The Molecular Chaperone, Atp12p, from Homo sapiens

*A molecular chaperone is a protein that assists in the folding of other proteins. In this context, Atp12p is shown to prevent aggregation of unassembled subunits during the assembly of the F1 subunit of the mitochondrial ATP synthase. This chaperone activity is crucial for maintaining the physical integrity of the protein and ensuring its correct function.*

Ayana Hinton†, Domenico L. Gatti‡, and Sharon H. Ackerman§‡§

From the §Department of Surgery and ‡Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

Work in *Saccharomyces cerevisiae* has shown that Atp12p binds to unassembled α subunits of F1 and in so doing prevents the α subunit from associating with itself in non-productive complexes during assembly of the F1 moiety of the mitochondrial ATP synthase. We have developed a method to prepare recombinant Atp12p after expression of its human cDNA in bacterial cells. The molecular chaperone activity of HuAtp12p was studied using citrate synthase as a model substrate. Wild type HuAtp12p suppresses the aggregation of thermally inactivated citrate synthase. In contrast, the mutant protein HuAtp12pE240K, which harbors a lysine at the position of the highly conserved Glu-240, fails to prevent citrate synthase aggregation at 43 °C. No significant differences were observed between the wild type and the mutant protein as judged by sedimentation analysis, cysteine titration, tryptophan emission spectra, or limited proteolysis, which suggests that the E240K mutation alters the activity of HuAtp12p with minimal effects on the physical integrity of the protein. An additional important finding of this work is that the equilibrium chemical denaturation curve of HuAtp12p shows two components, the first of which is associated with protein aggregation. This result is consistent with a model for Atp12p structure in which there is a hydrophobic chaperone domain that is buried within the protein interior.

Atp12p was first identified in studies of *Saccharomyces cerevisiae* mutants that are respiratory-deficient due to a defect in mitochondrial F1 assembly. The core structure of F1 is a hexameric unit of alternating α and β subunits that surrounds a rod-shaped γ subunit (1). Yeast *atp12* mutants fail to assemble the α,β,γ oligomer, and instead accumulate F1 α subunits as large, insoluble aggregates in the matrix of the organelle (2). This particular phenotype is observed also in yeast mutants that lack a functional Atp11p, another molecular chaperone of the mitochondrial F1 assembly pathway (3). There is also F1 protein aggregation in yeast null mutants that are missing either the α subunit or the β subunit, as such strains accumulate the lone β or α subunit as aggregated proteins inside mitochondria (2). Because lack of the γ subunit does not produce aggregation of α and β subunits (4), it is believed that γ assembles into the F1 structure after Atp11p- and Atp12p-mediated steps have secured the formation of a soluble αβ intermediate (3).

The amount of yeast Atp12p in the mitochondrial matrix is roughly 100 times smaller than the amount of F1 protein (5). This molar ratio seems appropriate in consideration of the fact that Atp12p does not bind to α subunits that are part of the F1 oligomer but, instead, associates with unassembled α subunits. Because unassembled F1 proteins do not accumulate to a significant degree in the cell (6), the Atp12p concentration in mitochondria may be comparable with the concentration of free F1 α subunit protein. Therefore, the amount of Atp12p is not likely to be a limiting factor in mitochondrial F1 biogenesis under normal conditions in the cell. However, reducing the level of active Atp12p in mitochondria is anticipated to correlate directly with a decreased flux through the F1 assembly pathway. Such considerations underscore the important role of human Atp12p (HuAtp12p) and its potential ties to mitochondrial disease.

We have now developed a method to purify recombinant HuAtp12p in high yield from a bacterial expression system, and this has enabled us to expand the analysis of the physical and functional properties of the molecular chaperone beyond what was formerly achieved in studies of the yeast protein in mitochondrial samples. One significant aspect of the work has been to evaluate wild type HuAtp12p in comparison with a mutant form of the protein that harbors a Glu → Lys substitution at a conserved position in the amino acid sequence. In particular, we show that the wild type HuAtp12p behaves *in vitro* as a molecular chaperone, whereas the mutant HuAtp12pE240K is defective in this capacity. Despite this clear functional difference, the wild type and mutant proteins are reported to be similar with respect to sedimentation properties, intrinsic fluorescence under native conditions, cysteine accessibility, and protease sensitivity. Spectroscopic analysis of HuAtp12p unfolding in guanidine hydrochloride is also presented, which suggests the presence of a hydrophobic subdomain in the protein structure.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Recombinant plasmids for the production of human Atp12p (HuAtp12p) in *E. coli* were constructed in the His-tag vector pPROEX HTa (Invitrogen) and were designed to produce the mature form of the protein without the mitochondrial leader peptide. The DNA for wild type HuAtp12p was prepared using the PCR to amplify an 806-bp fragment inclusive of HuAtp12p codons 55–288, start and stop codons, and flanking restriction sites for cloning. The forward and reverse primers were, respectively, 5'-CCGGAATTCTAGATCCGTCCTCCAGCCTGGGCTTAC-3' and 5'-CCGAGCTCACGTCCTCCTAGGACCTTGTC-3' and are annotated to highlight the EcoRI and KpnI restriction sequences (underlines), ATG and TGA codons (double underlines), and HuAtp12p coding sequences (bold) contained within. The plasmid template was pCUPHUATP12HYP (7). The PCR product and pPROEX HTa vector were each digested with a

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†To whom correspondence should be addressed: Dept. of Surgery, Wayne State University School of Medicine, 421 E. Canfield Ave., Detroit, MI 48201. Tel.: 313-577-8645; Fax: 313-577-7642; E-mail: sackerm@med.wayne.edu.
combination of EcoRI and KpnI, and the linear fragments were ligated and used to transform E. coli strain RRI. The resultant new plasmid (pPROEX/Atp12h) was used to prepare wild type HuAtp12p for this work and also served as the template for PCR-mediated site-directed mutagenesis via the Stratagene QuikChange method to introduce a Glu → Lys change at position 240 in the recombinant protein. The two complementary oligonucleotides used for mutagenesis were sense-strand primer 5'-GCCGTGCCTGCTGTTAGATGAAAAGGAGATTAC- CAGATC-3' and antisense-strand primer 5'-GACTGTGATTTACCAC- CGACCAGGC-3' and encompassed HuAtp12p DNA sequence 697–735 with three silent mutations (underline) to create a XbaI site for screening purposes and a G → A (bold) mutation to convert Glu240 to lysine. The PCR reaction (50 μl) contained 20 mm Tris-HCl, pH 8.8, 10 mM KCl, 10 mM NH₄(SO₄)₂, 2 mM MgSO₄, 0.1% Triton X-100, 0.1 mM bovine serum albumin, 50 μg of pPROEX/Atp12-12 h 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 25 mM dNTPs, and 2 μl of Taq polymerase (Promega) were added, and the tube was incubated at 72 °C for 5 min. Next, PCR was performed for 18 cycles (95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 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 of the digestion mixture was used to transform E. coli strain RRI. Following the mutagenesis (called pPROEX/Atp12E240K) were identified by restriction analysis in an EcoRI/XbaI digest and tested in small scale experiments for isopropyl β-thiogalactopyranoside-induced production of recombinant protein. One such expressing clone was selected and used for large scale production/purification of the mutant Atp12p protein (HuAtp12p<sub>E240K</sub>). DNA sequencing confirmed that only the desired mutation is present in the plasmid insert of pPROEX/ Atp12pE240K.

Purification of Recombinant HuAtp12p Wild Type and Mutant Proteins—HuAtp12p and HuAtp12p<sub>E240K</sub> were overproduced in E. coli RRI cells and purified according to the following method. An overnight LB culture of E. coli strain RRI harboring the mutant plasmid (called pPROEX/Atp12E240K) were lysed by sonication in 50 mM Tris-HCl, pH 8.0, under a nitrogen atmosphere. The lysate was brought to 1 M NaCl, and the cell debris was removed by centrifugation. The supernatant was dialyzed against buffer containing 20 mM Tris-HCl, pH 7.5, and 0.1% Triton X-100. Linear-extrapolation model (14) according to Equation 2, $\Delta G_{\text{binding}} = \Delta G^{\text{D240L}} - m[D] - RT \ln K_{\text{binding}}$ (Eq. 2)

**Aggregation Suppression Experiments**—Measurements of thermally induced aggregation of citrate synthase (CS) were carried out essentially as described (9). Porcine heart CS (Sigma) was diluted to 0.075 μM in 1 ml of 40 mM Hepes, pH 7.5, buffer that was prewarmed to 43 °C in a thermostatted cell of a SLM Amino-Bowman Series 2 fluorescence spectrometer. Right angle light scattering was recorded over time at 43 °C with excitation and emission wavelengths at 465 nm and slit widths at 4 μm. HuAtp12p (wild type or mutant) or other additives were included in the incubation at the concentrations indicated for each experiment. CS concentration was determined using the extinction coefficient of 1.78 for a 1 mg/ml solution of the dimer at 280 nm (10).

**Proteolytic Digestion**—For analytical experiments 5 μg of wild type or mutant HuAtp12p were mixed with serial dilutions of trypsin or chymotrypsin ranging from 0.01 to 3% in 20 μl of 10 mM Tris-HCl, pH 7.5. Proteolysis was allowed to proceed for 30 min at 37 °C, at which point the reactions were stopped with the addition of trichloroacetic acid to 10%. Precipitated protein was collected by centrifugation in a microcentrifuge, suspended in SDS gel buffer, and resolved in a 12% SDS-polyacrylamide gel. To identify the principal proteolytic fragment of highest molecular weight, a 100-μg sample of HuAtp12p was first digested with either trypsin or chymotrypsin at 1% in 6 μl and after the reaction was quenched, the acid-precipitated protein was resolved in an SDS gel, and then transferred to polyvinylidene difluoride membrane (Schleicher & Schuell, WESTRAN®) as described (11). The amino-terminal residues of the immobilized proteolytic fragment were identified through the Edman degradation protocol using the facility of the Department of Genetics at the University of Georgia.

**Chemical Denaturation Studies and Data Analysis**—Wild type or mutant HuAtp12p (1 μg) was incubated in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl supplemented with increasing amounts of guanidine hydrochloride (GdnHCl) at 23 °C for 3 h. Equilibrium was reached during this time period. The concentration of GdnHCl in each experimental sample (0–5 M range) was determined from its refractive index (12). Intrinsic fluorescence emission spectra were recorded between 300 and 400 nm after excitation at 280 nm. Slit widths were set at 4.0 nm.

The red shift in intrinsic fluorescence emission spectra at increasing GdnHCl concentrations were quantified as the intensity-averaged emission wavelength, $\lambda_{\text{avg}}$ (13), calculated according to Equation 1,

$$\lambda_{\text{avg}} = \frac{\sum f(\lambda)}{\sum f(\lambda)}$$ (Eq. 1)

where $\lambda_i$ and $f(\lambda_i)$ are the emission wavelength and its corresponding fluorescence intensity at that wavelength, respectively. Base-line and transition-regions data for the first and second component of the HuAtp12p GdnHCl equilibrium denaturation curve were fitted to a two-state linear-extrapolation model (14) according to Equation 2,

$$\Delta G_{\text{binding}} = \Delta G^{\text{D240L}} - m[D] - RT \ln K_{\text{binding}}$$ (Eq. 2)

where $\Delta G_{\text{binding}}$ is the free energy change for unfolding at a given denaturant (GdnHCl) concentration, $\Delta G^{\text{D240L}}$ is the free energy change for unfolding in the absence of denaturant, $m$ is the slope of a linear-extrapolation model (14), which equates the change in $\Delta G_{\text{binding}}$ per unit concentration of GdnHCl, $R$ is the gas constant (1.987 cal mol⁻¹ K⁻¹), $T$ is the temperature (296.15 K), and $K_{\text{binding}}$ is the equilibrium constant for unfolding. The linear extrapolation model expresses the signal ($\lambda_{\text{avg}}$) in a function of GdnHCl concentration according to Equation 3 (15),

$$\lambda_{\text{avg}} = \frac{\sum f(\lambda)}{\sum f(\lambda)}$$ (Eq. 1)

where $\lambda_i$ is the observed signal, $f(\lambda_i)$ is the base-line intercepts corresponding to, respectively, native and denatured proteins, $m$, and $m_i$ are the corresponding base-line slopes, [X], is the denaturant concentration corresponding to the i-th addition, $m$ is the slope of a 4G unfolding versus [X] plot, $R$ and $T$ are the gas constant and temperature as defined above, $[D]_{150}$ is the denaturant (GdnHCl) concentration at the midpoint of the transition, in which there is an equal amount of the folded and unfolded protein ($K_{\text{binding}} = 1$). Therefore, from Equation 2, it follows that

$$\Delta G_{\text{binding}} = \Delta G^{\text{D240L}} - m[D]_{150} - RT \ln K_{\text{binding}}$$ (Eq. 2)

The change in free energy of unfolding on mutation, $\Delta G^{\text{D240L}}$, between wild type and mutant HuAtp12p can be calculated from the

4 The abbreviations used are: CS, citrate synthase; GdnHCl, guanidine hydrochloride.
corresponding glutamic acid (Glu-240) of HuAtp12p to lysine
tagenesis of the pPROEx/Atp12ph plasmid insert to convert the
HuAtp12pE240K eluate from cobalt column (3.7 µg) to sonically
irradiated cells (Fig. 1, lane 2) . The protein is purified
in vitro effect of this mutation
purification. The first step in this project has been to
Atp12p function to experiments that explore features of the
HuAtp12p were recorded between 300 nm and 400 nm for 1-ml samples
of Lowry
immuno-decorated Atp12p by chemiluminescence was as described (5).
Sedimentation of molecular weight standards as described under “Experimental
Purification of recombinant HuAtp12p proteins. SDS-polyacrylamide gel of total bacterial protein (26 µg) from isopropyl
β- D-thiogalactopyranoside-induced phenimidbearing cells (lane 1), soluble protein fraction (16 µg) recovered after cell breakage (lane 2), pooled wild type HuAtp12p eluate from cobalt column (3.3 µg) (lane 3); pooled HuAtp12pE240K eluate from cobalt column (3.7 µg) (lane 4). The migration
of molecular weight standards is shown on the left-hand side. The arrow indicates the position of HuAtp12p in the protein gel.

RESULTS
Purification of Recombinant HuAtp12p—The complete cDNA for HuAtp12p encodes a protein with 289 amino acids, which includes a mitochondrial targeting sequence at the amino terminus (7). Cleavage of the precursor to the mature form is estimated to occur at/near the junction of Thr-32 and Ile-33 following its passage into mitochondria (7).

In previous work we have shown that the polypeptide fragment of HuAtp12p encompassing Ile-33 through Glu-289 closely approximates the true mature form of the protein since this fragment rescues the respiratory defect of a yeast Atp12 deletion strain and binds to the F1 α subunit in a yeast two-hybrid screen (7).

In the present work we have extended our studies of Atp12p function to experiments that explore features of the purified protein. The first step in this project has been to subclone codons 33 – 289 of HuAtp12p cDNA in a bacterial expression vector to create the plasmid pPROEx/Atp12p (see “Experimental Procedures”). Induction of expression from pPROEx/Atp12p leads to high production of His-tagged HuAtp12p (MF = 32,770) in E. coli (Fig. 1, lane 1) of which 60–70% partitions to the soluble fraction after centrifugation of sonically irradiated cells (Fig. 1, lane 2). The protein is purified to near homogeneity after successive chromatographic steps through DEAE and cobalt columns (Fig. 1, lane 3).

Previous work with yeast Atp12p identified a Glu → Lys mutation that inactivates the protein in vivo (8). To study the effect of this mutation in vitro we performed site-directed mutagenesis of the pPROEx/Atp12p plasmid insert to convert the corresponding glutamic acid (Glu-240) of HuAtp12p to lysine

(see “Experimental Procedures”). Highly pure preparations of the His-tagged mutant protein (HuAtp12pE240K) (Fig. 1, lane 4) are obtained following the same method described above for wild type recombinant HuAtp12p purification.

Molecular Chaperone Assays with HuAtp12p—We have proposed that the true substrate of Atp12p in mitochondria is a folded F1 α subunit monomer (5). Atp12p is believed to act by temporarily shielding a hydrophobic element of the α subunit surface that will ultimately be buried at the non-catalytic site interface with an adjacent β subunit of the assembled α3β3 oligomer. To study this activity in vitro we initially tried to obtain recombinant F1 α subunit as a soluble monomer. Because there are no Atp12p homologs in E. coli and there is no evidence from yeast two-hybrid screens of mitochondrial Atp12p interaction with E. coli F1 α, our work was focused on constructing plasmids for the expression of yeast and bovine mitochondrial F1 α. Unfortunately, forms of these recombinant proteins that accumulated either in the cytosol or in the periplasm (via a leader peptide) were not soluble; the former produced inclusion bodies, and the latter were found to be tightly associated with the membrane, requiring harsh alkaline conditions for extraction. Ultimately, efforts to recover the α subunit from urea-denatured inclusion bodies in a conformation that would be bound by Atp12p in vitro were not successful. As for using surrogate protein substrates to study the molecular chaperone activity of HuAtp12p and assess the effect

D. Sheluho and S. H. Ackerman, unpublished studies.
of mutations in vitro, we found that, for example, HuAtp12p did not inhibit the aggregation of reduced insulin b-chains, an assay that had proved particularly useful in assessing in vitro the molecular chaperone activity of yeast Atp11p (20). Instead, in our screen of potential substrates we identified mitochondrial CS as a suitable client protein for HuAtp12p. CS is a dimeric protein of 2 identical 48-kDa subunits that catalyzes the formation of citric acid and CoA from oxaloacetate and acetyl-CoA. In the absence of substrates CS is inactivated and rapidly aggregates after exposure to 43 °C, a property that has made it popular as a model substrate for studies of molecular chaperone activity in vitro (9, 21–24). We monitored thermally induced aggregation of CS at 43 °C by measuring the apparent fluorescence due to light scattering at 465 nm of solutions containing the enzyme in the absence or presence of other proteins. The presence of wild type HuAtp12p in the mixture has different effects on the CS aggregation phenomenon depending on the molar ratio of molecular chaperone (monomer) relative to CS dimers. At low molar ratios (3:1 and below), the degree of sample light scattering is exacerbated over what is observed for solutions of CS alone (Fig. 3A, data shown for 1:1 and 2:1 ratios). Molar ratios of HuAtp12p:CS in the range of 4:1–7:1 provide conditions under which the rate of CS aggregation is reduced, although the final degree of light scattering is the same as in the absence of HuAtp12p (Fig. 3A, data shown for 4:1 and 5:1 ratios). Raising the level of HuAtp12p to 8-fold molar excess over CS reduces the final degree of sample light scattering to ~40% of the maximum observed in the absence of effector protein (Fig. 3A, open triangles). Increasing the molar ratio of HuAtp12p:CS above 8:1 does not further improve the level of protection (data not shown). The amount of CS and HuAtp12p protein recovered in the particulate fraction after centrifugation of 1:1 and 8:1 protein mixtures exposed to 43 °C is shown in the inset of Fig. 3A. Bovine serum albumin fails to prevent thermally induced aggregation of CS when the effector protein is present at 8-fold or even 16-fold molar excess relative to CS dimers (Fig. 3B). Companion studies examined HuAtp12p for an affect on preventing loss of CS enzymatic activity at 43 °C. We found that the kinetics of CS inactivation is unaltered in the presence of the chaperone (Fig. 3C).

In contrast to the wild type protein, the mutant HuAtp12pE240K does not prevent the aggregation of CS at 43 °C when present in an 8-fold molar excess relative to the enzyme dimer concentration in the cuvette (Fig. 4). Increasing the molar ratio to 16:1 increased the time necessary to observe maximal CS aggregation, but the final degree of light-scattering observed for the sample was the same as in the absence of HuAtp12p.

Physical Characterization of Wild Type and Mutant HuAtp12p—Several parameters were measured for wild type HuAtp12p and HuAtp12pE240K to determine whether gross alterations of the three-dimensional structure of the two proteins could explain the loss of activity in the mutant. Titration of free sulphydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid) gave similar results with both proteins; of the three cysteines in mature HuAtp12p, one is accessible to the reagent under non-denaturing conditions, whereas all three react with the probe after denaturation of either the wild type or mutant protein in 8 M urea. The tryptophan emission spectra of the probe after denaturation of either the wild type or mutant indicated that samples were of CS alone (●) or of CS in combination with 0.60 μM (○) (8:1 ratio) or 1.2 μM (□) (16:1 ratio) bovine serum albumin. C, influence of wild type HuAtp12p on the inactivation of CS at elevated temperature. Inactivation of CS (0.075 μM) is shown at 43 °C in the absence (●) or in the presence of 0.60 μM HuAtp12p (△) (8:1 ratio). Molar ratios refer to amount of effector protein relative to CS dimer.

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significant difference in the amplitude or wavelength of maximal fluorescence (data not shown). We also examined the recombinant proteins for sensitivity to proteolytic digestion (Fig. 5). Wild type HuAtp12p (Fig. 5, upper panels) and HuAtp12pE240K (Fig. 5, lower panels) were exposed to increasing amounts of trypsin or chymotrypsin and then loaded on SDS-polyacrylamide gels to analyze the products of digestion. The digestion patterns for wild type and mutant HuAtp12p are essentially the same. Both proteases yield one principal digestion product of ~29 kDa, which correlates well with the size of the mature HuAtp12p protein encoded by the expression plasmid. Amino-terminal sequencing of the 29-kDa fragments showed that they result from either trypsin or chymotrypsin cleavage of 7 or 9 amino acids, respectively, downstream from the start of HuAtp12p-coding region (Fig. 5, bottom). Hence, the limited proteolysis only removes the tag sequence at the amino terminus of the recombinant proteins and does not disclose the presence of hypersensitive proteolytic sites in either the wild type or mutant proteins.

Chemical Unfolding Studies—GdnHCl denaturation curves were obtained for wild type and mutant HuAtp12p. Purified recombinant protein was incubated with increasing GdnHCl concentrations at 23 °C for 3 h. Exposure to the chaotrope resulted in changes of the intrinsic fluorescence emission from the HuAtp12p proteins that were manifest as a red-shift in maximal emission wavelength. The intensity-averaged emission wavelength (λ_{avg}), an integral measurement that is negligibly influenced by the noise, was calculated for each sample (Equation 1 under “Experimental Procedures”) and plotted versus GdnHCl concentration (Fig. 6). Wild type and mutant HuAtp12p both show two distinct and unequal components in the equilibrium denaturation curve, suggesting that either HuAtp12p consists of two domains or that there is a stable unfolding intermediate. Light-scattering measurements indicate that there is protein aggregation at the concentrations of GdnHCl (0–2.2 M) corresponding to the first component of the equilibrium denaturation curve (Fig. 6, inset) but not to the second (data not shown). Thermodynamic parameters associated with the two parts of the curve were calculated for HuAtp12p and HuAtp12pE240K (Table I) from a fit to the data of a model consisting of the sum of two components, each representing a direct transition from folded to denatured state (Equation 3 under “Experimental Procedures”). Because of the high standard errors for the data corresponding to low denaturant concentrations (Table I), a quantitative analysis of the thermodynamic parameters is reliable only for the part of the equilibrium denaturation curve corresponding to high denaturant concentrations. A ΔΔG^H_C (E240K-wt) = −1.38 kcal/mol (indi-
cating the mutant is less stable than the wild type) was calculated for this part of the curve. This value of $\Delta G^\alpha$ at any denaturant concentration to that of $\Delta G^\alpha$, can be easily appreciated between wild type and mutant in the slope of the transition region of the second component of the denaturation curve (Fig. 6). The smaller m value of the E240K mutant (1.47 kcal/mol M$^{-1}$) versus the wild type (2.21/mol M$^{-1}$) suggests that in the mutant there may exist a folding intermediate that is more exposed to solvent (14).

**DISCUSSION**

Previous studies from our laboratory measured the physical properties for Atp12p as it exists in the yeast mitochondrial matrix. We found that the protein is part of an oligomer of $\sim 70$–80 kDa (25), that such oligomerization is dependent on sequence at the carboxyl terminus of the protein (8), that it is the oligomeric form of Atp12p, which is functional in vivo (8), and that the oligomeric state of Atp12p is dependent on the absence or presence of the $F_1\alpha$ subunit (5). It has not been possible to extend the studies of yeast Atp12p to in vitro analysis because the protein forms inclusion bodies after overproduction in bacteria, and there are doubts about the functional integrity of the refolded protein (8). In contrast human Atp12p (HuAtp12p) remains largely soluble when produced in E. coli, and this has opened the door to experiments that probe the structural and functional properties of this protein in greater detail. A principal objective of the current work has been to analyze a Glu $\rightarrow$ Lys substitution that knocks out Atp12p action in vivo (8). Site-directed mutagenesis was used to change the relevant glutamic acid of HuAtp12p (residue 240) to lysine, and the mutant and wild type recombinant proteins were purified from bacterial host (Fig. 1) and studied in parallel.

The Atp12p gene product is a monomeric species as illustrated by the sedimentation profile obtained for purified HuAtp12p (both wild type and E240K mutant) in experiments that were designed to resolve monomers from dimers in the linear portion of a sucrose gradient and to capture high molecular weight assemblies, if present, at the 20%:80% concentration interface (Fig. 2). On this basis we conclude that the molecular weight for Atp12p is shifted toward a lower value in samples heterogeneous in nature. It is of interest that the molecular mass of recombinant Atp12p increases with all other amino acids (with the exception of proline) in either an $\alpha$-helix or $\beta$-sheet (see Table 17.3 in Ref. 14). A change in m, which is a constant of proportionality correlating the value of $\Delta G^\text{unfolding}$ to any denaturant concentration to that of $\Delta G^\alpha$, can be easily appreciated between wild type and mutant in the slope of the transition region of the second component of the denaturation curve (Fig. 6). The smaller m value of the E240K mutant (1.47 kcal/mol M$^{-1}$) versus the wild type (2.21/mol M$^{-1}$) suggests that in the mutant there may exist a folding intermediate that is more exposed to solvent (14).

**TABLE I**

| Protein          | First component* | Second component* |
|------------------|------------------|-------------------|
|                  | $\Delta G^\alpha$ | $m$ | $\Delta G^\alpha$ | $m$ |
| HuAtp12p$^{WT}$  | 2.85 ± 5.17      | 6.73 ± 9.13      | 0.43 ± 0.27 | 5.41 ± 0.41 |
| HuAtp12p$^{E240K}$ | 3.39 ± 2.20 | 5.82 ± 3.60 | 0.58 ± 0.08 | 4.93 ± 0.79 |

*Values of thermodynamic parameters (±S.E.) were obtained by non-linear regression analysis of the data according to Equations (3) and (4).
an aggregation-prone form of the F₁ α subunit in a protected state until the conditions are ready for the next step in the assembly pathway.

The phenotype of atp12 yeast mutants has led us to suggest that Atp12p serves a specialized role in the F₁ assembly pathway in mitochondria (3), as opposed to being a "general purpose" chaperone with a broad substrate clientele. In fact, recombining HuAtp12p shows selectivity even with model proteins as it is completely ineffective in preventing the aggregation of reduced insulin, which is instead a surrogue client protein for Atp11p in vitro (20, 28). At this time we do not know the common features between thermally denatured CS and unassembled F₁ α subunits that enable these two proteins to be bound by HuAtp12p. Perhaps, during the thermal denaturation of dimeric CS, hydrophobic regions of the monomers that are hidden from solvent at the dimer interface produce an uncontrolled aggregation of the monomers, which is prevented by the shielding properties of HuAtp12p. We also do not have a good explanation for why the maximal effect of HuAtp12p requires that it be present in 8-fold molar excess over the CS dimer. It suffices, however, that the CS aggregation assay has provided us with a tool to assess Atp12p function in vitro and to examine the effect of mutations on Atp12p molecular chaperone activity. To this end we show that the E240K mutation severely alters the function of HuAtp12p. There was no protection by HuAtp12p E240K against CS aggregation at 43 °C when the mutant protein was included in 8-fold molar excess (Fig. 4). At best, a 16-fold molar excess HuAtp12p E240K (Fig. 4) slows down aggregation in a manner that is comparable with what is observed with 4- or 5-fold molar excess wild type HuAtp12p (Fig. 3A). These results obtained in vitro are in agreement with the observation that the corresponding yeast mutant Atp12p (E289K) is inactive in vitro (8) and substantiate the point that our in vitro studies reflect the physiological function of Atp12p.

Wild type and E240K mutant HuAtp12p were found to be similar with respect to several measured physical parameters, including accessibility of cysteines to labeling with 5,5'-dithio bis(2-nitrobenzoic acid) under native and denaturing conditions (data not shown), intrinsic fluorescence spectra obtained under native conditions (data not shown), and sensitivity to limited tryptic digestion (Fig. 5). The latter work established that there is no proteolytic hot spot in HuAtp12p that could define the boundary between subdomains in the protein. However, the GdnHCl equilibrium denaturation curves for both wild type and mutant HuAtp12p were found to be biphasic (Fig. 6), suggesting the existence of a stable intermediate between the native and fully denatured protein, possibly associated with the unfolding of a subdomain of HuAtp12p. The first phase of the denaturation curves is also associated with some protein aggregation, as indicated by an increase in light scattering (Fig. 6, inset). This aggregation might be caused by the exposure of a hydrophobic subdomain that is buried within HuAtp12p under native conditions. We further speculate that this subdomain may be the "active" surface of the chaperone that shields the exposed hydrophobic surfaces of unassembled F₁ α subunits (3) or thermally denatured CS (Fig. 3A). Under this point of view, the perturbation of HuAtp12p structure observed at low concentrations of GdnHCl may mimic a conformational switch that occurs naturally when HuAtp12p interacts with a substrate protein.

The second phase of the equilibrium denaturation curve of both the wild-type and mutant HuAtp12p was fitted to a two-state cooperative (all or none) transition between native and denatured states. The appreciable difference between wild type and mutant in the values of m (the slope around the curve mid-point) suggests the existence of a state R in equilibrium with the native state N. In the mutant E240K the equilibrium N ↔ R may be shifted toward R, which could be a different conformation of the protein with a higher solvent exposure and a correspondingly lower value of m (14). The extremely low concentration of R at equilibrium may be the reason why the individual components of the GdnHCl equilibrium denaturation curves of HuAtp12p are fitted reasonably well by Equation 3, which describes a two-state cooperative transition. In this view the existence of two components in the equilibrium chemical denaturation curve of HuAtp12p and the different slope of the second component of this curve in the inactive mutant E240K are consistent with the R state being the conformation of the chaperone that binds to the hydrophobic surfaces of the target proteins. The Glu → Lys substitution might increase the probability of this state to occur but decrease its intrinsic chaperone activity.

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