A Purified Subfragment of Yeast Atp11p Retains Full Molecular Chaperone Activity*

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Atp11p is a molecular chaperone of the mitochondrial matrix that participates in the biogenesis pathway to form F1, the catalytic unit of the ATP synthase. Affinity tag pull-down assays and yeast two-hybrid screens have shown that Atp11p binds to free β subunits of F1 (Wang, Z. G., and Ackerman, S. H. (2000) J. Biol. Chem. 275, 5767–5772). This binding action prevents the β subunit from associating with itself in non-productive complexes and fosters the formation of a (αβ)3 hexamer. Following the premise that Atp11p action is mediated primarily through a surface (as opposed to specific amino acids, as in an enzyme active site), solving its three-dimensional structure so that we may learn how the shape of the protein influences its function is a high priority. Recombinant yeast Atp11p has proven refractory for such analysis because of the presence of a disordered region in the protein. In this article, we show that removal of 67 residues from the amino terminus of recombinant Atp11p yields a subfragment of the protein (called Atp11pTRNC) that retains molecular chaperone function as determined in vitro with both a surrogate substrate (reduced insulin) and the natural substrate (F1 β). Moreover, preliminary 15N-1H heteronuclear single quantum coherence spectra obtained with Atp11pTRNC indicate that the truncated protein is well ordered and amenable to structure determination by nuclear magnetic resonance.

Atp11p was first identified in studies of Saccharomyces cerevisiae mutants that are respiratory deficient because of a defect in mitochondrial F1 assembly (1). In normal cells, F1 is formed of five different types of subunits that assemble an α3β3γδε oligomer (2). In this structure, the α subunits and β subunits are arranged, in alternate position, in a hexamer that accounts for ~90% of the mass of F1 (3, 4). The αβ2 structure is not observed to any significant degree in mitochondria from Atp11 mutants, instead, such yeast accumulate F1 β subunits as insoluble aggregates in the matrix of the organelle (1). This particular phenotype is observed also in yeast cells deficient for Atp12p, another protein of the F1 bioenergetic pathway (1). Information about the steric and chemical nature of surfaces in the protein that mediate this binding interaction will be forthcoming once the three-dimensional structure of Atp11p is solved. Progress in this area has been hampered by our inability to crystallize Atp11p from preparations of the recombinant protein. In this article, we report the purification and characterization of an Atp11p subfragment that exhibits molecular chaperone activities with model and natural substrates that are comparable with those of the full-length protein. Atp11pTRNC prevents the aggregation of insulin B-chains that are liberated when the disulfide bonds of the hormone are reduced. In other work, the interaction of Atp11pTRNC with the F1 β subunit is revealed in protein affinity blotting studies. Finally, we report that Atp11pTRNC shows great promise for structure determination by nuclear magnetic resonance as indicated by 15N-1H HSQC spectral data that have been obtained with isotopically labeled protein.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The Escherichia coli expression plasmid pATP11/Xa, produces yeast Atp11p with a His6-tag at the amino terminus and a Factor Xa cleavage site inserted between Lys106 and Ser107 in the protein sequence (sequence numbering as reported in ref. 7). The ATP11 DNA was subcloned from plasmid pTRC/ATP11 (8); pTRC/ATP11 carries codons 41–318 of Atp11p, corresponding to the mature protein without its mitochondrial leader sequence, ligated in-frame to an initiator ATG provided by the plasmid. In the first step, pTRC/ATP11 was digested partially with NcoI, and the pool of 5-kb linearized plasmid DNA was gel purified. The partially cut DNA was next digested to completion with BanHI, and the 860-bp NcoI-BanHI product of this digestion was cloned in pBlueScript. PCR was used to insert a Factor Xa recognition site inside the ATP11 coding sequence of pPROEX/ATP11. Two complementary oligonucleotides were constructed and used as primers for PCR mutagenesis following the QuikChange method (Stratagene). The sense and antisense primers, 5′-CTTGTGATGACGAAAATCCGAGGTCGTCTCAGAAGTCTCTCGATCTCA-3′ and 5′-TGAGCATGAGGAGGTCTTGATCGAATCCGTTCTCTCGATCTCAATGC-3′, respectively, included ATP11 nucleotides 304–318, followed by 12 nucleotides (bold) coding for the Factor Xa recognition sequence IEGR, followed by ATP11 nucleotides 319–339. The primers also encoded a silent (A→T) bp change (underlined) in the codon for Ser107, which introduced an XbaI restriction site unique to the mutant plasmid. The PCR reaction (50 μl) contained 20

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‡ The abbreviations used are: HSQC, heteronuclear single quantum coherence; TBST, Tris-buffered saline/Tween 20; NTD, N-terminal domain; NC, nitrocellulose.
Fig. 1. Schematic map of domains in yeast Atp11p. Different shading is used to indicate the span of Atp11p primary sequence in each of four protein domains: mitochondrial leader sequence (MLS), cross-hatched; N-terminal domain (NTD), dark gray; functional domain (FD), white; C-terminal domain (CTD), light gray. The numbers above the map indicate the first amino acid of each domain and the last amino acid in the protein.

Fig. 2. Genetic engineering and purification of Atp11pTRNC. A, schematic protein maps showing how the original DNA for recombinant yeast Atp11p (RcaAtp11p (see Ref. 8)) was modified to include a His6-tag sequence (black fill) and a Factor Xa cleavage site (IEGR) to enable the purification of the subfragment Ser-107–Asn-318 (Atp11pTRNC) to be purified from the N-terminal domain (dark gray shading) of the protein (see text for details). B, SDS-polyacrylamide gel of uncleaved (lane 1) and cleaved (lane 2) His6Atp11pTRNC and of purified Atp11pTRNC (lane 3). The migration of molecular mass standards is shown at right.

MW Tris-HCl, pH 8.8, 10 mM KCl, 10 mM NH₄SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 50 ng pPROEX/ATP11 plasmid (DNA template), and 125 ng of each primer. After the reaction was incubated at 95 °C for 2 min and then allowed to slowly cool to room temperature, 3 μl of QuikSolution (Stratagene), 6 μl of 2 mM dTTP mixture, and 1 μl of Pfu Turbo (2.5 units) were added to the tube, and the tube was incubated at 68 °C for 5 min. Next, PCR was performed for 18 cycles (95 °C for 1 min, 55 °C for 1.5 min, 68 °C for 12 min). The resultant mixture was digested with DpnI at 37 °C for 1 h to destroy the original plasmid DNA template, after which 10 μl was used to transform competent E. coli cells. Bacterial colonies harboring the mutant plasmid (called pATP11/Xa) were identified by restriction analysis with XbaI and tested in small-scale experiments for isopropyl-1-thio-β-D-galactopyranoside-induced production of recombinant protein. One such expressing clone was selected and used for large-scale production/purification of the modified Atp11p protein (His₆Atp11pTRNC). DNA sequencing confirmed the correct position of the inserted Factor Xa cleavage site and showed there were no codon changes elsewhere in the gene for His₆Atp11pTRNC.

Purification of Recombinant Atp11p and Preparation of a Factor Xa Cleavage Product, Atp11pTRNC—E. coli strain RRI served as host for the production of unlabeled recombinant proteins. Unmodified Atp11p was produced from the expression vector pTRC/ATP11 (8) and His₆Atp11pTRNC was produced from plasmid pATP11/Xa (described above). Growth of bacterial cultures in 2 l yeast tryptone medium, induction of plasmid expression with isopropyl-1-thio-β-D-galactopyranoside, cell breakage by sonic irradiation, and purification of Atp11p proteins from clarified cell extracts following sequential chromatographic steps through DEAE and CM Fast Flow-Sepharose were as described by White and Ackerman (8) with the following modifications: the concentration of isopropyl-1-thio-β-D-galactopyranoside used for induction was 0.6 mM; the buffer employed up to and including the DEAE column was 20 mM Tris-HCl, pH 7.5, 1 mM EDTA; lysozyme was omitted from the cell-breaking step; the CM column was run with a continuous gradient of phosphate buffer (50 to 500 mM KPO₄, pH 7.5, 1 mM EDTA). Highly purified Atp11p or His₆Atp11pTRNC was prepared from His₆Atp11pXa in the following manner. Factor Xa was added directly to the CM column eluate at a protein/protein ratio of 1:2500 Factor Xa/His₆Atp11pXa and the solution was dialyzed against 4 liters of 20 mM Tris-HCl, pH 8.0, overnight at 4 °C. The dialysate was adjusted to pH 8.0 (if necessary) and applied to a cobalt column (1.5 ml bed volume) (TALON resin; Clontech) that had been pre-equilibrated with 20 mM Tris-HCl, pH 8.0. The column was washed with 60 ml of the equilibration buffer, and the flow-through was collected and passed through a p-amino-benzamidine column (1 × 2.5 cm; ~9 ml bed volume) (Sigma) to remove Factor Xa from the protein preparation. Purified preparations of unmodified Atp11p and Atp11pTRNC were concentrated to 10 to 15 mg/ml using a Centricon-10, flash-frozen in liquid N₂, and stored at −80 °C.

Preparation of ¹³⁵N-Atp11p and ¹³⁵N-Atp11pTRNC—Bacterial strain BL21 was used to propagate expression plasmids for preparation of recombinant Atp11p proteins labeled with ¹⁵N. Unmodified Atp11p was modified to include a His₆-tag sequence (black fill) and a Factor Xa cleavage site (IEGR) to enable the purification of the subfragment Ser-107–Asn-318 (Atp11pTRNC) to be purified from the N-terminal domain (dark gray shading) of the protein (see text for details). B, SDS-polyacrylamide gel of uncleaved (lane 1) and cleaved (lane 2) His₆Atp11pTRNC and of purified Atp11pTRNC (lane 3). The migration of molecular mass standards is shown at right.

Nuclear Magnetic Resonance—Two-dimensional ¹³⁵N-¹H HSQC spectra and one-dimensional ¹³⁵T₂ and ¹³⁵T₂* experiments were collected on a Varian 800 MHz INOVA spectrometer at a sample temperature of 25 °C.

Protein Affinity Blots—The subunits of purified yeast F₂ were resolved in an SDS-polyacrylamide gel as described previously (9) and transferred electrophoretically to nitrocellulose membrane. The blot was washed three times for 10 min each with 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% Tween 20 (TBST) and then blocked for 30 min with a 1:2000 dilution in 10 ml TBST/milk and allowed to react for 1 h. After removal of the blocking solution, the blot was washed with TBST three times for 10 min each. Next, polyclonal antibody against yeast Atp11p was added to the blot at 1:2000 dilution in 10 ml TBST/milk and allowed to react for 1 h. After this step, the blot was washed with TBST and then challenged with horseradish-conjugated goat anti-rabbit secondary antibody in TBST/milk for 30 min. After this reaction, the blot was washed with TBST, and immunologically reactive protein was detected with chemiluminescent reagents (Amersham Biosciences).

Miscellaneous Procedures—The light scattering assay for insulin B-
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RESULTS AND DISCUSSION

Preparation of a Recombinant Atp11p Subdomain (Atp11pTRNC)—In previous work (12), we assigned the boundaries of four domains in the polypeptide chain of Atp11p, which include the mitochondrial leader sequence (residues 1–39), the N-terminal domain (NTD, residues 40–111), the functional domain (residues 112–183), and the C-terminal domain (residues 184–318) (Fig. 1). Our standard preparation of recombinant yeast Atp11p purified from bacteria lacks the targeting sequence and encompasses codons 41–318 of the primary gene product (8). Such preparations are highly pure and active in biochemical assays of Atp11p activity (see ref. 10 and below). However, the recombinant yeast protein does not crystallize and this has hampered our efforts to pursue the determination of its three-dimensional structure by x-ray diffraction methods. In preliminary work to determine whether nuclear magnetic resonance might be an avenue to study Atp11p structure, $^{15}$N-$^1$H HSQC spectra obtained with $^{15}$N-Atp11p (279 amino acids) showed that only 110 of 264 observable resonances are resolved outside an overlapped region of the spectrum (data not shown). This result is consistent with the failed crystallization experiments because the high degree of overlapped resonances suggests that the Atp11p preparations are not uniformly well ordered.

A strategy based on known features of Atp11p was developed to allow the preparation of a stable subfragment that might be more amenable to structure analysis. The decision was made to remove the protease-hypersensitive NTD (Fig. 1) from the protein (see ref. 12). Complementation studies have shown that the NTD is not required for the chaperone action of Atp11p in yeast cells (12), and the extreme hydrophilic character of this region was considered to be the principle factor causing disorder of the protein structure. Efforts to produce a truncated protein directly from an expression vector did not yield detectable levels of the protein in bacterial cell lysates. This finding is consistent with the observation that although N-terminally truncated forms of Atp11p retain function, they are much less physically stable in vivo relative to the full-length protein (12). To overcome this problem, recombinant Atp11p was engineered with a Factor Xa cleavage site (IEGR$^7$) near the end of the NTD, in-between Lys106 and Ser107 (Fig. 2A). The protein was also modified with a His$_6$-tag at the amino terminus. In the purification scheme (see “Experimental Procedures”), His$_6$Atp11pXa is prepared from bacteria, and after digestion with Factor Xa, truncated Atp11p (Atp11pTRNC, Ser-107 through Asn-318) is purified from the His$_6$NTD and from any undigested protein after passage of the digested mixture through a cobalt column, which binds the His$_6$ sequence (Figs. 2, A and B). Subsequent chromatography of Atp11pTRNC through p-amino benzydine agarose removes Factor Xa from the preparation and yields the highly purified subfragment Atp11pTRNC (Fig. 2B, lane 3).

Atp11pTRNC Prevents Aggregation of Reduced Insulin B-chains—The molecular chaperone activity of Atp11p can be assessed in vitro using reduced insulin as a surrogate substrate (10). In this method, light scattering is used to follow the progression of insulin B-chain aggregation that occurs when the disulfide bonds in the hormone are reduced with dithiothreitol (13). Atp11pTRNC provides >60% protection against aggregation to reduced insulin B-chains in this assay (Fig. 3). This result can be compared with the value of 70% protection that is obtained with full-length recombinant Atp11p in similar studies (10). Atp11pTRNC is just five residues shorter at the amino terminus than Atp11p (40–111) (12), a plasmid-borne variant of Atp11p that has been shown to confer respiratory competence to a Atp11p yeast mutant in previous work. Hence, there is excellent correlation of results obtained in vivo and in vitro to support the argument that removal of the Atp11p NTD does not interfere significantly with the molecular chaperone activity of the protein.

Interactions between Atp11p and F$_1$ β Subunits Studied by Affinity Blotting—Atp11p binds to the β subunit of F$_1$ in yeast cells (6) but not to a preparation of recombinant mitochondrial β subunit that is solubilized from bacteria with alkaline buffer after export to the periplasmic face of the plasma membrane. A likely reason for this observation is that the alkaline-extracted protein poorly reflects the character of the β subunit that is presented to Atp11p during F$_1$ assembly. Protein affinity blotting has provided an alternative method to study Atp11p interaction with its natural substrate in vitro. In this work, purified yeast F$_1$ was run in an SDS gel under conditions that maximize resolution between α and β subunits (9), after which the proteins were transferred to nitrocellulose (NC) membrane (Fig. 4). The NC was first incubated in TBST (see “Experimental Procedures”) and then treated with TBST/milk to reduce nonspecific protein binding. Next, the blocked NC was incubated with purified Atp11p (full-length or truncated subdomain) in TBST/milk, and after the protein overlay solution was removed, it was immunoblotted with antiserum against Atp11p. Atp11p (31 kDa) and Atp11pTRNC (24 kDa) were detected with the antibody at the position of F$_1$ β protein

Fig. 4. Affinity blotting of F$_1$ β subunit using purified Atp11p or Atp11pTRNC as the probes. NC, Ponceau Red-stained nitrocellulose showing the positions of resolved α and β subunits of F$_1$ transferred from a 10% SDS gel. X-ray film, lanes 1–3, affinity blotting of the NC paper with full-length Atp11p (lanes 1 and 3) or with Atp11pTRNC (lane 2), followed by immunoblotting with anti-Atp11p serum (lanes 1 and 2) or with preimmune rabbit serum (lane 3). Lane 4, direct immunoblotting with anti-Atp11p serum of NC-immobilized F$_1$ subunits without prior incubation of the blot with Atp11p.

Fig. 3. Aggregation of reduced insulin in the absence and presence of Atp11pTRNC. Insulin (34 µM) was reduced with dithiothreitol and aggregation of the B-chain was monitored by light scattering at 465 nm as described under “Experimental Procedures” in the absence of Atp11pTRNC (○) or in the presence of 34 µM Atp11pTRNC (□).

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5(55 kDa) in the blot (Fig. 4, lanes 1 and 2). The Atp11p protein signals were not detected if immunoblotting was done using pre-immune rabbit serum instead of anti-Atp11p serum (Fig. 4, lane 3). Moreover, under conditions in which the incubation step with purified Atp11p was omitted from the experiment and the NC was instead exposed directly to Atp11p antiserum, there was no immunoreactive signal (Fig. 4, lane 4). Such findings illustrate that anti-Atp11p does not recognize epitopes under the following solution conditions: 0.8 mM protein and 25 mM sodium phosphate buffer, pH 7.0.

The subsequent washing of the NC membrane. A corollary finding of partially after electrophoretic transfer from the denaturing gel and not completely denatured in the SDS gel or it renatures partially from an SDS gel to NC paper, which retains or regains sufficient higher order structure to support an interaction with nucleic acid (15) or protein (16, 17) applied as an overlay solution to the blot. It is possible that either the F1 beta subunit, which binds Atp11p in this assay, has been resolved from other F1 subunits in an SDS gel is not inconsistent with our model for Atp11p action, which suggests the chaperone recognizes a structural element in its target protein (14). There are examples in the literature of protein, transferred from an SDS gel to NC paper, that retains or regains sufficient higher order structure to support an interaction with nucleic acid (15) or protein (16, 17) applied as an overlay solution to the blot. It is possible that either the F1 beta subunit is not completely denatured in the SDS gel or it renatures partially after electrophoretic transfer from the denaturing gel and subsequent washing of the NC membrane. A corollary finding of this analysis is that the Atp11p:F1 beta binding interaction occurs in the absence of other proteins or cofactors. The dependence of chaperone release on other factors remains to be determined.

Nuclear Magnetic Resonance Studies with Atp11pTRNC. 15N-labeled Atp11pTRNC (212 amino acids) yields a very well resolved 15N-1H HSQC spectrum at 25 °C from which 200 strong main-chain cross peaks can be easily counted (Fig. 5). Their peak intensity and line-width is rather uniform. Lower level plots reveal another 10 resolved low intensity peaks. Together, the data are indicative of a structured protein, without mobile tails, and with few exchange broadened peaks. Using one-dimensional variants of standard 15N relaxation experiments, 15NT2 = 0.043 ± 0.005 s, and 15NT1 = 1.4 ± 0.2 s at 25 °C were found for resonances with a 1H chemical shift at 9 ppm (i.e. resonances belonging to the structured core of the protein). This yields a value of 14 ± 2 ns for the protein’s rotational correlation time, corresponding to a molecular mass of 28 ± 4 kDa, which is near the value of 24 kDa calculated from the sequence of Atp11pTRNC. It may be inferred from the spectral data that the protein is mono-disperse at the concentrations (24 mg/ml) used in the experiment. The prospects for NMR assignment and solution structure determination of Atp11pTRNC, once uniformly triple-labeled with 15N, 2H, and 13C, are excellent. Having demonstrated here that the purified subfragments of Atp11p shows functional activities comparable with the full-length protein, there is every reason to believe that solving the solution structure of Atp11pTRNC will provide relevant information about the mechanism of this molecular chaperone.

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Fig. 5. 15N-1H HSQC NMR spectrum of Atp11pTRNC. Data were collected for 30 min on a Varian 800 MHz INOVA spectrometer at 25 °C under the following solution conditions: 0.8 mM protein and 25 mM sodium phosphate buffer, pH 7.0.