The second metal-binding site of 70 kDa heat-shock protein is essential for ADP binding, ATP hydrolysis and ATP synthesis

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The chaperone activity of Hsp70 (70 kDa heat-shock protein) in protein folding and its conformational switch, including oligomeric and monomeric interconversion, are regulated by the hydrolysis of ATP and the ATP–ADP exchange cycle. The crystal structure of human ATPase domain shows two metal-binding sites, the first for ATP binding and a second, in close proximity to the first, whose function remains unknown [Sriram, Osipiuk, Freeman, Morimoto and Joachimiak (1997) Structure **5**, 403–414]. In this study, we have characterized the second metal-binding motif by site-directed mutagenesis and the kinetics of ATP and ADP binding, and found that the second metal-binding site, comprising a loop co-ordinated by His-227, Glu-231 and Asp-232, participates both in ATP hydrolysis and ATP-synthetic activities, in co-operation with the first metal-binding site. The first metal-binding site, a catalytic centre, is essential for ATP binding and the second site for ADP binding in the reactions of ATP hydrolysis and ATP synthesis.

Key words: ATP hydrolysis, ATP synthesis, calcium-/magnesium-binding site, chaperone, nucleotide binding, nucleoside diphosphate kinase.

INTRODUCTION

The Hsp70s (70 kDa heat-shock proteins) comprise a family of molecular chaperones in eukaryotes involved in various cellular functions, such as protein folding, translocation and degradation [1–3]. Hsp70s recognize misfolded proteins and facilitate the conversion to their functional conformations. Efficient refolding by Hsp70s depends on repeated cycles of binding/release of substrates coupled to ATP hydrolysis, and an ATP–ADP exchange co-ordinated by their conformational changes including oligomeric and monomeric interconversion [4–6]. Co-chaperones, such as DnaJ and GrpE, accelerate this ATP–ADP exchange cycle to drive the chaperone activities of the Hsp70 family proteins efficiently [7,8].

We have previously reported that, in the presence of physiological concentrations of $\approx$5 mM ATP and $\approx$0.5 mM ADP, Hsp70 catalyses both in ATP hydrolysis and ATP-synthetic activities by transfer of γ-phosphate between ATP and ADP, through an acid-labile autophosphorylated intermediate, whereas in the absence of ADP it only catalyses the hydrolysis of ATP [9]. A NDP (nucleoside diphosphate)-dependent dephosphorylation of this intermediate is observed in the ATP-synthetic reaction. These observations suggest that Hsp70 catalyses the intra- or intermolecular ATP synthesis by transferring γ-phosphate, probably co-ordinated by a conformational switch as well as conversion between oligomers and active monomers in Hsp70s. However, the molecular mechanisms of the conformational switch and catalytic site of the ATP synthesis have not been clarified.

Conformational changes of uridylate kinase (EC 2.7.1.48) with a different pair of nucleotide-binding sites [10], intramolecular two-nucleotide-binding sites for phosphate donor and acceptor in adenosine kinase (EC 2.7.1.20) and adenylate kinase (EC 2.7.4.3) [11,12], and a new C-terminal ATP-binding site, other than the N-terminal ATP-binding site of Hsp90 for molecular switch mechanisms [13], have been reported. Within the ATPase domain of human Hsp70, a new metal-binding site other than the first magnesium/calcium site in the catalytic pocket was found by high-resolution crystal structure analysis using synchrotron radiation at 120 K [14], although its function remains unknown. In the present study, we analysed the role of the second and first metal-binding motifs of human Hsp70 in the reaction of ATP hydrolysis and ATP synthesis and in the interconversion of oligomeric and monomeric Hsp70s by site-directed mutagenesis and kinetics of ATP and ADP binding, and discuss the role of the second metal-binding site in the catalytic activities of Hsp70 in co-operation with the first metal-binding site. In addition, we have carefully excluded the contamination of NDP kinase (EC 2.7.4.6) from *Escherichia coli* in the fraction of recombinant Hsp70, since it has been reported that a weak physical interaction exists between DnaK or Hsc70 and NDP kinase [15,16].

MATERIALS AND METHODS

Biochemicals

ATP– and ADP–agarose, and various nucleotides were purchased from Sigma. [8-^{14}C]ADP (56.7 mCi/mmol), [8-^{14}C]ATP (56 mCi/mmol) and [^{35}S]ATP[S] (adenosine 5′-[γ-[^{35}S]thio]triphosphate; 320 mCi/mmol) were obtained from NEN Life Science Products. All other reagents were commercial products of the highest grade available.

Site-directed mutagenesis of human Hsp70

Mutagenesis of human Hsp70 [17] was performed according to the instructions of the manufacturer of a QuikChange™ site-directed mutagenesis kit from Stratagene. The sense oligonucleotides used for mutagenesis were as follows: 5′-TTTGATGCCGCACGACTTATTGGA-3′ for K71A, 5′-CTGGGCGGGGGCGCCTTCGACGTGTC-3′ for T204A, 5′-GGCCGGGGACACCAGCCTGGGTGGGGAGGA-3′ for H227S, 5′-GGCCGGGGACACCCAACTGGGTGGGGAGGA-3′ for H227Q, 5′-GACACCCACCTGGGTGGGTCGGACTTTGACAACAGG-3′ for

Abbreviations used: Hsp70, 70 kDa heat-shock protein; NDP, nucleoside diphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; WT, wild type.

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E231S, and 5′-ACCTGGGTGGGGAGGCCTTTGACAACAGG-C-3′ for D232A. The altered codons are underlined. The presence of the expected mutations without any artifact was confirmed by DNA sequencing. The recombinant genes encoding the WT (wild-type) Hsp70 and its mutants were cloned into the pET21 His-tagged plasmid (Qiagen) 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.

Purification of human recombinant WT Hsp70, mutant Hsp70s, bovine Hsp70 and NDP kinase from *E. coli*

The human recombinant WT and mutant Hsp70s were purified on a HisTrap™ Ni^{2+}–agarose column (Amersham Biosciences) [18], then on a TSK gel DEAE 3SW anion-exchange column (7.5 mm × 75 mm) eluted with a narrow salt gradient of 0–0.2 M KCl in buffer A (25 mM Tris/HCl, pH 7.2, 1 mM EDTA and 1 mM dithiothreitol) to eliminate contaminating proteins with affinity for ATP and ADP [9,19]. These materials were further purified on a gel-permeation TSK gel G3000SW column (7.5 mm × 600 mm) in buffer A. The NDP kinase from *E. coli* BL21(DE3) was purified according to the method of Ohtsuki et al. [20,21], with an additional final step consisting of DEAE 3SW column chromatography. Hsp70 from bovine brain purchased from Sigma was further purified to homogeneity by DEAE 3SW column chromatography as described in [9]. Each purified Hsp70 and NDP kinase was > 99 % pure, as ascertained by silver-stained SDS/PAGE.

Assay of ATP hydrolysis and ATP synthesis

ATP hydrolysis and ATP-synthesis activities of Hsp70s in the presence of ATP and ADP in the reaction mixture were analysed separately by measuring the conversion of [^{14}C]ATP to [^{14}C]ADP for ATP hydrolysis, and the conversion of [^{14}C]ADP to [^{14}C]ATP for ATP synthesis [9].

Thermal stability

Aliquots of purified NDP kinase and recombinant WT human Hsp70 at a concentration of 0.3 mg/ml were incubated at 60 °C in buffer A containing 1 mg/ml BSA [22]. After incubation, the samples were cooled on ice, and the residual ATP synthesis activity was measured.

Binding of the human recombinant WT Hsp70, mutant Hsp70s and bovine Hsp70 to ATP– and ADP–agarose

The purified human WT Hsp70, mutant Hsp70s and bovine Hsp70 (30 μg) were applied to ATP–agarose (C_8-linked; Sigma catalogue no. A2767) and ADP–agarose (Sigma catalogue no. A2810) affinity columns (500 μl), equilibrated with buffer B (25 mM Hepes/KOH, pH 7.6, containing 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA and 10 mM 2-mercaptoethanol). The columns were washed with 5 ml of buffer B plus 0.5 M NaCl, followed by 2 ml of buffer B, then eluted with 5 ml of buffer B plus 5 mM ATP for ATP–agarose and 5 mM ADP for ADP–agarose columns. The protein concentration in each 250 μl fraction was measured by the method of Bradford [23]. The column recoveries of the human WT Hsp70, mutant Hsp70s and bovine Hsp70 were between 92 and 94 % on ATP–agarose and between 79 and 85 % on ADP–agarose. The non-specific binding of each Hsp70 to these columns was below the detection level.

Nucleotide-binding properties

Characterization of the ATP and ADP binding to the purified recombinant human WT Hsp70, mutant Hsp70s and bovine Hsp70 was performed by equilibrium gel penetration assay [24]. The reaction mixture (1.0 ml) comprised buffer C (100 mM Hepes/KOH, pH 7.8, and 5 mM $MgCl_2$), 70 μg of Hsp70 or a mutant, 0.5–4 μM [^{14}C]ATP diluted with unlabelled ATP (to 35.1 μCi/ml), 50 units of creatine kinase (EC 2.7.3.2) and 10 mM creatine phosphate for ATP binding or 0.5–4 μM [^{14}C]ADP diluted with unlabelled ADP (to 15.3 μCi/ml), and 50 units of hexokinase (EC 2.7.1.1) and 10 mM glucose for ADP binding. After the addition of each Hsp70 to the reaction mixture, the mixture was equilibrated for 10 h, then transferred to 70 mm × 10 mm plastic tubes, each containing 80 mg of dry Sepharose G-50 (coarse) powder. The tubes were stored at 4 °C and gently agitated for 14 h to allow swelling of the gel and equilibration of free ligand with the solvent inside the gel. After equilibration, the tubes were centrifuged, and 50 μl aliquots of the supernatant were collected for measurements of radioactivity. A solution of Blue Dextran in the same buffer was equilibrated with the gel to measure the excluded volume. The quantity of ligand bound to protein was calculated by the equation described in [24]. Dissociation constants (K_d) were determined by the Scatchard plot method.

Size exclusion and nucleotide contents of Hsp70s as oligomers and monomers

WT and mutant Hsp70s (200 μg of each) in buffer B (100 μl) were reacted with 150 μM [^{35}S]ATP[S] (320 mCi/mmol) and [^{14}C]ADP (32 mCi/mmol) for 30 min at 37 °C, then exposed to HPLC on a TSK gel G3000SW column with 100 mM sodium phosphate buffer, pH 6.8, and 100 mM Na_2SO_4. Protein concentrations and radioactivity of the eluate were monitored. The fractions of protein peaks were freeze-dried, dissolved with 1 M perchloric acid for 30 min, and the supernatant containing the released nucleotides was applied to a TLC plate (Macherey-Nagel) after neutralization with 1 M KOH as described previously [9]. The radioactivity in the resolved spots was measured with a Bio Imaging Analyser BAS 1500 (Fuji Photo Film Co., Tokyo, Japan).

RESULTS

Separation of recombinant Hsp70 free from *E. coli* NDP kinase and difference in the thermal stability of ATP synthetic activity between Hsp70 and NDP kinase

We have reported that the purified Hsp70 exhibits intrinsic ATP synthetic activity in the presence of ATP and ADP in the reaction mixture with a formation of acid-labile auto-phosphorylated intermediates, in addition to the canonical ATPase activity [9]. To confirm the intrinsic ATP synthetic activity and its catalytic site of Hsp70, we mutated putative catalytic sites of human Hsp70, including the second metal-binding sites. *E. coli* contains NDP kinase abundantly and the enzyme shows high turnover rates of ATP hydrolysis and ATP synthesis [25], suggesting that NDP kinase contamination masks these intrinsic catalytic activities of Hsp70. To verify the separation of recombinant human Hsp70 from NDP kinase, purified NDP kinase from *E. coli* BL21(DE3) was premixed with recombinant WT human Hsp70 for 30 min on ice, applied to a DEAE 3SW anion-exchange column, then separated with a narrow salt gradient of 0–0.2 M KCl (Figure 1). No increase in the ATP synthetic activity in the fraction of Hsp70 was observed, and the activities of Hsp70 and NDP kinase were almost completely recovered stoichiometrically in each separate protein peak. This is consistent with a previous report by other authors [15] and with our earlier observations on the separation of bovine Hsp70 and NDP kinase on a Mono Q column [9]. The result suggests either the effective separation of recombinant Hsp70 from

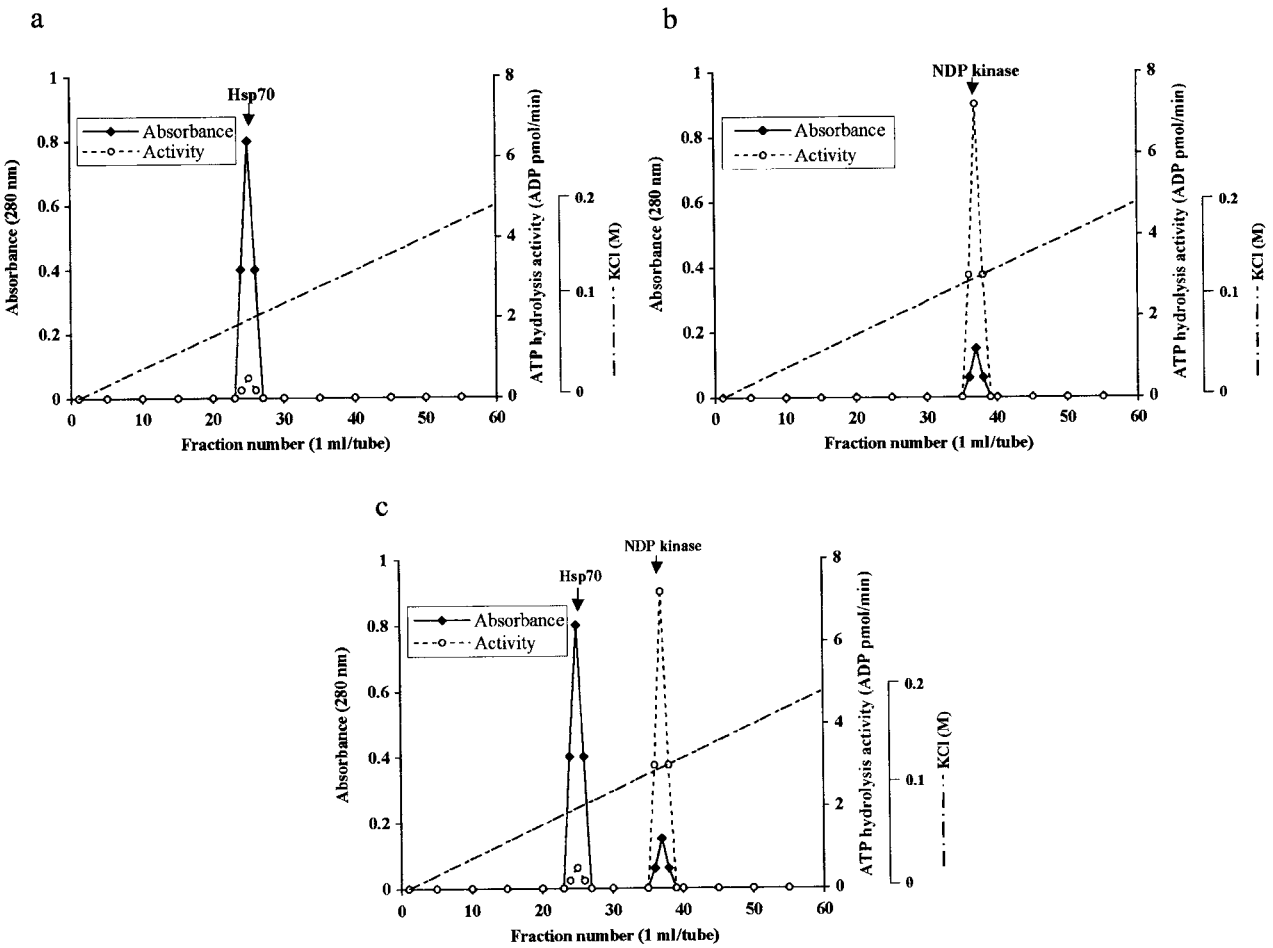

Figure 1 Separation of recombinant human WT Hsp70 from NDP kinase

Purified recombinant WT Hsp70 (50 μg; **a**), *E. coli* NDP kinase (10 μg; **b**) and a mixture of the recombinant WT Hsp70 (50 μg) and *E. coli* NDP kinase (10 μg) preincubated on ice for 30 min (**c**) were applied to a TSK gel DEAE 3SW column, then eluted with a narrow salt gradient of 0–0.2 M KCl in buffer A.

NDP kinase or the ineffective complete dissociation from Hsp70 of the pre-attached trace amount *E. coli* NDP kinase in the fraction of Hsp70 on the anion-exchange column. The latter, however, was mostly excluded by the significant difference between Hsp70 and NDP kinase in the thermal stability of ATP synthetic activity (Figure 2). The activities of bovine and *E. coli* NDP kinases purified on a DEAE 3SW anion-exchange column remained remarkably stable at 60 °C for 1 h, as previously reported [22], whereas the activities of purified bovine and recombinant Hsp70 on a DEAE column were nearly abolished at 60 °C for 30 min. This observation indicates that the ATP synthetic activity eluted in the fraction of Hsp70 does not originate from contaminated NDP kinase. These results were further confirmed by the near abolition of both the canonical ATPase and ATP synthetic activities by the mutation of the first and second metal-binding sites of Hsp70.

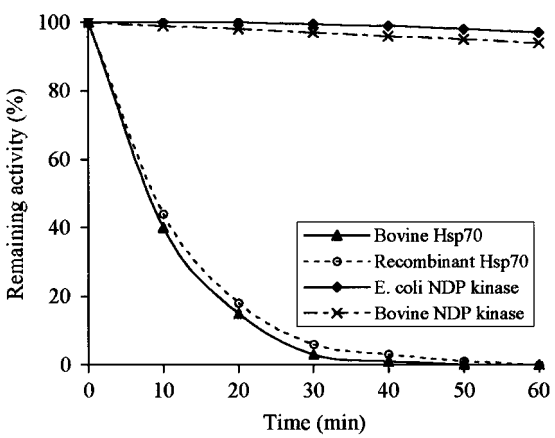

Figure 2 Thermal stability of ATP synthetic activities of NDP kinase and human recombinant Hsp70

Aliquots of bovine and *E. coli* NDP kinases and bovine and recombinant Hsp70s at 0.3 mg/ml were incubated at 60 °C in buffer A containing 1 mg/ml BSA. Residual ATP synthetic activities were measured as described in the Materials and methods section.

Mutations of the second metal-binding site of Hsp70 selectively diminished ADP binding and the activities of ATP hydrolysis and ATP synthesis

Hsp70 has two metal-binding sites: the first, a calcium-/magnesium-binding site, binds within the canonical catalytic pocket, bridging ADP and P_i, the products of ATP hydrolysis; the second is tightly co-ordinated on the protein surface by Glu-231, Asp-232 and the carbonyl of His-227 [14]. To identify the role of the second metal-binding site in the catalytic activity of Hsp70, we examined the effects of mutations of these amino acids on the ATP

Table 1 Enzymic activities of WT and mutant Hsp70s

ATP-hydrolysis and ATP-synthesis activities were analysed by measuring the conversion of [^{14}C]ATP to [^{14}C]ADP for hydrolysis, and conversion of [^{14}C]ADP to [^{14}C]ATP for synthesis in the presence of both nucleotides in the reaction mixture ($n = 6$).

	Enzymic activity (pmol of ADP or ATP/min per pmol of protein)	
Protein	ATP hydrolysis	ATP synthesis
WT	0.85 ± 0.18	0.96 ± 0.20
K71A	0.01 ± 0.005	0.01 ± 0.005
T204A	0.01 ± 0.006	0.02 ± 0.009
H227S	0.01 ± 0.07	0.02 ± 0.007
H227Q	0.28 ± 0.11	0.32 ± 0.15
E231S	0.50 ± 0.14	0.56 ± 0.16
D232A	0.01 ± 0.004	0.02 ± 0.006

hydrolysis and ATP synthesis in presence of ATP and ADP, and on the binding of ATP and ADP to the recombinant WT Hsp70, mutant Hsp70s and bovine Hsp70. Although the substitution of Lys-71 or Thr-204, located in the catalytic pocket and co-ordinated by a salt bridge with the P_i group, nearly abolished the ATPase activity, an observation consistent with previous reports [26,27], it also abolished the ATP-synthetic activity of Hsp70 (Table 1). It is particularly noteworthy that point mutations in the second metal-binding motif, H227S and D232A, eliminated ATP hydrolysis and ATP synthesis, and that these activities by the H227Q and E231S mutants were markedly weakened or nearly abolished. These observations suggest that the second metal-binding motif co-ordinates the catalytic reactions of ATP hydrolysis and ATP synthesis.

To clarify the role of the second metal-binding site in the ATP hydrolysis and ATP synthesis, we analysed the binding characteristics of WT Hsp70, mutant Hsp70s and bovine Hsp70 to ATP– and ADP–agarose columns, and compared the dissociation constants (K_d) for ATP and ADP in mutant Hsp70s versus WT and bovine Hsp70s. The Hsp70s were applied to ATP– and ADP–agarose and, after extensive washing with binding buffer B, the proteins bound to the agarose were eluted with 5 mM ATP or 5 mM ADP respectively. Recombinant WT Hsp70 and bovine Hsp70 avidly bound to ATP–agarose, and 95 % of applied proteins were eluted with ATP. However, the K71A mutant exhibited a prominent loss of binding to ATP–agarose. Mutation of Thr-204 in the canonical catalytic pocket of Hsp70, a critical element in ATP hydrolysis, did not modify binding to ATP–agarose (Figure 3a), an observation consistent with a previous report [28]. In contrast, almost no reduction of binding to ATP–agarose was observed for the H227Q and E231S mutants in the second metal-binding motif, and a slight reduction was detected for the H227S and D232A mutants. These results indicate that ATP binds preferentially to the first metal-binding site in the canonical catalytic pocket, and has almost no interaction with the second site.

The recombinant WT Hsp70, bovine Hsp70, and the K71A and T204A mutants bound avidly to ADP–agarose and eluted efficiently with ADP, whereas a prominent loss of binding was observed for the H227S, H227Q, E231S and D232A mutants in the second metal-binding motif (Figure 3b). The binding of mutant H227S to ADP–agarose was mostly abolished, and the other mutants in the second metal-binding motif, such as E231S and D232A, significantly reduced the binding but retained weak ADP-binding properties.

We measured the apparent K_d values of ^{14}C-labelled ATP and ADP in recombinant WT Hsp70, bovine Hsp70 and mutant Hsp70s by equilibrium gel penetration [24]. The apparent K_d values of the recombinant WT Hsp70 for ATP and ADP were 57 ± 3 and 66 ± 3 nM respectively, being in a similar range to those of bovine Hsp70 (Table 2). Among the Hsp70 mutants tested, the K_d value of K71A for [^{14}C]ATP had selectively and significantly increased, $\approx$33-fold, compared with that of WT Hsp70, whereas the K_d values for the other mutants were unchanged or only slightly increased. In contrast, the K_d values of all Hsp70 mutants for [^{14}C]ADP in the second metal-binding motif were prominently increased 5–15-fold compared with the value of WT Hsp70. No significant increases in the values for ADP of the mutants in the first metal-binding site, K71A and T204A, were observed. These data support the observations presented in Figure 3, which suggest that the second metal-binding motif predominantly influences ADP binding, as opposed to ATP binding in the case of the first magnesium-/calcium-binding site.

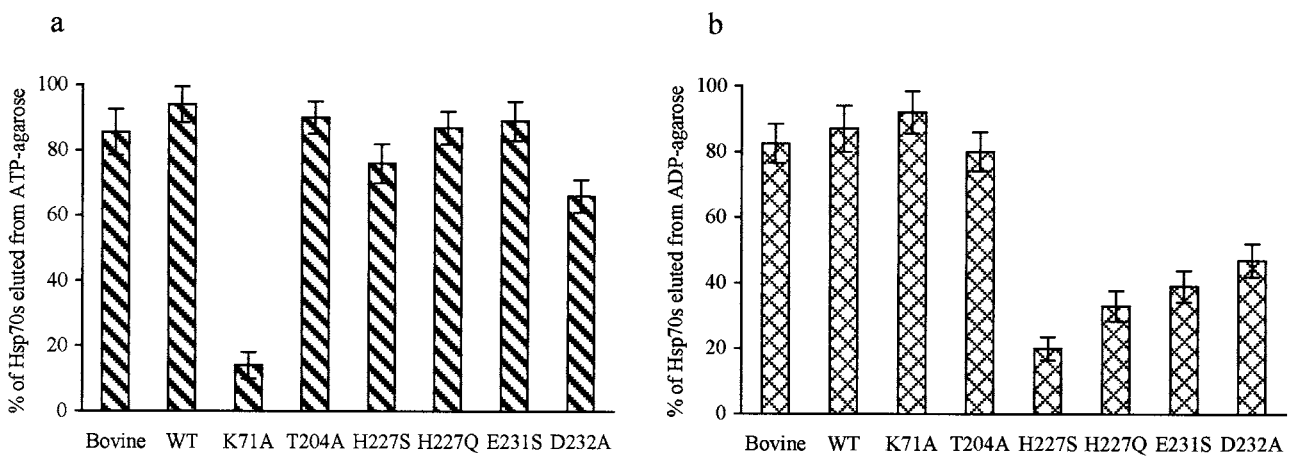

Figure 3 Binding properties of bovine Hsp70, WT Hsp70 and mutant Hsp70s to ATP–agarose (a) and ADP–agarose (b)

Bovine Hsp70, WT Hsp70 and mutant Hsp70s (30 μg) in binding buffer A were chromatographed on an ATP–agarose or ADP–agarose column (500 μl). The percentages of Hsp70s eluted from ATP–agarose by 5 mM ATP and from ADP–agarose by 5 mM ADP were calculated from the total amounts of Hsp70s recovered from the columns taken as 100 %. Each bar represents the mean $\pm$ S.D. from four measurements.

Table 2 Nucleotide-binding properties of bovine Hsp70, WT Hsp70 and mutant Hsp70s

Dissociation constants (K_d) were determined by the Scatchard plot method after equilibrium gel penetration assays with various protein concentrations (at least five different peptide concentrations) as described in the Materials and methods section.

	K_d (nM)	
Protein	ATP	ADP
Bovine	77 ± 6	85 ± 6
WT	57 ± 3	66 ± 3
K71A	1860 ± 60	73 ± 4
T204A	61 ± 4	71 ± 4
H227S	160 ± 10	1000 ± 30
H227Q	80 ± 5	810 ± 30
E231S	63 ± 4	360 ± 15
D232A	100 ± 7	370 ± 20

Mutations of the second metal-binding site of Hsp70 affect its oligomerization

The Hsp70 family of proteins are present in nucleotide-dependent and interconvertible oligomeric and monomeric forms [6,19]. Bovine Hsp70 exists as a mixture of monomers and dimers in the absence of nucleotide or in the presence of ADP, and as active monomers only in the presence of ATP [29]. Size-exclusion chromatographic studies of the recombinant WT Hsp70 in the presence of 150 μM [^{35}S]ATP[S] and [^{14}C]ADP showed a mixture of dimers with a molecular mass of 140 kDa and monomers with a mass of 70 kDa in an ≈3:2 molar ratio, calculated from the number of Hsp70s as monomers (Figure 4). [^{35}S]ATP[S] bound ≈50% of the monomers, and [^{14}C]ADP bound the remainder of the monomer and nearly all dimers. K71A mutant lost its ATP binding, though retained its native ADP-binding properties, and was present as a mixture of dimers and monomers in an ≈3:1 molar ratio. Nearly all K71A monomers and dimers bound [^{14}C]ADP. In contrast, H227S mutant lost its ADP binding, retained its native ATP-binding properties, and was present only as monomers. These data indicate that the first and second metal-binding sites of Hsp70 selectively affect ATP and ADP binding respectively, as well as its oligomerization and ADP–ATP exchange activity.

DISCUSSION

Hsp70 has a second metal-binding site besides the first magnesium-/calcium-binding site in the catalytic pocket in the ATPase domain, and the motif of the second site, His-Leu-Gly-Gly-Glu-Asp, which is in close proximity to the first (Figure 5), is conserved among Hsp70, Hsc70, Grp78 and adenylate cyclase [14,31]. The shortest distances between the second calcium ion and the ADP β-phosphate oxygen and the P_i in the catalytic site are 10.72 and 11.54 Å (where 1 Å = 0.1 nm) respectively [14]. For mutants in the second metal-binding site, such as H227S, H227Q, E231S and D232A, the affinity for ATP was not, or only mildly,

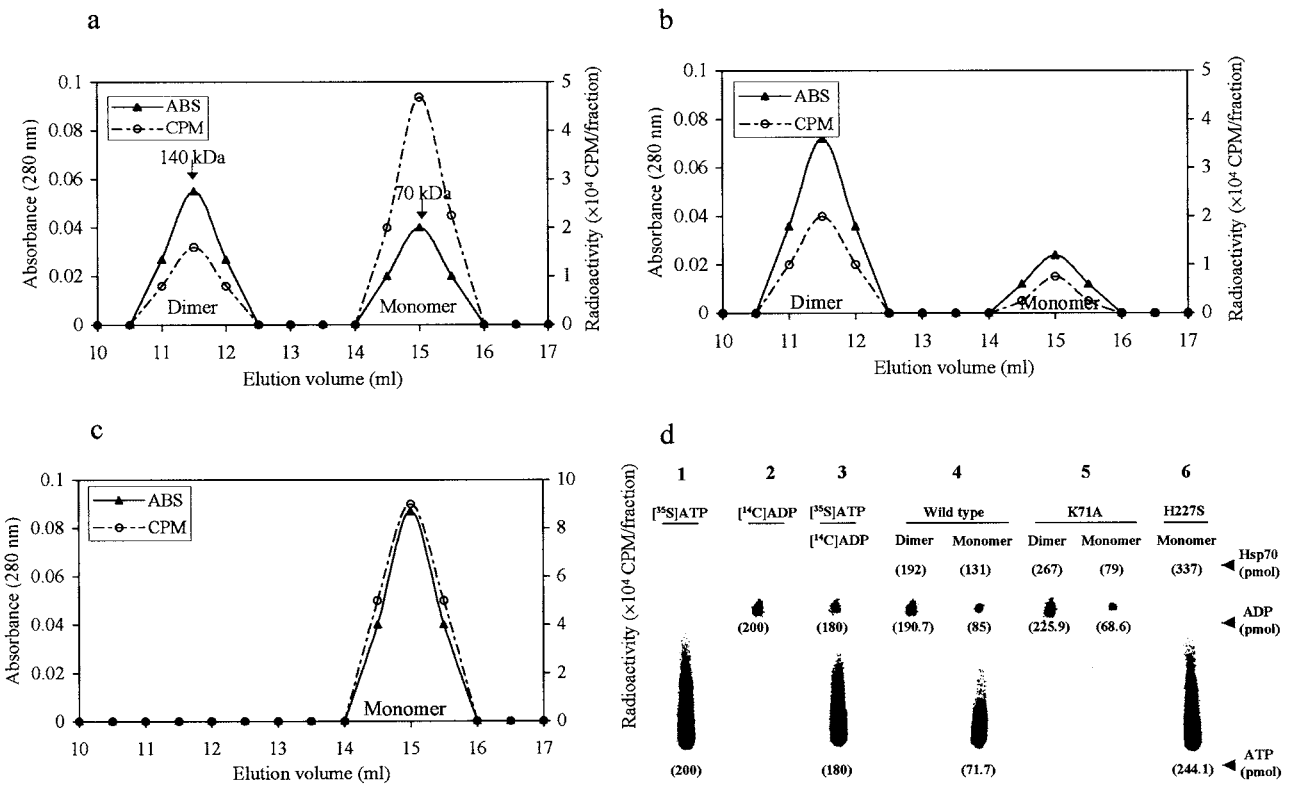

Figure 4 Size exclusion and nucleotide contents of Hsp70s as dimers and monomers

Size-exclusion chromatography on a TSK gel G3000SW column (7.5 mm × 600 mm) of WT Hsp70 (**a**), K71A mutant (**b**) and H227S mutant (**c**) (200 μg of each) after reaction with 150 μM [^{35}S]ATP[S] and [^{14}C]ADP for 30 min at 37 °C was performed as described in the Materials and methods section. Absorbance (ABS) at 280 nm (▲) and radioactivities (○) of the eluate were monitored. (**d**) The amounts (pmol) of bound radioactive ATP and/or ADP in dimers and monomers of the WT Hsp70 (lane 4), K71A mutant (lane 5) and H227S mutant (lane 6) were measured as described in the Materials and methods section. [^{35}S]ATP[S] (200 pmol; lane 1), [^{14}C]ADP (200 pmol; lane 2) and a mixture of [^{35}S]ATP[S] (180 pmol) and [^{14}C]ADP (180 pmol; lane 3) were used as standards.

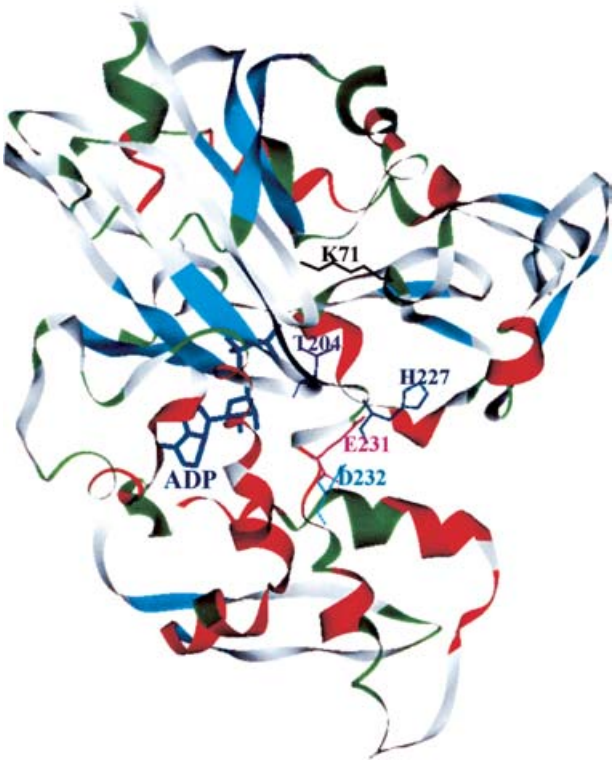

Figure 5 Interactive proximity between the first and second metal-binding sites of Hsp70

Crystal structure of the human Hsp70 ATPase domain with bound ATP (Protein Data Bank code for Hsp70 ATPase domain, 1hjo [30]). Product of ATP hydrolysis, ADP, in the canonical catalytic site and the second metal-binding site (His-227, Glu-231 and Asp-232) are in close proximity to the catalytic site and ADP.

affected while the affinity for ADP was selectively reduced, with significant weakening of the activities of ATP hydrolysis and ATP synthesis, suggesting that the second metal-binding site plays an essential role in ADP binding by affecting residues in the canonical catalytic pocket that interact with β-phosphate and ribose, or that the site itself is a binding site for ADP. However, the incomplete inhibition of the binding of these mutants, except for H227S, to ADP–agarose, suggest that a single amino acid mutation of the second metal-binding motif, except H227S, is insufficient to inhibit completely the ADP binding. On the basis of these observations, we propose that the second metal-binding site is an essential motif of Hsp70 affecting the hydrolysis of ATP, the intrinsic ATP synthetic activity and ADP binding, closely co-ordinating the canonical catalytic pocket and its conformation.

NDP kinase with ATPase and ATP synthesis activities (ADP kinase) is sometimes co-purified in the preparation of *E. coli* DnaK [15] and Hsp70s [16], with the characteristic initial burst of activities [16], which may hide the intrinsic activities of ATP synthesis, as well as ATP hydrolysis of Hsp70. However, obvious differences have been reported in the enzymic properties of Hsp70 versus NDP kinase. The k_{cat} values of ATP hydrolysis and ATP synthesis of NDP kinase [25] are significantly higher than those of Hsp70 [9,15]. The ADP kinase activity of NDP kinase is highly thermostable [22], whereas the activity of Hsp70 was thermolabile and was almost completely abolished at 60 °C for 30 min (Figure 2). Nucleotide specificity of NDP kinase is non-specific with respect to phosphate donor and acceptor nucleotides, whereas Hsp70 strictly prefers ATP as a phosphate donor, and ADP, UDP and CDP as phosphate acceptors, as we have previously reported [9]. These selective properties guarantee that the preparation of Hsp70 purified on a DEAE 3SW or Mono Q anion-exchange column is free from contaminated NDP kinase. In addition, the significant decrease in both the ATP hydrolysis and ATP synthesis activities of Hsp70 by mutations in the first and second metal-binding sites also support the intrinsic ATP synthetic activity of Hsp70. Furthermore, NDP kinase forms a tetramer with a molecular mass of 64 kDa [20,21], and its nucleotide-dependent oligomeric and monomeric interconversion, similar to that observed in Hsp70, has not been reported. Analyses of ATP and ADP binding in the conversion between the oligomer and monomer of WT Hsp70 and its mutants (Figure 4) reveal that the second metal-binding site plays a role in ADP binding and the interconversion. Taking these results together, we conclude that the ATP synthetic activity of Hsp70 was intrinsically enzymic, and not caused by contaminated NDP kinase from *E. coli*.

The turnover number of ATPase activity for the purified WT Hsp70 (0.85 min^{-1}), determined by measuring the conversion of [^{14}C]ATP to [^{14}C]ADP (Table 1), was relatively high compared with previously published values [27,32], which were determined by measuring the release of inorganic phosphate from ATP. Since Hsp70 exerts both ATP hydrolysis and ATP synthesis activities by γ-phosphate transfer reaction, in the presence of ATP and ADP in the reaction mixture [9], the enzymic activities and the kinetic constants for Hsp70 ATPase determined by measurement of inorganic phosphate may not be accurate. Although K_m values of ATP for the ATP hydrolysis have been reported in the range of 0.7–1.4 μM in the absence of ADP [27,33], we analysed ATP hydrolysis activities with 5 mM ATP and 0.5 mM ADP instead of ATP in a micromolar range, since the K_m value of ATP for the hydrolysis of ATP in the presence of 0.5 mM ADP, and the K_m value of ADP for the ATP synthesis of Hsp70 are 4.3 and 0.31 mM respectively [9].

Further studies of the second metal-binding site in the molecular latch mechanism of Hsp70 in peptide binding and release, which may be coupled to the γ-phosphate-transfer reaction, and in the nucleotide-dependent oligomeric and monomeric interconversion are currently in progress.

This work was supported, in part, by a Grant-in-Aid (13470026) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Received 4 November 2003/9 December 2003; accepted 10 December 2003
Published as BJ Immediate Publication 10 December 2003, DOI 10.1042/BJ20031680