ATPase Activity and Molecular Chaperone Function of the Stress70 Proteins

Jan A. Miernyk* and G. Thomas Hayman 2

Phytoproduts Research Unit, United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, 1815 North University Street, Peoria, Illinois 61604

The codons for the amino acid residues making up the proposed ATP-binding sites of the maize (Zea mays L.) endoplasmic reticulum and tomato (Lycopersicon esculentum) cytoplasmic Stress70 proteins were deleted from their respective cDNAs. The deletions had little effect on the predicted secondary structure characteristics of the encoded proteins. Both wild-type and mutant proteins were expressed in Escherichia coli and purified to electrophoretic homogeneity. The mutant recombinant proteins did not bind to immobilized ATP columns, had no detectable ATPase activity, and were unable to function in vitro as molecular chaperones. Additionally, the inability to bind ATP was associated with changes in the oligomerization state of the Stress70 proteins.

One important class of molecular chaperone proteins is the 70-kD family of heat-shock or stress-related proteins (Craig et al., 1994). The structure of the Stress70 proteins can be divided into three regions: a C-terminal peptide recognition and binding domain, a short linker sequence, and an N-terminal ATPase domain (McKay, 1993; Wang et al., 1993). A stable 44-kD N-terminal ATPase can be prepared from native Stress70 by partial proteolysis (Chappell et al., 1987). The 44-kD fragment has been crystallized and a low resolution three-dimensional structure solved (Flaherty et al., 1990). The roles of ATP binding and hydrolysis in chaperone function remain somewhat controversial, and despite the advances in understanding the structure of the Stress70 complex requires K+ binding, not ATP hydrolysis (Palleros et al., 1993). Models of chaperone function have been presented that accommodate each school of thought (Hubbard and Sander, 1991).

Although the three-dimensional structures of plant Stress70c proteins have not yet been solved, there is a high degree of primary sequence homology with mammalian and microbial Stress70c proteins (Gupta and Golding, 1993). Based on prior results with DnaK (Cegielska and Georgopoulos, 1989), it was proposed that the sequence $A_{135}E_{Ax}LG_{x}T_{xx}N_{AVVVTV}$ makes up at least part of the ATP-binding site of tomato (Lycopersicon esculentum) Stress70c (Lin et al., 1991). Homologous sequences are present in other plant Stress70 proteins (Fontes et al., 1991). Using recombinant DNA methods, we have deleted the proposed ATP-binding sites of maize (Zea mays) Stress70er and tomato Stress70c, expressed the mutant proteins in E. coli, and then evaluated their ability to bind ATP and function as molecular chaperones in vitro.

MATERIALS AND METHODS

Reagents

All buffers were from Research Organics, Inc. (Cleveland, OH). The purified Escherichia coli stress proteins DnaK, DnaJ, and GrpE were from Epicentre Technologies (Madison, WI). DNA-modifying enzymes were from New England BioLabs (Beverly, MA) and were used according to the manufacturer’s recommendations. Malachite green carbinol base was from Aldrich. Unless otherwise indicated, other reagents were from Sigma and were of the highest purity available.

Abbreviations: ds, double stranded; MBP, maltose-binding protein, product of the mne gene; Stress70, any member of the M, 70,000 family of stress-related proteins; Stress70c, cytoplasmic Stress70; Stress70er, ER-resident Stress70.
**Plasmid Construction**

The coding region for tomato Stress70c was removed from pLeHsc70-2 by digestion with NarI and EcoRI. The resulting 2033-bp fragment was directionally cloned into XmnI × EcoRI-digested pMal-c2. This plasmid was designated pJAM103 and drives bacterial expression of the chimeric MBP-Stress70c protein. Then, pJAM103 was digested with SaeI, and the resulting fragment was inserted into the unique SaeI site of pUC19. To delete the region of the DNA sequence encoding the proposed ATP-binding site of the Stress70c protein, the pUC19 construct was digested with BglII and BamHI. The oligonucleotides 5'-GATCTCATCTATGGTGCCTGCT-3' and 5'-AGTAGATACCACGAGC-3' were annealed, the dsDNA was mixed with BglII × BamHI-digested plasmid, and the mixture was ligated together. Finally, the modified SaeI fragment was removed from the pUC19 intermediate construct and re-inserted into pJAM103. The amino acid sequence 130-MKEIAEAFLGTlVKNA (numbered from the N terminus; the proposed ATP-binding site removal is underlined) was deleted from the poly peptide-reading frame. This plasmid was designated pJAM105 and encodes a chimeric protein consisting of the MBP, a factor Xa cleavage site, and the mature Stress70c-coding region minus the proposed ATP-binding site (Stress70cΔATP).

To delete the sequence encoding the proposed ATP-binding site of Stress70c, p9/25PCR1 (Fontes et al., 1991) was first digested with SaeI and MstI. This resulted in removal of a 111-bp fragment. The SaeI-MstI fragment was replaced with synthetic dsDNA, which had a SaeI cohesive end and an MstI blunt end. This fragment consisted of two annealed oligonucleotides: a 30-mer, 5'-TGAGGAGATCATGCCATATTCTTGCCGC-3'; and a 27-mer, 5'- GCCGCAAGAATCATGGCAGTACTCCCTC-3'. The final construction had 84 bp deleted from the Stress70c-coding region. Thus, the amino acid sequence K130MKEIAEAFLGTlVKNA (numbered from M1 of the signal sequence; the proposed ATP-binding site is underlined) was deleted from the polypeptide-reading frame. This plasmid was designated pJAM106.

A plasmid was constructed for bacterial expression of mature maize Stress70er fused to the MBP. A 2.2-kb BamHI-HindIII fragment including all but six codons of the complete coding region was removed from p9/25PCR1 and inserted into pMAL-c2. This plasmid was designated pJAM159. To bring the MBP and Stress70er into frame and to position the factor Xa cleavage site immediately upstream from the mature Stress70er-coding region, pJAM159 was digested with SaeI and AccI. The SaeI-AccI fragment was replaced with synthetic dsDNA, which had SaeI and AccI cohesive termini. This fragment consisted of two annealed oligonucleotides: an 85-mer, 5'-CGATCGAGGGAAGGAAAGGAGACCAAGAACCTCGGGACCCTGATCGATCTTGGTAACCACCTACTCTCTFTGTTGCTGTTG-3'; and a 91-mer, 5'-AGACACCGACACAGGAGTTAGTGGTACAAAAAGTCGATACCGATACCGTGCCCAGCTTCTTCTTGCTCCCCTATCCGACAGCT-3', that abutted the first codon (Val) of processed Stress70er to the reselected 3' end of malE and restored the factor Xa cleavage site immediately upstream of this codon. This plasmid was designated pJAM161.

pJAM161 was digested with SaeI and HindIII, which resulted in removal of a fragment of approximately 1.7 kb. Similarly, pJAM106 was digested with SaeI and HindIII. The Sae1-HindIII fragment from pJAM106, which has the proposed ATP-binding site deletion, replaced the corresponding fragment from pJAM161. This plasmid was designated pJAM163. The final construction encodes a chimeric protein consisting of the MBP, a factor Xa cleavage site, and the mature Stress70er-coding region minus the proposed ATP-binding site (Stress70erΔATP).
trated and then dialyzed overnight against 400 volumes of Fast Protein Liquid Chromatography column buffer (20 mM Tes-NaOH, pH 7.0, containing 0.1 mM EDTA). Clarified dialysates were loaded onto 1-mL Pharmacia Mono Q anion-exchange columns that were washed with column buffer and then eluted at 0.5 mL min⁻¹ with a linear gradient of 0 to 0.5 mM KCl in equilibration buffer.

The Stress70-containing fractions were concentrated to a volume of less than 2 mL by ultrafiltration and then stored at −80°C until used.

Immobilized ATP columns (Welch and Feramisco, 1985; Miernyk et al., 1992a) were equilibrated with 20 mM Tes-NaOH, pH 7.5, containing 20 mM KCl and 3 mM MgCl₂. After the samples were loaded, the columns were washed with equilibration buffer and then successively eluted with equilibration buffer containing 0.5 mM KCl, 1 mM GTP, and 3 mM ATP. Samples of each fraction were taken for SDS-PAGE, western blotting, and quantitation by laser densitometry (Miernyk et al., 1992a).

Other Methods

ATPase activity was measured using the malachite green procedure (Baykow et al., 1988; Geladopoulos et al., 1991). Sedimentation analyses and measurements of molecular chaperone activity were conducted exactly as previously described (Miernyk et al., 1992b). Protein levels were quantitated by the method of Bradford (1976), using fraction V BSA as the standard. Protein secondary structure predictions were analyzed according to the method of Garnier et al. (1978), using the PC/GENE software from IntelliGenetics, Inc. (Mountain View, CA).

RESULTS AND DISCUSSION

The method described for purification of recombinant Stress70 resulted in yields of approximately 250 µg of electrophoretically homogeneous protein per 100 mL of bacterial culture. Although substantially more fusion protein was synthesized in the cultures, most of the material in a typical preparation was refractory to factor Xa digestion. Changing digestion time, temperature, or physical conditions (pH, ions, etc.) resulted in little improvement.

The experiments described herein were originally undertaken in response to a report that deletion of a large internal fragment from mammalian Stress70 apparently did not interfere with the ability of the protein to protect cells from what would normally be a lethal heat shock (Li et al., 1992). This deletion included all of the proposed ATP-binding site of this protein. Removal of this much of the protein-coding sequence results in a major alteration in the predicted 2° structure. Despite the magnitude of structural disruption, some molecular chaperone functions for the resulting protein might still be possible (Hubbard and Sander, 1991). We wished to directly test the involvement of ATP binding and hydrolysis in chaperone activity using a protein with relatively minor changes in 2° structure. A predicted 2° structure comparison of the wild-type and ATP-binding site mutant proteins used in these studies revealed no significant differences (Table I).

<table>
<thead>
<tr>
<th>Chaperone Protein</th>
<th>Helical</th>
<th>Extended</th>
<th>Turn</th>
<th>Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato Stress70c</td>
<td>45.1</td>
<td>35.8</td>
<td>7.4</td>
<td>11.4</td>
</tr>
<tr>
<td>Tomato Stress70cΔATP</td>
<td>45.3</td>
<td>35.7</td>
<td>7.5</td>
<td>11.4</td>
</tr>
<tr>
<td>Maize Stress70er</td>
<td>51.3</td>
<td>35.2</td>
<td>5.8</td>
<td>7.5</td>
</tr>
<tr>
<td>Maize Stress70erΔATP</td>
<td>51.1</td>
<td>35.3</td>
<td>5.8</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Characteristic of the Stress70 molecular chaperone proteins is a low-level basal ATPase activity (Hendrick and Hartl, 1993; Craig et al., 1994; Georgopoulos and Welch, 1994; Szabo et al., 1994). Both the basal catalytic activity of recombinant Stress70c and the extent of stimulation by the E. coli DnaJ and GrpE co-chaperone proteins (Fig. 1) were comparable to previous observations with the native proteins (Zhou and Miernyk, 1996). The Stress70ΔATP protein had virtually zero basal ATPase activity, and there was no detectable stimulation by the co-chaperone proteins (Fig. 1). Essentially identical results were observed with comparisons of Stress70er and Stress70erΔATP (data not presented).

ATPase activity can be separated into three major component reactions: substrate binding, catalysis, and product release. The ability of Stress70 proteins to bind specifically to immobilized ATP was recognized early in their study, and this property is routinely exploited during protein purification (Welch and Feramisco, 1985). Recombinant wild-type Stress70er bound quantitatively to an immobilized ATP column and could be eluted specifically with 3 mM ATP (Fig. 2). In contrast, Stress70erΔATP failed to bind to the immobilized ATP column (Fig. 2). A variety of changes in pH, ionic strength, and cation concentrations had no effect on the inability of Stress70erΔATP to bind to the ATP column. Results essentially identical with those obtained with Stress70er and Stress70erΔATP were seen with Stress70c and Stress70cΔATP (data not presented).

Based on deduced amino acid sequence analyses (Gaut and Hendershot, 1993; Wilbanks et al., 1994; Hendershot et al., 1995) and crystallographic studies (Flaherty et al., 1990), a structural model of the ATP-binding site of Stress70 has been proposed (McKay, 1993). This model invokes complex multiple interactions between amino acid side chains and various portions of the ATP molecule but is amenable to testing both in vivo (Hendershot et al., 1995) and in vitro (Gaut and Hendershot, 1993; Wilbanks et al., 1994). In particular, the hydrolytic attack of the phosphodiester bonds has been analyzed (McKay, 1993). A number of mutant proteins have been prepared; however, most have dealt with effects on catalysis rather than on nucleotide binding. The prediction of residues involved in ATP binding was based on in vivo analysis of E. coli partial loss of function mutants (Cegielska and Georgopoulos, 1989; Wild et al., 1992). Mutant proteins such as we have prepared for
tomato Stress70c and maize Stress70er have not previously been characterized.

It has been suggested that Stress70 might have two ATP-binding sites (Schmid and Rothman, 1985; McKay, 1993). Our results with the mutant Stress70 proteins support the prediction of residues involved in ATP binding (Cegielska and Georgopoulos, 1989; Lin et al., 1991; Wild et al., 1992). They do not provide any support for the proposal of more than one class of nucleotide-binding site. In our in vitro analysis system, the mutant proteins failed completely to bind to an ATP column (Fig. 2). It remains conceivable, however, that a putative second class of ATP-binding site would require prior occupation of the other site.

It is clear that the Stress70 proteins can exist in a variety of oligomeric structures in vivo (Hendrick and Hartl, 1993; Craig et al., 1994; Georgopoulos and Welch, 1994; Szabo et al., 1994). It has been proposed that the active form of Stress70 is the monomer (McKay, 1993), but this remains a point of controversy. Guy and associates have studied the oligomeric structure of spinach Stress70c (Anderson et al., 1994). They observed three different forms of the chaperone: monomers, dimers, and a high-molecular-weight oligomeric fraction. Addition of ATP converted a portion of the high-molecular-weight fraction to monomers and dimers and separately converted the dimer fraction to monomers and oligomers (Anderson et al., 1994). Thus, ATP binding has the potential to control biological activity.

We also observed that maize Stress70er (Fig. 3A) and tomato Stress70c (data not presented) can exist as monomers, dimers, and oligomers. It is interesting that, when the Stress70ΔATP proteins were analyzed by sedimentation, none of the dimer form was observed (Fig. 3B). Furthermore, there was an increase in the relative amount of oligomers and possibly an increase in the size of some components of the oligomer fraction. These results suggest that ATP binding or a structural change resulting from ATP binding is essential for Stress70 dimer formation. At this time, the possibility that the deletions somehow subtly change global protein structure such that oligomerization is promoted cannot be ruled out.

The in vitro molecular chaperone system used in these studies measures the membrane translocation and cotranslational modification of a model secretory precursor (Miernyk et al., 1992b). We previously established that Stress70c is essential for this membrane translocation. The relative chaperone activities of native maize Stress70c and the E. coli DnaK protein are similar to those we previously

**Figure 1.** Comparison of the ATPase activities of recombinant wild-type tomato Stress70c and Stress70cΔATP. Lanes 1, Stress70; lanes 2, Stress70 plus E. coli DnaJ; lanes 3, Stress70 plus DnaJ plus GrpE.

**Figure 2.** Immobilized ATP affinity chromatography of wild-type maize Stress70er (●) and Stress70erΔATP (△). Fractions were analyzed by SDS-PAGE, western blotting, and laser densitometry (Miernyk et al., 1992a).

**Figure 3.** Sedimentation analyses of the maize Stress70er proteins. A, Wild-type Stress70er; B, Stress70erΔATP. Fractions were analyzed by SDS-PAGE, western blotting, and laser densitometry. Suc concentrations were determined by refractometry. The positions of standard proteins are indicated by carats (‡). BSA, 68 kD; alcohol dehydrogenase (ADH), 150 kD; apoferritin (AFT), 443 kD; thyroglobulin (TGB), 669 kD.
observed (Fig. 4; Miernyk et al., 1992b). Molecular chaperone activity of the recombinant tomato Stress70c is indistinguishable from that of the native maize protein. In the present experiments we observed that the mutant Stress70cΔATP protein, which has no detectable ATPase activity, failed to promote membrane translocation of the precursor protein (Fig. 4). This result corroborates our earlier observations that reducing or eliminating ATPase activity through the use of slowly hydrolyzable ATP analogs or by autophosphorylation of the chaperone proteins causes a parallel reduction in chaperone activity.

Our results are in general agreement with most results reported by others both in vitro (Rothman, 1988; Gaut and Hendershot, 1993; Szabo et al., 1994) and in vivo (Cegielska and Georgopoulos, 1989; Wild et al., 1992; Hendershot et al., 1995). However, they do not agree with the results of Li et al. (1992) or the proposal by Palleros et al. (1993). One possible explanation for this is that different degrees of chaperone activity are necessary for different target proteins. In some instances simply binding the chaperone to the target might provide all of the necessary molecular assistance. This would be analogous to the "plucking" model described by Hubbard and Sander (1991). In others, the relatively small conformational changes brought about by K+ binding might be adequate for chaperone function. Finally, in instances in which the need for chaperone assistance is greater, nucleotide binding and hydrolysis, perhaps mediated by co-chaperone proteins (Liberek et al., 1991; Szabo et al., 1994; Zhou et al., 1995), might be required. Since a relatively small number of molecular chaperone types must deal with an enormous number of different target proteins (Hendrick and Hartl, 1993; Craig et al., 1994; Georgopoulos and Welch, 1994), it would not be surprising if the chaperones have evolved to use more than one mode of action.

**CONCLUSIONS**

Deletion of the proposed ATP-binding sites resulted in Stress70 proteins having little predicted secondary structure disruption but that were unable to bind to immobilized ATP and had no detectable ATPase activity. The mutant Stress70c protein with no ATPase activity also had no molecular chaperone activity in vitro. These results support the proposal that, at least in the instance of membrane translocation of a secretory precursor, ATPase activity is essential for Stress70 chaperone function.

**ACKNOWLEDGMENTS**

B.M. Manarelli provided technical assistance during the early stages of the research. R.S. Boston generously provided the plasmid p9/25PCR1.

Received June 30, 1995; accepted October 30, 1995.

Copyright Clearance Center: 0032-0889/96/110/0419/06.

**LITERATURE CITED**


