchange domain of Hsp70. Purified bovine Hsp70 shows intrinsic ATP–ADP exchange activity, i.e. the transfer of γ-phosphate in the presence of ATP and ADP in the reaction mixture with the formation of an acid-labile autophosphorylated intermediate, in addition to canonical ATPase activity [15, 16]. Since the ATP–ADP exchange reaction of Hsp70 consists of coordinated ATP hydrolysis and ATP synthesis reactions, which can be monitored as the conversion of [^{14}C]ATP to [^{14}C]ADP and [^{14}C]ADP to [^{14}C]ATP, respectively, we postulated the functional domain of the ATP–ADP exchange reaction was in the 44-kD ATPase domain of Hsp70, which is prepared by limited hydrolysis with α-chymotrypsin [23]. To examine our hypothesis, we treated purified bovine brain Hsp70 with TLCK-treated α-chymotrypsin for 0-2 h and then separated the degradation products by HPLC on a Mono Q anion-exchange column. α-Chymotrypsin cleaves Hsp70 at the carboxyl sides of Ala546 and/or Gly402, resulting in two N-terminal fragments with molecular masses of 60 and 44 kD, respectively [23]. The N-terminal 44-kD fragment has been identified as the ATPase domain [23]. SDS-PAGE analysis of the degradation products revealed a rapid decrease in the intact Hsp70 protein, with the concomitant appearance of the 60-kD fragment and a steady increase in the 44-kD fragment with increasing incubation period (Fig. 1a). The 44-kD fragment was resistant to further degradation. These 44- and 60-kD fragments could be separated from NDP kinase on a Mono Q column, as

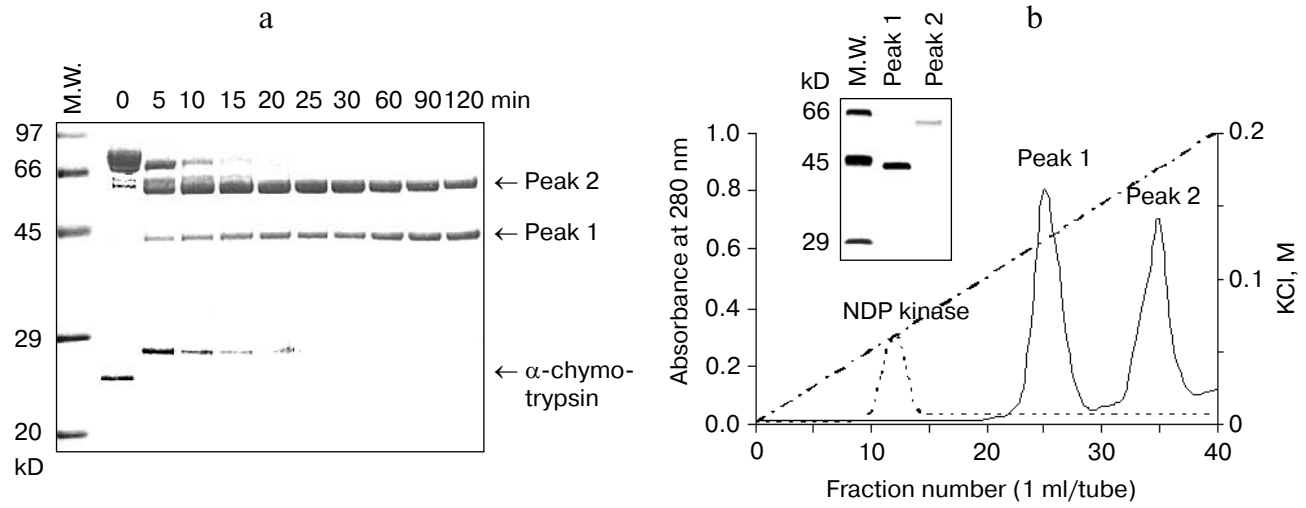

Fig. 1. Chymotryptic digestion of bovine brain Hsp70 and separation of digested fragments. a) Purified bovine brain Hsp70 (4 μg) was digested with 2 μg TLCK-treated α-chymotrypsin for 0-120 min at 37°C. The digested fragments were separated by 10-20% gradient SDS-PAGE, followed by Coomassie brilliant blue staining. MW, molecular weight markers. b) Bovine brain Hsp70 (100 μg) was digested with 50 μg TLCK-treated α-chymotrypsin for 2.5 h at 37°C. The resulting fragments were separated on a Mono Q column (0.5 × 5 cm) and then eluted with a salt gradient of 0-0.2 M KCl as described under "Materials and Methods". The collected protein peak fractions were analyzed by SDS-PAGE, followed by silver staining.

shown in Fig. 1b. The N-terminal amino acid sequences of the purified 44- and 60-kD fragments confirmed they are N-terminal side fragments of Hsp70 and that there was no detectable contamination by NDP kinase of these fragments (data not shown). The purified 44-kD ATPase fragment of Hsp70 exhibited ATP–ADP exchange activities, i.e. ATP hydrolysis and ATP synthesis, in the presence of 5 mM ATP and 0.5 mM ADP in a similar manner to these activities of undigested Hsp70, as reported [15, 16] (Figs. 2a and 2b). The ATP hydrolysis activity of the 44-kD fragment was stimulated by 3.6-fold in the presence of 0.5 mM ADP and no ATP synthesis activity was observed in the absence of ADP, similar to in the case of Hsp70. Both enzymatic activities increased linearly with the dose, and no initial bursts of ATP hydrolysis and ATP synthesis activity [24] were observed. No enzymatic activities were observed for the control protein, BSA. The specific activities of the 44-kD fragment for ATP hydrolysis and ATP synthesis were 0.73 molecule of ADP per min per 44-kD fragment monomer and 0.97 molecule of ATP per min per 44-kD fragment monomer, respectively.

Autophosphorylation of the 44-kD fragment of Hsp70. NDP kinase is autophosphorylated at an active site histidine residue with high efficiency as an intermediate in the process of the catalytic phosphate transfer between nucleoside triphosphates and nucleoside diphosphates [25-27], and is also autophosphorylated at a serine residue in its molecule, probably through transfer from the acid-labile phosphohistidine to Ser [28]. We previously detected an acid-labile autophosphorylated intermediate of Hsp70 during the ATP–ADP exchange reaction and its nucleoside diphosphate-dependent dephosphory-

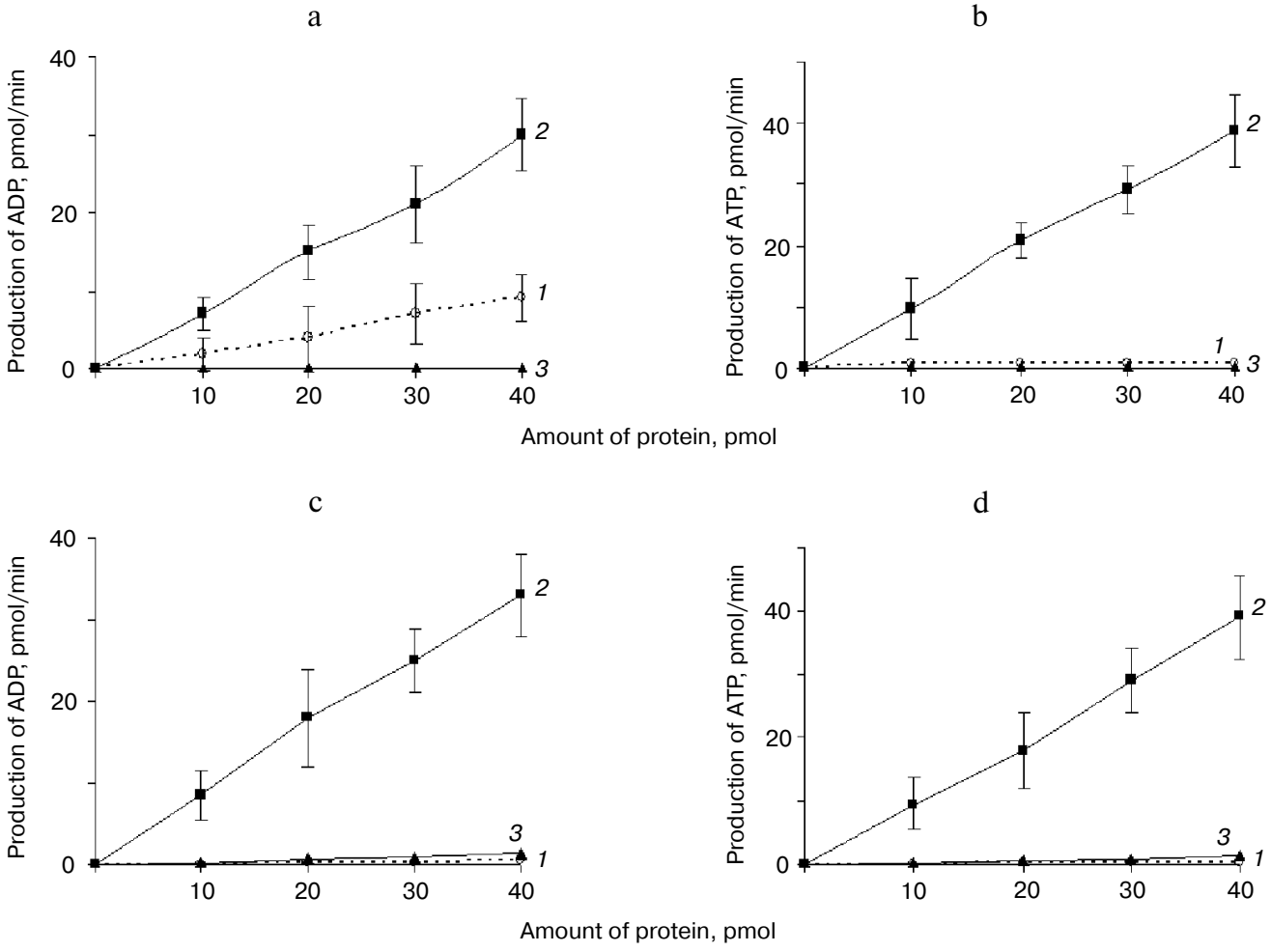

Fig. 2. ATP–ADP exchange activity of the 44-kD fragment of Hsp70. The ATP hydrolysis (a) and ATP synthesis (b) activities of the 44-kD fragment were analyzed by measuring the conversion of [^{14}C]ATP to [^{14}C]ADP and [^{14}C]ADP to [^{14}C]ATP, respectively, in the reaction mixture, as described under "Materials and Methods", without (*1*) or with 0.5 mM ADP (*2*). The activity of BSA (*3*) as a negative control was analyzed with 0.5 mM ADP. The ATP hydrolysis (c) and ATP synthesis (d) activities of the human recombinant wild-type Hsp70 (*2*), Lys71Ala mutant (*1*), and Thr204Ala mutant (*3*) were also analyzed. The reported values are the means of at least six independent experiments.

lation, similar to that in the case of NDP kinase [15, 16]. In addition, an acid-stable autophosphorylation at Thr204 of Hsp70 in the phosphate transfer reaction of ATP hydrolysis and almost complete abolition of both ATP hydrolysis and its acid-stable autophosphorylation of Thr204Ala mutant have been reported [17]. As shown in Figs. 2c and 2d, the substitution of Lys71 and Thr204, located in the catalytic pocket and coordinated by a salt bridge with the γ-phosphate of ATP, nearly abolished the ATPase activity as reported [17, 29] and the ATP synthesis activity of Hsp70. These results indicate that the ATP synthesis activity of Hsp70 is strictly coupled with ATP hydrolysis activity, and the ATP–ADP exchange activity of Hsp70 does not originate from contaminating NDP kinase.

Next we analyzed acid-stable autophosphorylation (Fig. 3) and its phosphorylated residue of the 44-kD fragment (Fig. 4). We also analyzed autophosphorylation of recombinant wild-type Hsp70 and its mutants of Lys71Ala and Thr204Ala. The wild-type Hsp70 and 44-kD fragment exhibited acid-stable autophosphorylation, but no autophosphorylation was observed in the Lys71Ala and Thr204Ala mutants (Fig. 3a), the latter findings being consistent with previous reports [17, 29]. The autophos-

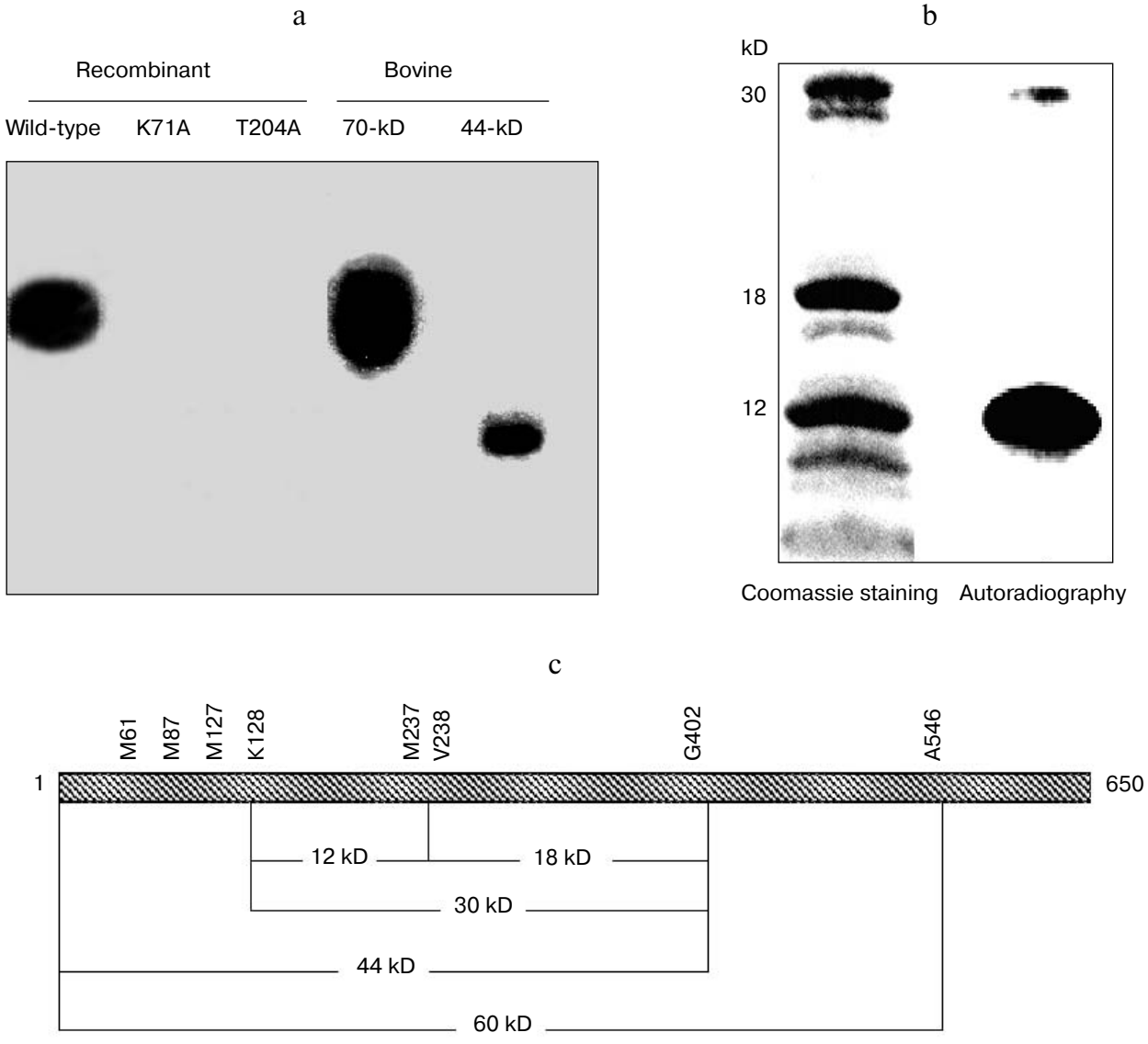

Fig. 3. Autophosphorylation and separation of the phosphorylated peptide fragment. a) Hsp70 (bovine brain and human recombinant) (7 μg of each) and the 44-kD fragment of bovine Hsp70 (4.4 μg) were incubated at 37°C for 2 h with [γ-^{32}P]ATP and then analyzed by SDS-PAGE and subjected to autoradiography as described under "Materials and Methods". b) The phosphorylated 44-kD fragment (20 μg) was treated with CNBr, and the resulting cleavage products were separated by SDS-PAGE, transferred to a Problot™ sequence membrane, and then stained with Coomassie brilliant blue and visualized by autoradiography. c) Diagram showing bovine Hsp70 (650 residues) and fragments generated on treatment with α-chymotrypsin and CNBr.

phorylated 44-kD fragment was cleaved with CNBr at four putative Met cleavage sites under acidic conditions, i.e. Met61, Met87, Met127, and Met237. Coomassie brilliant blue staining of the hydrolytic products revealed three major protein bands corresponding to molecular masses of 30-, 18-, and 12-kD, and four additional minor protein bands (Fig. 3b). Analysis of the twenty-five residues of the N-terminal amino acid sequence of each of the major fragments was performed and the arrangement of each fragment was identified, as shown in Fig. 3c. Among them, the 12-kD fragment of Lys128-Met237, but not the 18-kD one, exhibited autophosphorylation. To determine the phosphorylated residue of the 44-kD fragment, the fragment was digested with lysyl endopeptidase, followed by separation by reversed-phase HPLC. A single radioactive peptide, Val189-Lys220, was isolated (Fig. 4a), and the amino acid sequence and radioactivity measurement of the N-terminal 22 residues revealed that Thr204, but not Ser208, was phosphorylated (Fig. 4b). The identified residue was consistent with that previously reported by McCarty and Walker [17]. Since an acid-labile autophosphorylated intermediate readily loses radioactivity during the process of peptide separation under acidic conditions and amino acid sequence determination, we could not determined the acid-labile phosphorylated residue under these conditions.

α-Chymotrypsin digestion of bovine liver NDP kinase. NDP kinase is abundantly distributed in mammalian cells and *E. coli*, and weakly binds to DnaK and Hsp70s [19, 20]. To evaluate the possibility of NDP kinase contamination of the 44-kD fragment, we examined the difference in susceptibility to α-chymotrypsin digestion between Hsp70 and NDP kinase. Despite the limited cleavage of Hsp70 (as shown in Fig. 1), 16-kD NDP kinase was readily degraded into many small fragments of below 10-kD during incubation for 120 min at 37°C (Fig. 5). This may be supported by the finding of 15 aromatic amino acids among the 152 amino acids of NDP kinase A and NDP kinase B [30]. Proteolytic digestion of an equimolar mixture of Hsp70 and NDP kinase with α-chymotrypsin followed by Mono Q anion-exchange chromatography gave no detectable peaks derived from NDP kinase other than the 44- and 60-kD fragments of Hsp70. In addition, no significant increases in the ATP hydrolysis and ATP synthesis activities of the 44-kD fragment generated from the equimolar mixture of Hsp70 and NDP kinase with α-chymotrypsin were observed in comparison with these activities of the 44-kD fragment generated from only Hsp70 (Fig. 6). Also, no initial burst activity was observed even for the 44-kD fragment generated from the mixture of Hsp70 and NDP kinase.

DISCUSSION

In the present study, we found that the 44-kD ATPase domain of Hsp70 is the functional domain of its ATP–ADP exchange activities, i.e. ATP hydrolysis and ATP synthesis activities. Since it has been reported that a weak interaction exists between DnaK or Hsc70 and NDP kinase with characteristic initial burst activity [19, 20], we carefully examined the possibility of NDP kinase contamination throughout our experiments. To avoid NDP kinase contamination, Hsp70 purchased from Sigma was further purified on a Mono Q anion-exchange column, which is able to separate an additive of bovine

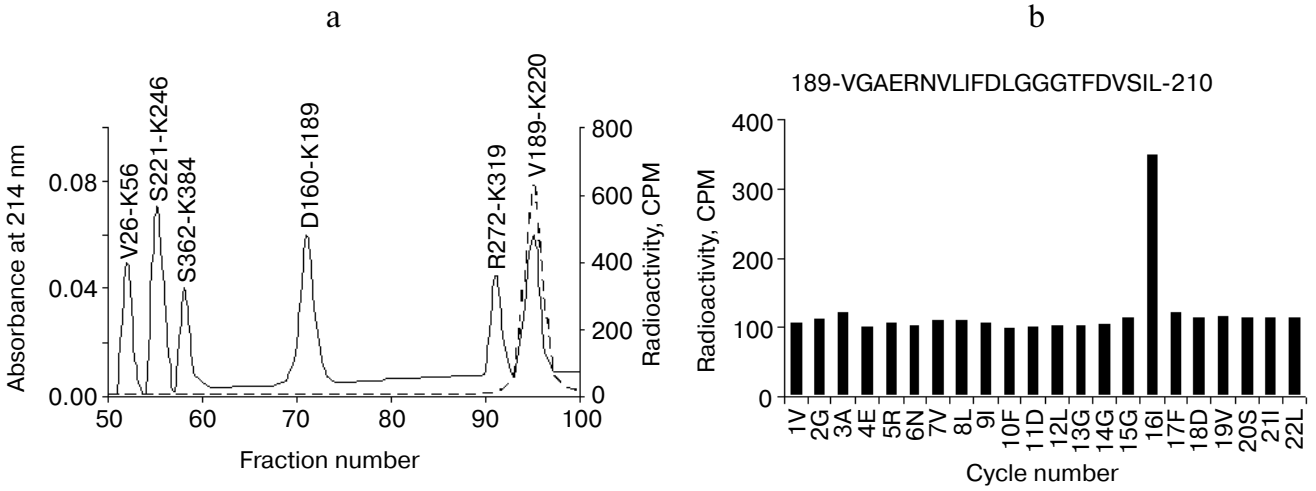

Fig. 4. Identification of the phosphorylated residue in the 44-kD fragment of Hsp70. a) The phosphorylated 44-kD fragment (44 μg) was S-pyridylethylated and then digested with lysyl endopeptidase as described under "Materials and Methods". The resulting peptides were separated on a reverse phase Cosmosil 5C4-300 column with a linear gradient of acetonitrile (30-45%) and then the radioactivities of the peptide peaks were counted. b) The N-terminal 22 amino acid sequence was determined, and the radioactivity of the Edman degradation product at each cycle was monitored.

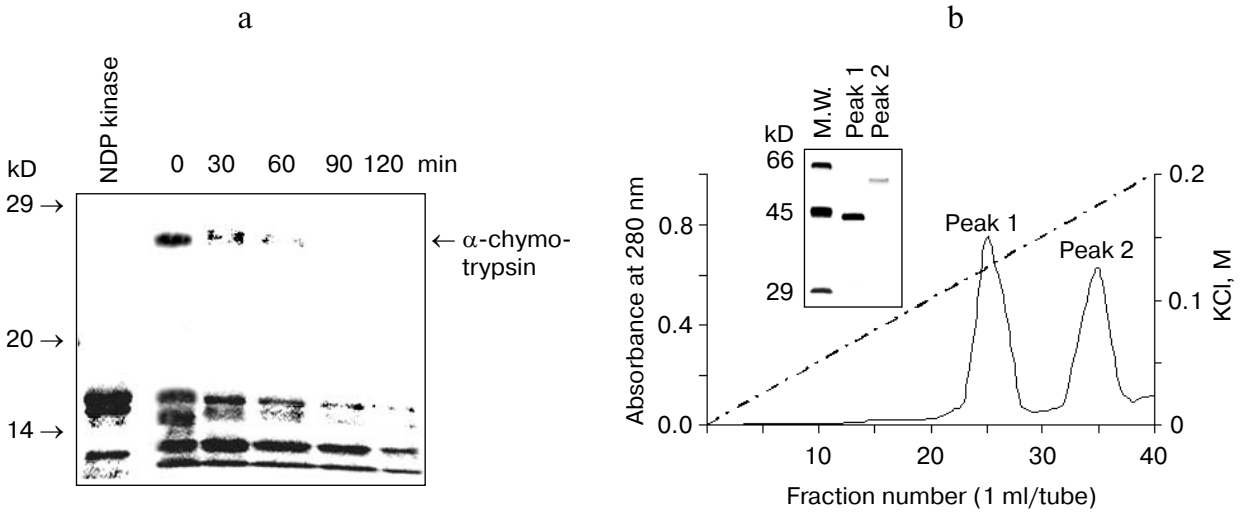

Fig. 5. α-Chymotryptic digestion of Hsp70 with or without NDP kinase, and separation of the resulting fragments. a) Bovine liver NDP kinase (6 μg) was digested with 2 μg TLCK-treated α-chymotrypsin for 0-120 min at 37°C and the resulting fragments were separated by SDS-PAGE. b) An equimolar mixture of NDP kinase (23 μg) and Hsp70 (100 μg) was digested with 50 μg TLCK-treated α-chymotrypsin for 2.5 h at 37°C, followed by separation on a Mono Q column as described in the legend to Fig. 1.

NDP kinase from bovine brain Hsp70 [15, 16] and also from the N-terminal 44-kD Hsp70 generated on α-chymotrypsin digestion (Fig. 1b). The purified N-terminal 44-kD fragment showed ATP hydrolysis and ATP synthesis activities without an initial burst, in a similar manner to in the case of Hsp70 reported [15, 16]: the weak ATP hydrolysis activity was stimulated by 3.6-fold on the addition of 0.5 mM ADP, although ADP is a product inhibitor of common ATPase; the reverse reaction of ATP hydrolysis, i.e. ATP synthesis, of the 44-kD fragment was observed with a similar catalytic turnover number to that for ATP hydrolysis.

These results suggest that the basic machinery of the ATP–ADP exchange reaction is located in the 44-kD ATPase domain. Furthermore, no increases in the ATP hydrolysis and ATP synthesis activities of the 44-kD fragment were observed even on the addition of an equimolar mixture of NDP kinase and Hsp70, followed by α-chymotrypsin digestion (Fig. 6).

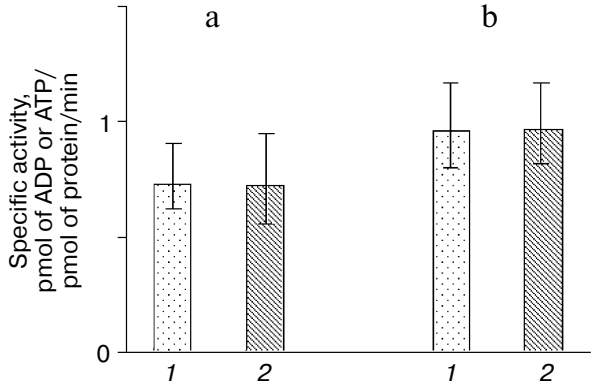

Fig. 6. Comparison of the ATP hydrolysis (a) and ATP synthesis (b) activities between the 44-kD fragments of Hsp70 generated with α-chymotrypsin in the absence (*1*) and presence of NDP kinase (equimolar mixture) (*2*). The reported values are the means of at least six independent experiments.

Taking these results together, we concluded that the ADP–ATP exchange activity of Hsp70 is intrinsically enzymatic, and not caused by contaminating NDP kinase. Although the molecular mechanisms of the phosphate transfer reaction have not been fully clarified, autophosphorylation of the 44-kD ATPase domain might be involved in the intrinsic phosphate transfer process. In the process of ATP–ADP exchange, an acid-labile autophosphorylated intermediate of Hsp70 and its nucleoside diphosphate-dependent dephosphorylation have been reported [16]. Furthermore, acid-stable autophosphorylation of conserved Thr204 and its abolition on Thr204 mutation (Figs. 3 and 4), which is coupled to the nearly complete abolition of the ATP hydrolysis and ATP synthesis activities, were observed. These findings are supported by the crystal structure of the human ATPase domain which is similar to that of the bovine ATPase domain: the distance between the P_i-phosphorus atom and the Thr204 γ-oxygen is 3.50 Å, and that between the phosphate oxygen and the Thr204 γ-oxygen is 2.60 Å, suggesting that the threonine residue is in position for in-line nucleophilic attack on the γ-phosphate and the formation of phosphothreonine [31]. On the basis of the present data, we propose that the 44-kD ATPase domain is the functional domain of the ATP–ADP exchange reaction involving the transfer of γ-phosphate in cooperation with autophosphorylation.

REFERENCES

1. Nelson, R. J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M., and Craig, E. A. (1992) *Cell*, **71**, 97-105.
2. Beckmann, R. P., Mizzen, L. E., and Welch, W. J. (1990) *Science*, **248**, 850-854.
3. Skowyra, D., Georgopoulos, C., and Zylicz, M. (1990) *Cell*, **62**, 939-944.
4. Kang, J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A., and Pfanner, N. (1990) *Nature*, **348**, 137-143.
5. Shi, Y., and Thomas, J. O. (1992) *Mol. Cell Biol.*, **12**, 2186-2172.
6. Wagner, I., Arlt, H., van Dyck, L., Langer, T., and Neupert, W. (1994) *EMBO J.*, **13**, 5135-5145.
7. Stone, D. E., and Craig, E. A. (1990) *Mol. Cell Biol.*, **10**, 1622-1632.
8. Bukau, B., and Horwich, A. L. (1998) *Cell*, **92**, 351-366.
9. Lewis, M. J., and Pelham, H. R. (1985) *EMBO J.*, **4**, 3137-3143.
10. Hartl, F.-U. (1996) *Nature*, **381**, 571-579.
11. Blond-Elguindi, S., Fourie, A. M., Sambrook, J. F., and Gething, M. J. (1993) *J. Biol. Chem.*, **268**, 12730-12735.
12. Freeman, B. C., Myers, M. P., Schumacher, R., and Morimoto, R. I. (1995) *EMBO J.*, **14**, 2281-2292.
13. Hohfeld, J., and Jentsch, S. (1997) *EMBO J.*, **16**, 6209-6216.
14. Bimston, D., Song, J., Winchester, D., Takayama, S., Reed, J. C., and Morimoto, R. I. (1998) *EMBO J.*, **17**, 6871-6878.
15. Wu, X., Yano, M., Washida, H., and Kido, H. (2004) *Biochem. J.*, **378**, 793-799.
16. Hiromura, M., Yano, M., Mori, H., Inoue, M., and Kido, H. (1998) *J. Biol. Chem.*, **273**, 5435-5438.
17. McCarty, J. S., and Walker, G. C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 9513-9517.
18. Munoz-Dorado, J., Inouye, S., and Inouye, M. (1990) *J. Biol. Chem.*, **265**, 2707-2712.
19. Barthel, T. K., and Walker, G. C. (1999) *J. Biol. Chem.*, **274**, 36670-36678.
20. Leung, S. M., and Hightower, L. E. (1997) *J. Biol. Chem.*, **272**, 2607-2614.
21. Yano, M., Mori, S., and Kido, H. (1994) *J. Biol. Chem.*, **274**, 34375-34382.
22. Kido, H., Yokogoshi, Y., and Katunuma, N. (1988) *J. Biol. Chem.*, **263**, 18104-18107.
23. Chappell, T. G., Konforti, B. B., Schmid, S. L., and Rothman, J. E. (1987) *J. Biol. Chem.*, **262**, 746-751.
24. Ha, J. H., and McKay, D. B. (1994) *Biochemistry*, **33**, 14625-14635.
25. Biondi, R. M., Walz, K., Issinger, O. G., Engel, M., and Passeron, S. (1996) *Analyt. Biochem.*, **242**, 165-171.
26. Deville-Bonne, D., Sellam, O., Merola, F., Lascu, I., Desmadril, M., and Veron, M. (1996) *Biochemistry*, **35**, 14643-14650.
27. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.
28. Almaula, N., Lu, Q., Delgado, J., Belkin, S., and Inouye, M. (1995) *J. Bacteriol.*, **177**, 2524-2529.
29. O'Brien, M. C., Flaherty, K. M., and McKay, D. B. (1996) *J. Biol. Chem.*, **271**, 15874-15878.
30. Abdulaev, N. G., Karaschuk, G. N., Ladner, J. E., Kakuev, D. L., Yakhyaev, A. V., Tordova, M., Gaidarov, I. O., Popov, I., Fujiwara, J. H., Chinchilla, D., Eisenstein, E., Gilliland, G. L., and Ridge, K. D. (1998) *Biochemistry*, **37**, 13958-13967.
31. Sriram, M., Osipiuk, J., Freeman, B., Morimoto, R. I., and Joachimiak, A. (1997) *Structure*, **5**, 403-414.