Lysine 71 of the Chaperone Protein Hsc70 Is Essential for ATP Hydrolysis*

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It has been proposed that lysine 71 of the bovine 70-kDa heat shock cognate protein might participate in catalysis of ATP hydrolysis by stabilizing an H2O molecule or an OH− ion for nucleophilic attack on the γ-phosphate of the nucleotide (Flaherty, K. M., Wilbanks, S. M., DeLuca-Flaherty, C., and McKay, D. B. (1994) J. Biol. Chem. 269, 12899–12907; Wilbanks, S. M., DeLuca-Flaherty, C., and McKay, D. B. (1994) J. Biol. Chem. 269, 12893–12898). To test this hypothesis, lysine 71 of the ATPase fragment 70-kDa heat shock cognate protein has been mutated to glutamic acid, methionine, and alanine, and the kinetic and structural properties of the mutant proteins have been determined. All three mutant proteins are devoid of measurable ATP hydrolysis activity. Crystal structures of the mutant proteins have been determined to a resolution of 1.7 Å; all three have ATP in the nucleotide binding site. These data identify lysine 71 as a residue that is essential for chemical hydrolysis of ATP.

The bovine 70-kDa heat shock protein (Hsc70)1 is thought to "chaperone" protein folding by, at a minimum, binding to nascent or misfolded segments of polypeptides, thereby inhibiting their aggregation (for reviews, see Refs. 3 and 4). ATP-induced release of peptides then allows the opportunity to fold or refold correctly. Hsc70 has an ATPase activity (5), a somewhat diverse peptide binding activity (6–8), and a mechanism for coupling the two. The nucleotide regulates the peptide binding activity; with ADP bound, Hsc70 binds peptides or denatured proteins tightly, but when ATP binds, peptides are released (9).

Within the 650-residue sequence of Hsc70, the ATPase activity is localized in the amino-terminal 385 residues (10), and the function of the carboxyl-terminal 100 residues remains unclear. The ATPase domain can be isolated as an independent, fully functional entity either as a proteolytic fragment from full-length protein (10, 12) or as a recombinant expression product (2, 13). This allows the option of studying the ATPase mechanism on this domain alone, thereby circumventing the complexities encountered in the full-length protein where the ATPase activity is coupled to peptide binding and release.

We have tested this hypothesis by mutating Lys-71 to alanine (K71A), methionine (K71M), and glutamic acid (K71E) in the Hsc70 ATPase fragment. We report the structural and kinetic properties of the three mutant proteins below.

MATERIALS AND METHODS

Construction of Mutant Plasmids—A cDNA clone coding for the 44-kDa ATPase domain of bovine Hsc70 has been described (2). The mutagenic oligonucleotides were 5′-TTTGATGCCACTTTGAGGA for K71E, 5′-TTTGATGCCACGACTTTGAGGA for K71A, and 5′-TTTGATGCGCCGACTTTGAGGA for K71M, where the mismatched bases are underlined. Mutagenesis was done with the reagents and protocols of the Muta-Gen kit from Bio-Rad. The presence of the correct mutation was confirmed in all constructs by dideoxy DNA sequencing (16).

Expression and Purification of Mutant Proteins—Mutant proteins were expressed in Escherichia coli as described previously (2, 13). The proteins were purified over hydroxylapatite and ATP-agarose (Sigma) gravity-flow columns and then over Mono P (chromatofocusing) and Superdex-75 (gel filtration) fast protein liquid chromatography columns (Pharmacia Biotech Inc.) as described in detail elsewhere (13). Yields of purified proteins ranged from 7 to 21 mg/liter of E. coli cell culture.

ATPase Assays—ATPase reactions were carried out at 37 °C in Buffer B (40 mM HEPEs, pH 7.0, 75 mM KCl, 45 mM CH3COO) Mg with 400 nM enzyme, 0.025 μCi/l[γ-32P]ATP, and varying amounts of cold ATP in a total volume of 20 μl. Aliquots (0.6 μl) of the reactions were spotted on polyethyleneimine-cellulose thin-layer chromatography plates. ADP was separated from ATP by development in 1.0 M LiCl, 0.5 mM formic acid (17). The plates were dried, and radioactivity in resolved spots of ADP and ATP was quantitated with a PhosphorImager (Molecular Dynamics, Inc.). The percentage of the total counts/lane
found in the ADP spot was used to calculate the amount of cold ATP hydrolyzed at each time point. The hot ATP contained a small amount of hot ADP (typically 4–7%) that did not increase with time in control experiments that had no enzyme.

Crystallization and Data Collection—Mutant proteins were crystallized in the presence of 1 mM MgATP as described (12) and then adapted to a cryosolution (20% ethylene glycol, 20% polyethylene glycol, 1.0 M KCl, 50 mM HEPES, pH 7.0, 1 mM MgATP) at 4 °C with successive 30-min soaks in incremental steps of increasing ethylene glycol concentration (5, 10, 15, and 20%) (15). Crystals were then flash-frozen to 100 K in a stream of cold N2 gas. Data were collected with a Rigaku R-axis image plate system using copper Kα radiation. Data collection and processing were effected as described (15). Data statistics are summarized in Table I.

Model Refinement—Standard crystallographic computations were performed using PROTEIN (18). Model building was initiated with the program CHAIN (Rice version ESVAIN 2.2) (19) on an Evans and Sutherland ESV. XPLOR (20) was used for model refinement by positional restrained least squares minimization and simulated annealing. K+ ions were identified from anomalous difference Fourier maps and surrounding ligands as described (15). Refinement statistics are summarized in Table I. Coordinates for the mutant structures have been deposited in the Protein Data Bank, Brookhaven National Laboratory.

RESULTS

Mutagenesis of Lys-71 of the recombinant 44-kDa ATPase fragment of bovine Hsc70 to glutamic acid, alanine, and methionine was carried out, and the enzymatic properties and crystallographic structures of the mutant proteins have been determined.

ATPase Activity—All three mutants were assayed in the presence of 1 μM ATP, 0.4 μM enzyme for a total time of 320 min. Under these conditions, even a single turnover would have been detected, yet no ATP was produced by any of the three mutant proteins. In order to test for an altered Km of the mutants, ATPase assays were performed in the presence of 50 or 100 μM ATP, also for a total time of 320 min. Again, no enzyme activity was detected. These experiments establish an upper boundary for the ATPase activities of the mutant proteins of <0.003 min⁻¹, at least 3 orders of magnitude slower than that of wild-type protein. The fact that we have observed no activity whatsoever with these proteins combined with the fact that we have previously made reliable measurements of ATPase activities with other mutant proteins whose turnover rates were 100-fold slower than wild-type protein argues strongly that all three Lys-71 mutants are devoid of ATPase activity.

Crystallographic Structures—The starting model for refinement of K71M and K71A was a high resolution structure of the wild-type ATPase fragment refined to 1.7-Å resolution with data collected from a frozen crystal (15). The active site nucleotide was truncated to AMP, and residue 71 was replaced with alanine. Inorganic phosphate, potassium and magnesium ions, and all solvent molecules within a 10-Å radius of the inorganic phosphate site were removed from the initial model. A rigid body refinement was performed, followed by three alternating rounds of positional and B-factor refinements. For K71E, since it was apparent in the initial maps that the structure was similar to the other two mutants, the K71A structure was used as the starting model for rigid body refinement. The crystallographic R-factors after the first round of refinement were 0.23 for K71E, 0.23 for K71A, and 0.27 for K71M. Free R-factor values were 0.27, 0.26, and 0.31, respectively, at this point. Electron density corresponding to the mutated side chains was clearly visible at residue 71 in the Fo − Fc and 2Fo − Fc maps for K71E and K71M; the respective residues were therefore built into the models. In all three mutants, electron density corresponding to an ATP molecule, a magnesium ion, and several H2O molecules was observed and modeled. Anomalous difference Fourier maps revealed three strong peaks that were interpreted as K+ ions in each structure (Fig. 1); two of the three K+ ions superimpose on the two K+ ions found earlier in the wild-type structure. Refinement of the structures continued with further cycles of model building and positional refinement until the R-factors showed no further improvement and the models had no significant deviations from acceptable structural parameters. Refinement statistics are summarized in Table I.

The overall tertiary structure of the mutant proteins shows little difference from that of the wild-type ATPase fragment; root mean square differences of backbone atomic positions from those of the wild-type protein after superposition were 0.15, 0.16, and 0.32 Å for K71E, K71A, and K71M, respectively. Compared with the wild-type structure with ADP and P, additional H2O molecules are present in the nucleotide binding site, including one near the position of the &epsilon;-amino group of the lysine side chain of the wild-type protein. We presume the H2O molecules compensate to fill the void left by the smaller size of the side chains introduced at residue 71 by the mutagenesis.

All three mutant proteins have an ATP molecule in the nucleotide binding site. This contrasts with the wild-type ATPase fragment that has ADP and P, the products of hydrolysis, bound to it (1, 15). In K71E and K71M, ATP forms a β,γ-bidentate complex with the Mg2+ ion. The axial ligand...
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**Table I**

| Data collection | K71E | K71A | K71M |
|-----------------|------|------|------|
| Unit Cell      | 143.5 | 143.7 | 143.9 |
| a (Å)          | 63.8  | 64.2  | 64.6  |
| b (Å)          | 46.1  | 46.2  | 46.2  |
| c (Å)          | 1.70  | 1.70  | 1.70  |
| Resolution (Å) | 134.447 | 101.059 | 116.095 |
| Measurements   | 42,307 | 40,944 | 38,288 |
| Completeness   | 0.894 | 0.866 | 0.810 |
| R-factor^a     | 0.041 | 0.043 | 0.033 |

^a R_w= \frac{\sum |I_{obs} - |I_{calc}| |}{\sum |I_{obs}| |}

^b R-factor = \frac{\sum |F_o - F_c|}{\sum F_o} where F_o = observed structure factor amplitude = |I|^{1/2}; F_c = structure factor amplitude calculated from model.

c rms, root mean square.

Discussion

All three of the proteins in which the lysine at position 71 has been mutated to a different residue are devoid of measurable ATPase activity. This contrasts with the results of earlier work where mutagenesis of any of the four acidic residues in the active site region (Asp-10, Glu-175, Asp-199, and Asp-206) resulted in a 10–100-fold lower ATPase turnover rate, but in all cases, measurable activity was still observed (2). Furthermore, ATP is bound in crystals of all three mutant proteins at neutral pH in the presence of 1 M KCl. Although we previously observed ATP bound on other mutants (specifically D10N, D10S, D199N, and D199S) in K71A, at pH 9 in the presence of 1 M NaCl (1), adapting crystals of at least one of these mutants (D199N) to 1 M KCl at neutral pH (prompted by the observation that the ATPase activity depends on the type of monovalent ion present (22, 23)) results in hydrolysis to ADP + P_i. Taken together, the results reported here demonstrate that Lys-71 is essential for ATP hydrolysis. It is notable that all 70-kDa heat shock proteins for which the sequences are known (GenBank™, release 92.0) have a lysine at this position. We presume that the essential function of this lysine is conserved throughout the heat shock protein family.

The two conformations of ATP observed in structures of the mutant proteins are consistent with and may represent two steps along the pathway previously proposed for nucleotide hydrolysis (1). The manner in which ATP binds in K71A, with an interstitial H_2O molecule between the γ-phosphate and the Mg^{2+} ion, is similar to that observed with AMPPNP bound to wild-type ATPase fragment and to that of ATP seen in particular mutant proteins (D10N, D10S, D199N, and D199S) in the presence of 1 M NaCl (1). In some of the structures reported previously, an H_2O molecule is also apparent in an interstitial position between the Mg^{2+} and the ATP γ-phosphate. In others, the electron density in that area was too noisy to unambiguously resolve the Mg^{2+}-ligand details.

Three K^+ ions are also seen in the nucleotide binding cleft of each of the mutant structures. Two of these (labeled #1 and #2 in Fig. 2) correspond to the two K^+ ions found in the wild-type structure with ADP and P_i (15), and one K^+ ion is at a distance ranging from 4.0 to 4.2 Å from K^+ #2 in the three mutant structures. It is coordinated by the carboxyl of Asp-206, the hydroxyl of Thr-204, the phenolic hydroxyl of Tyr-149, and at least one H_2O molecule in all three mutants. In the wild-type protein, this position is occupied by an H_2O molecule.

There are a few additional, subtle differences between the structures of the mutants. In K71M, the methionine side chain packs against the polypeptide backbone of the β strand that includes residues 10–13, and the side chain of T13 is rotated relative to its orientation in wild-type and other mutant proteins. These minor differences between K71M and the other two mutants do not give rise to any major differences in the interactions of the protein with the nucleotide or metal ions.

References

2 S. M. Wilbanks and D. B. McKay, unpublished data.
FIG. 2. Structures of wild-type and mutant proteins in the triphosphate binding region of the ATPase fragment. Phosphorus atoms are black, carbon atoms are white, and others are gray. Metal ions are labeled. Subsets of the interactions with Mg$^{2+}$ and K$^+$ are shown by dotted lines. A, K71E; B, K71M; C, K71A; D, active site region in the wild-type protein with ADP and P, bound.
H₂O molecule after hydrolysis but before ADP or Pᵢ release. We do not yet have information on whether a third K⁺ binds at this site in wild-type protein in the presence of nonhydrolyzable ATP analogs, so we cannot rule out this possibility. However, an alternative explanation for its presence is that it may provide electrostatic compensation for the removal of the buried positive charge of the lysine side chain. We have observed a precedent that can be rationalized as electrostatic compensation for removal of a buried charge by mutagenesis of the ATPase fragment. In the D199N mutant protein, which eliminates a carboxyl group (which would presumably be ionized at neutral pH), K⁺ #2 is absent.²

It has been demonstrated that binding of ATP induces a conformational change in Hsc70 that switches the protein from a high peptide affinity state to a low peptide affinity state (5, 22). It remains to be seen whether full-length Hsc70 protein with mutations at position 71 can still undergo the ATP-induced conformational change. If so, one would expect that ATP would permanently “lock” such an Hsc70 mutant protein into its peptide release state in the presence of excess ATP, both in vitro and in vivo. If not, one would expect Hsc70 with mutations at position 71 to be in its peptide binding state regardless of the ATP/ADP ratio. In either case, such mutants could prove valuable in characterizing the in vivo activities of representative 70-kDa heat shock proteins. Experiments are currently under way to determine how the ATP hydrolysis and/or knockout mutations at position 71 affect the conformational change that couples the ATPase cycle to peptide binding and release in Hsc70.

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