An Accessible Hydrophobic Surface Is a Key Element of the Molecular Chaperone Action of Atp11p

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Atp11p is a soluble protein of mitochondria that binds unassembled β subunits of the F1-ATPase and prevents them from aggregating in the matrix. In this report, we show that Atp11p protects the insulin B chain from aggregating in vitro and therefore acts as a molecular chaperone. The chaperone action of Atp11p is mediated by hydrophobic interactions. An accessible hydrophobic surface in Atp11p was identified with the environment-sensitive fluorescent probe 1,1’-bis(4-anilino-5-naphthalenesulfonic acid (bis-ANS). The spectral changes of bis-ANS in the presence of Atp11p indicate that the probe binds to a nonpolar region of the protein. Furthermore, the dye quenches the fluorescence of Atp11p tryptophan residues in a concentration-dependent manner. Although up to three molecules of bis-ANS can bind cooperatively to Atp11p, the binding of only one dye molecule is sufficient to virtually eliminate the chaperone activity of the protein.

Atp11p is one of two proteins (the other is Atp12p) required for assembly of the catalytic (F1) component of the mitochondrial ATP synthase. In normal cells, F1 assembly proceeds with the incorporation of five different protein subunits to form the oligomer α3β3γδε (1). In contrast, mitochondria from atp11 or atp12 mutants of Saccharomyces cerevisiae show the accumulation of the F1 α subunits and β subunits in an aggregated form (2). Notably, the “sticky” character of unassembled F1 α and β subunits is also apparent from the phenotype of yeast atp1α (α subunit) and atp2β (β subunit) null mutants; the β subunits aggregate in the mitochondria of cells lacking the α subunit, and the α subunits aggregate when there are no β subunits to combine with (2). The phenotypic similarity among atp1α, atp2β, atp11, and atp12 null strains suggests that Atp11p and Atp12p are important elements in determining the solubility status of the aggregation-prone, unassembled F1 α and β subunits. In line with this hypothesis are the findings that Atp11p interacts directly with the F1 β subunit (3) and that Atp12p interacts directly with the F1 α subunit (4).

The present work focuses on the molecular mechanism of Atp11p. Based on experiments with the yeast two-hybrid system (3), we have proposed that the substrate for Atp11p is the folded form of the F1 β subunit. Previous work also identified the middle third of Atp11p as its functional domain and showed that this region is characterized by two stretches of hydrophobic amino acids (5), the more distal of which is coincident with the PFXXXLPR consensus sequence common to Atp11p proteins from diverse sources (6, 7). The reason why folded β subunits aggregate in the absence of Atp11p probably lies in the fact that the unassembled protein exposes hydrophobic groups at the surface that are not meant to be in contact with solvent because they will ultimately be sequestered at contact points with adjacent α subunits in the F1 αβαβαβ hexamer (8, 9). Thus, Atp11p may function by providing a hydrophobic surface that is the steric complement of a nonpolar region of the unassembled F1 β subunit. An example of a chaperone that temporarily transmits structural information to its substrate is PapD; in the complex formed between PapD and the pilus subunit, PapK, the chaperone inserts a β-strand in the PapK structure to fill a groove in the protein and shield its hydrophobic core from the solvent (10).

The studies reported here provide evidence that a hydrophobic surface in Atp11p is indeed an important element of its mechanism. Because attempts to use the recombinant form of the mitochondrial F1 β subunit have not been successful, we have employed insulin as a model substrate. After reduction of the disulfide bonds in insulin, the A chain remains in solution, whereas the B chain aggregates (11). In a manner similar to that observed for other molecular chaperones including α-crystallin (12), Hsp90(2) (13), and SecB (14), Atp11p protects the insulin B chain from aggregation in vitro. In other work, we show that upon binding the fluorescent probe bis-ANS to a hydrophobic region of Atp11p, the protein loses the capacity to prevent the aggregation of the insulin B chain.

EXPERIMENTAL PROCEDURES

Materials—Insulin from bovine pancreas, dithiothreitol, and α-crystallin from bovine eye lens were purchased from Sigma. bis-ANS was purchased from Molecular Probes, Inc. (Junction City, OR). Concentrated stock solutions of bis-ANS were prepared in 100% methanol, and their concentration was verified spectrophotometrically at 25 °C by measuring absorbance at 395 nm using an extinction coefficient of 23,000 M–1 cm–1 (Molecular Probes). Working stock solutions of bis-ANS (1 mM) were prepared such that the concentration of methanol was 10%. All other chemicals were of the highest grade commercially available.

Preparation of Atp11p—Yeast Atp11p is overproduced as a soluble recombinant protein in Escherichia coli from plasmid pTRC99a (15). Purification of the recombinant protein was performed as described previously (15), with two exceptions. First, the supernatant recovered after precipitation with streptomycin sulfate was not diluted before loading the DEAE column, and second, Atp11p eluted from the carboxymethyl sepharose column was dialyzed against 40 mM KH2PO4, pH 7.5, and 1 mM EDTA. Final preparations of Atp11p were routinely concentrated to 15–20 mg/ml using a nitrogen pressure filtration unit.
(Amicon) fitted with a 20-kDa cutoff membrane, flash frozen in liquid nitrogen, and stored as 100-μl aliquots at −80 °C. The purity of this protein preparation is >99% as judged by SDS-polyacrylamide gel electrophoresis. Protein concentration was estimated as described by Lowry et al. (16).

Light Scattering Assay for Insulin B Chain Aggregation—The insulin aggregation assay was modified from Ref. 12. In a final volume of 1 ml, 0.2 mg of insulin was reduced with 20 mM dithiothreitol in the presence of the amounts of Atp11p indicated for each experiment (or in the presence of control proteins) in 50 mM KH₂PO₄, pH 7.5, and 0.1 mM NaCl. Aggregation of the insulin B chain was monitored by measuring light scattering with an Aminco-Bowman Series 2 spectrophotometer, using 465 nm as the wavelength for both the incident and scattered light. All light scattering and fluorescence measurements (see below) were made at 30 °C using a thermostated 1-cm quartz cuvette, with the excitation and emission band passes set at 4 nm. The data were collected directly on a Dell Pentium I computer connected to the spectrophotometer and analyzed using SigmaPlot.

Fluorescence Measurements—Fluorescence measurements were used to monitor the binding of bis-ANS to Atp11p. To obtain fluorescence spectra for bis-ANS in the absence and presence of Atp11p, samples were excited at 395 nm, and emission was recorded between 400 and 600 nm. For energy transfer experiments, an excitation wavelength of 295 nm was used to selectively excite tryptophan residues (17). Emission spectra were recorded between 300 and 580 nm; emission maxima for excited tryptophans and bis-ANS were at 328 and 490 nm, respectively. Emission data for Atp11p tryptophans were corrected for the absorption of bis-ANS by the relation \( F_{\text{corr}} = F_{\text{obs}} + OD_{\text{em}} \cdot \lambda(\text{OD}_{\text{em}} + OD_{\text{em}}) / \lambda(\text{OD}_{\text{em}}) \) (17); in this instance, \( OD_{\text{em}} \) and \( OD_{\text{em}} \) were the optical densities measured at 295 and 328 nm, respectively. To estimate the number of bis-ANS molecules that bind to Atp11p, the protein (0.5 μM) was titrated to saturation with increasing amounts of the dye (0–20 μM). The samples were excited at 395 nm, and emission from bis-ANS was measured at 490 nm. Observed fluorescence data were corrected for inner filter effects (see above) using the absorption data for bis-ANS measured at 395 nm (\( \lambda_{\text{exc}} \)) and 490 nm (\( \lambda_{\text{em}} \)) and analyzed by nonlinear least squares regression using the Hill equation, \( F_{\text{corr}} = F_{\text{obs}}\left[1 + \left(\frac{[S]}{K_{d}}\right)^n\right]^{-\frac{n}{n+1}} \), in which \( F_{\text{corr}}, F_{\text{obs}}, \lambda_{\text{exc}}, \lambda_{\text{em}}, \lambda_{\text{exc}}, \lambda_{\text{em}} \), and \([S]\) are, respectively, the observed fluorescence intensity, maximum fluorescence intensity, apparent binding constant, Hill coefficient, and bis-ANS concentration. The value of maximum fluorescence observed for the bis-ANS:Atp11p complex was determined by doing a reverse titration in which Atp11p was added incrementally to a solution of bis-ANS (2 μM) until saturation was achieved (data not shown). The value at saturation was used to convert bis-ANS fluorescence data into molar quantities of the dye.

Isolation of Atp11p Complexed with bis-ANS—Atp11p (544 μM in 62 μl) was mixed with 18.5 μl of a bis-ANS stock solution that was prepared by diluting 3.5 μl of 9.9 mM bis-ANS stock solution (in 100% MeOH) with 15 μl of reaction buffer (50 mM KH₂PO₄, pH 7.5, and 0.1 mM NaCl) to give a mixture of the protein and dye at 1:1 molar stoichiometry. A control sample of Atp11p was prepared in an identical manner, except that 100% replaced the concentrated bis-ANS stock solution. Each of the samples (81.5 μl) was passed through a G-25 centrifuge column before experiments with reduced insulin.

Gel Filtration Chromatography—Atp11p (0.3 mg/ml) was subjected to gel filtration chromatography on a Sephacryl S-100 column (1.5 × 70 cm; Amersham Pharmacia Biotech) at a flow rate of 0.5 ml/min in 50 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, and 0.05% NaN₃. The column was calibrated with proteins (0.3–0.5 mg/ml) selected from two different Gel Filtration Calibration Kits (Amersham Pharmacia Biotech catalogue numbers 17-0441-01 and 17-0442-01) and included ribonuclease A (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), and albumin (67 kDa).

RESULTS

Atp11p Protects the Insulin B Chain from Aggregation—Aggregation of the insulin B chain occurs concomitant with reduction of the disulfide bonds in the parent protein (11). The ability of Atp11p to reverse this reaction under controlled conditions has been exploited to study the chaperone action of several proteins (12–14). We have employed the insulin B chain aggregation assay to determine whether Atp11p displays molecular chaperone activity in vitro. Insulin B chain aggregation was monitored by measuring the apparent fluorescence due to light scattering at 465 nm as indicated in Fig. 1. In mixtures of equimolar insulin and Atp11p (Fig. 1A), Atp11p provides 70% protection against aggregation of the B chain. This level of protection is similar to that observed in equimolar mixtures of the chaperone domains of Hsp90 with reduced insulin (13). In contrast, ribonuclease A is not effective in preventing B chain aggregation after reduction of insulin (Fig. 1B). We have also employed this assay to reproduce the findings of other groups (12, 18) in experiments in which α-crystallin protects the reduced insulin B chain from aggregation (data not shown). On this basis, we conclude that the chaperone activity of Atp11p observed in our experiments is a real phenomenon.

Interaction of bis-ANS with Atp11p—The fluorescence quantum yield of bis-ANS increases upon binding to hydrophobic sites of proteins (19). This feature has gained the fluorescent dye popularity as a probe to monitor conformational changes in proteins and determine the accessibility of hydrophobic surfaces (20–24). bis-ANS is minimally fluorescent in aqueous buffer with an emission maximum at ~525 nm (Fig. 2, curve 1). In the presence of a substoichiometric amount of Atp11p, the fluorescence intensity of bis-ANS increases >10-fold, and the emission maximum is blue-shifted to 492 nm (Fig. 2, curve 2). The observed spectral changes are diagnostic of bis-ANS binding to a hydrophobic region (or regions) of Atp11p.

Energy Transfer from Atp11p Tryptophans to Bound bis-ANS—Overlap of the tryptophan emission spectrum of Atp11p with the excitation spectrum of bis-ANS was manifest in energy transfer from the donor (tryptophan) to the acceptor (bis-ANS) fluorophore. Emission spectra of Atp11p alone and of mixtures of Atp11p plus bis-ANS were obtained after excitation of the samples at 295 nm (Fig. 3). Without bis-ANS, Atp11p tryptophan residues emitted maximally at 328 nm. The addition of bis-ANS to the protein sample caused a decrease in tryptophan fluorescence at 328 nm coincident with the appearance of dye fluorescence at 492 nm. Evidence of energy transfer
is provided by the observed dose-dependent quenching of the tryptophan emission at 328 upon addition of bis-ANS (Fig. 3, inset). Because aqueous solutions of free bis-ANS do not exhibit fluorescence upon excitation at 295 nm (data not shown), we conclude that the 492 nm emission is entirely from bis-ANS that has become bound to Atp11p. However, it is likely that energy transfer is only one component that contributes to the excitation of the dye and that part of the 492 nm emission may result from the direct excitation of Atp11p-bound bis-ANS with the incident 295 nm beam.

**Titration of bis-ANS Binding Sites in Atp11p**—Titration of Atp11p with bis-ANS revealed cooperative (sigmoidal) binding of the dye (Fig. 4). The fluorescence values measured for bis-ANS were converted to molar units by dividing the numbers by the value of maximal dye fluorescence (0.655) that was determined for 2 μM bis-ANS fully saturated with Atp11p (see “Experimental Procedures”). The fluorescence data (Fig. 4, ○) were fit least-square to the Hill equation (solid line; defined under “Experimental Procedures”), yielding an apparent binding constant ($K_{0.5}$) of 3.65 μM, a Hill coefficient (n) of 1.87, and a maximal value of fluorescence ($F_{\text{max}}$) of 0.891. Conversion of the latter value to a molar quantity indicates that 2.72 molecules of bis-ANS are bound to Atp11p at saturation.

**Effect of bis-ANS Binding on the Chaperone Action of Atp11p**—The effect of preincubation of Atp11p with bis-ANS on the ability of the protein to prevent aggregation of insulin B chains was examined (Fig. 5). Untreated Atp11p showed a 70% protective effect (see also Fig. 1, A and B), whereas a complex formed at equimolar Atp11p:bis-ANS concentrations elicited only 16% protection. The tryptophan fluorescence spectrum of Atp11p in the presence of bis-ANS showed no significant change in the emission wavelength (see Fig. 3). Furthermore, there was no indication from light scattering measurements that the addition of bis-ANS in equimolar amounts to Atp11p caused precipitation of the protein, even after overnight incubation at 4 °C (data not shown). We conclude that the decreased chaperone action of Atp11p with bound bis-ANS is not caused by a gross perturbation of the protein structure but is rather due to the dye binding to a hydrophobic surface that is essential for Atp11p activity.

**DISCUSSION**

Until now, the proposal that Atp11p is a molecular chaperone has been based on plausible yet indirect evidence, including the phenotype of yeast atp11 mutants, which accumulate subunits of the F$_1$-ATPase in an aggregated form (2, 5), and the demonstration by two-hybrid screens and co-precipitation of interactions between Atp11p and the F$_1$ β subunit (3). However, the fact that purified mitochondrial F$_1$ β subunits fail to remain monodispersed in solution has hampered studies of the direct interaction of Atp11p with its natural substrate in vitro. Insulin, on the other hand, has proven to be an excep-
conformationally good model substrate for in vitro studies with Atp11p. In particular, Atp11p is not affected by the conditions used to induce aggregation of the insulin B chain. The aggregation of the B chain, which accompanies reduction of the insulin disulfide bonds (11), is likely to occur because amino acid side chains of the B chain, which are buried in the native structure, are exposed when the two insulin chains are separated. In this respect, an unassembled F1 β subunit is not dissimilar from the free insulin B chain because a free β subunit exposes at its surface hydrophobic residues that will be sequestered in the F1 subunit. Our demonstration that Atp11p protects the insulin B chain from aggregation (Fig. 1) provides, for the first time, direct evidence of the molecular chaperone activity of this protein.

The fluorescent dye bis-ANS has been instrumental in providing information about hydrophobic surfaces in many proteins, including those belonging to the superfamilies of molecular chaperones such as DnaK (20) (Hsp70 class), GroEL (24) (Hsp60 class), and α-crystallin (21, 23) and Hsp18.1 (25) (small heat shock protein class). In some cases, such work has been geared toward the detection of conformational changes in the chaperone proteins (20, 21, 24), whereas others have used this tool as a probe for hydrophobic domains with the intent of linking the findings to the chaperone action of the protein (23, 25). Our current work with bis-ANS follows the latter strategy. The spectral characteristics of the dye in an aqueous buffer containing Atp11p (Fig. 2) indicate that bis-ANS binds to an accessible hydrophobic surface of the protein. Further evidence for the existence of an Atp11p-bis-ANS complex is provided by the demonstration of energy transfer from tryptophan to the dye (Fig. 3). The recombinant yeast Atp11p used in this work contains three tryptophan residues at positions 144, 148, and 205 in the primary sequence (26). These amino acids collectively emit maximal fluorescence at 328 nm after excitation at 295 nm (Fig. 3), in contrast with free tryptophan, which emits at 350 nm (17). Thus, although we do not know whether all three tryptophans of Atp11p contribute to the observed fluorescence, maximal emission at 328 versus 350 nm is taken to be diagnostic of the fact that the fluorescing amino acid(s) is (are) buried in a nonpolar region of the protein (17).

Titration studies indicate that ~3 moles of bis-ANS bind per mole of Atp11p at saturation (Fig. 4). Furthermore, the binding data are indicative of positive cooperativity, which suggests that Atp11p may undergo a change in conformation upon binding bis-ANS in a manner similar to that observed when bis-ANS binds to DnaK (20) and to the apical domain of GroEL (24). Although we do not know the nature of the putative conformational change induced by bis-ANS, the spectral and solubility properties of Atp11p in the presence of stoichiometric amounts of the dye do not reveal a gross perturbation of the protein structure. However, it is clear that the protein function is greatly compromised by bis-ANS because at an equimolar dye:protein concentration, the chaperone action of the Atp11p is virtually eliminated (Fig. 5). The inability of an Atp11p-bis-ANS complex to provide significant protection against aggregation of the reduced insulin B chain (Fig. 5) provides evidence that the hydrophobic surface in Atp11p to which the dye binds is important for the chaperone activity of the protein.

Native mitochondrial Atp11p and the purified recombinant protein both sediment in linear sucrose gradients as a 31-kDa monomer (15). Gel filtration experiments performed as part of this study confirm that Atp11p remains a monomer under the conditions of the insulin aggregation assay (data not shown). In accord with the proposal that Atp11p is active as a monomer is the fact that the hydrophobic surface required for its function is highly accessible to bis-ANS under physiological conditions (pH 7.5, 30 °C) (see Fig. 2). In this respect, Atp11p is distinctly different from oligomeric chaperone proteins such as GroEL/Hsp60 (24) and α-crystallin/small heat shock protein (21), which show low accessibility of the hydrophobic surface in the native state relative to perturbed forms of the proteins.

The ability of Atp11p to prevent aggregation of the insulin B chain occurs in the absence of ATP hydrolysis (Fig. 1). Although our studies do not address the possibility that ATP is required for Atp11p action with its natural substrate in vivo, our results are in accord with the fact that there is no indication from the primary sequence (26) or from equilibrium binding experiments to suggest that Atp11p binds ATP. In a similar vein, there is no evidence that ATP hydrolysis is involved in the chaperone actions of PapD (10) or small heat shock proteins such as α-crystallin (12). There are other properties of the latter two chaperones that share common ground with Atp11p. For example, PapD, which binds unassembled pilus subunits, and Atp11p, which binds unassembled F1 β subunits, are much more specific in their chaperone activities versus members of the Hsp60, Hsp70, and Hsp90 superfamilies (27). Also noteworthy is that just as small heat shock proteins (including α-crystallin) are characterized in part by a short hydrophobic stretch of amino acids (XGVXLXLXXPX) (25, 28), Atp11p proteins from yeast on up to humans are characterized by a hydrophobic

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3 Z.-G. Wang and S. H. Ackerman, unpublished observations.
consensus sequence, PXFXXXLPR (7). Future studies will be aimed at determining whether this Atp11p sequence is coincident with the bis-ANS binding site.

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