The Primary hyperoxaluria type I is a rare autosomal recessive disease caused by mutations in the alanine glyoxylate aminotransferase gene (AGXT). We have previously shown that Pro11Leu and Ile340Met polymorphisms together with Ile244Thr mutation (AGXT-LTM) represent a conformational disease that could be amenable to pharmacological intervention. Thus, the study of the folding mechanism of AGXT is crucial to understand the molecular basis of the disease. Herein, we provide biochemical and structural data showing that AGXT-LTM is able to form non-native folding intermediates. The three dimensional structure of a complex between the bacterial chaperonin GroEL and a folding intermediate of AGXT-LTM mutant has been solved by cryo-electron microscopy. The electron density map shows the protein substrate in a non-native extended conformation that crosses the GroEL central cavity. Addition of ATP to the complex induces conformational changes on the chaperonin and the internalization of the protein substrate into the folding cavity. The structure provides a three dimensional picture of an in vivo early ATP dependent step of the folding reaction cycle of the chaperonin and supports a GroEL functional model in which the chaperonin promotes folding of the AGXT-LTM mutant protein through forced unfolding mechanism.

Chaperonins are ATP dependent molecular machines that are able to bind misfolded protein substrates and promote their proper folding (1). Protein misfolding is the main mechanism involved in a large number of genetic diseases caused by missense mutations, giving rise to the concept of ‘conformational diseases’ (2, 3). The role of chaperones in the pathogenesis of conformational diseases has been recently reviewed (4). A deficiency of the hepatic enzyme alanine glyoxylate aminotransferase (AGXT, also known as AGT) is responsible for the rare hereditary disease primary hyperoxaluria type 1 (PH1, OMIM #259900). Insufficient AGXT activity leads to increased conversion of the substrate glyoxylate to the toxic ion oxalate, which subsequently accumulates as insoluble calcium salt in the kidney. In the later stages of the disease, calcium oxalate deposition becomes widespread and life-threatening unless liver and kidney transplantation is performed (5). Most of the PH1 alleles detected in the Canary Islands patients carry the Ile244Thr mutation together with the polymorphisms Pro11Leu and Ile340Met. These amino acid changes result in a misfolded protein (AGXT-LTM) that undergoes stable interaction with molecular chaperones and aggregation (6). A large number of the pathogenic variants of this protein are the result of missense mutations that are likely to result in misfolded proteins (7). Thus, the study of these folding intermediates and the effect of the point mutations on the structure of the protein are central to elucidate the molecular basis responsible for the PH1 disease.

The bacterial chaperonin-cochaperonin GroEL-GroES system is well characterized both at the...
biochemical and structural level (8, 9). GroEL is a large homo-oligomeric barrel structure formed by two rings each one comprising 7 subunits of a 57KDa polypeptide chains. Each GroEL ring displays a large central cavity lined by hydrophobic residues at its entrance, which are responsible for substrate binding (10). GroES is a 70 kDa homohexameric ring which caps both ends of the GroEL barrel and plays an important role in the folding cycle of GroEL (11). The GroEL folding cycle first includes binding of a protein substrate to the apical domains of one of the rings (the cis-ring) followed by binding of ATP and GroES. This triggers a conformational change that makes the central cavity hydrophilic and leads to the encapsulation of the protein substrate. Binding of GroES and ATP to the opposite ring (trans-ring) results in the release of a properly folded protein substrate. The protein molecules that fail to fold are re-captured by GroEL for another reaction cycle.

To investigate the AGXT-LTM conformational changes responsible for PH1 in our patients, we have used an E. coli strain that overexpresses GroEL-GroES system and AGXT-LTM protein. This enables to trap in vivo folding intermediates of mutant AGXT and GroEL. This complex was three-dimensionally reconstructed by cryoelectron microscopy (Cryo-EM) and image processing techniques, providing information of an early stage in the folding reaction cycle. The electron density map reveals that AGXT-LTM crosses the GroEL central cavity making contacts with the apical domains of distant GroEL subunits. An atomic model of AGXT derived from its x-ray structure (16) fits very well with the mass of the complex attributable to AGXT-LTM mutant protein suggesting that the mutant protein is able to partially fold. Such folding intermediates could be targeted by molecular chaperones or other drugs to rescue the native conformation. Our results support the hypothesis that the assisted folding of AGXT-LTM mutant is driven through the forced unfolding of a quasi-native state of the protein.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transformations.** Top10 Escherichia coli (Invitrogen) were transformed with AGXT constructs (6) and GroEL-ES plasmids (Takara Inc.), using standard protocols (17). They were grown at 37°C in LB broth. GroEL-GroES expression was induced with 2mg/ml L-arabinose at the beginning of the culture. AGXT expression was induced with 0.5 mM isopropyl-D-thiogalactoside (IPTG) when the O.D. was 0.4-0.6, and the culture was incubated for additional 4h at 25°C.

**AGXT Enzymatic Assay.** AGXT activity from the soluble extract was determined as described (6). Activity units are expressed as μmol pyruvate/hr/mg total protein.

**Western Blots.** Cell pellets from 1ml of culture were sonicated in 100μl (50 mM TrisHCl, pH 7.4; 150 mM NaCl, protease inhibitors (Complete, Roche; Gipf-Oberfrick, Switzerland) 0.05% NP-40 and 0.05% Triton X-100). The soluble fraction was separated by centrifugation and the protein concentration was measured with bicinchoninic acid. Equal amounts of each extract (1 μg) were analyzed by immunoblotting (17) with anti-hAGXT (1:5,000) (rabbit, raised against recombinant AGXT). Peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch) was used as secondary antibody (1:10,000), and the chemiluminescence substrate was from Pierce.

**Protein Purification.** Cells were harvested, and the His-tagged AGXT was purified using HisTrap FF columns (GE Healthcare) according to the manufacturer’s instructions. Soluble AGXT was batch purified under native conditions and it was eluted from the Ni²⁺ resin in a buffer with 500 mM imidazole. Imidazole was removed using a PD-10 column (GE Healthcare) and protein was concentrated by filtration (UltraFree 0.5, Millipore Corporation, Bedford, MA, USA). GroEL was copurified with AGXT due to protein-protein interactions. Purified proteins were subjected to a size exclusion chromatography (Bio-Silect SEC 250, BioRad) in order to isolate the complex. The purified complex was used to test the effect of ATP in the interaction between both proteins after 20 minutes of incubation.
Electron Microscopy. For electron microscopy of negatively stained samples, 5 μl aliquots of a solution containing 0.05 mg/ml GroEL:AGXT-LTM complexes buffered with 10mM Tris HCl pH 7.5, 50mM NaCl were applied to glow-discharged carbon coated collodion grids for 1 min and then stained for 1 min with 2% uranyl acetate. Images were recorded in a JEOL 1200EX-II electron microscope operated at 100 kV and recorded at 20,000 nominal magnification. For cryoelectron microscopy, 5 μl aliquots were applied to holey carbon grids (Quantifoil) for 2 min, blotted for 5 seconds and frozen rapidly in liquid ethane. Images were recorded under minimum dose conditions in a FEI G2 FEG electron microscope operated at 200 kV using a Gatan cold stage and recorded on Kodak SO-163 film at 50,000 nominal magnification between 1.5 and 3.0 μm underfocus.

Image Processing, Two-Dimensional Averaging and Three-Dimensional Reconstruction. Micrographs were digitized in a Zeiss Photoscan scanner with a final sampling window corresponding to 3.5 Å /pixel for negatively stained samples and 2.8 Å per pixel for vitrified samples. Contrast transfer function (CTF) of micrographs was estimated using CTFFIND software (18) and corrected using BSOFT (19). Images were 2D processed and aligned using maximum likelihood reference free methods using the XMIPP package (20, 21). To analyze the heterogeneity of the particles, the images were subjected to Kohonen’s self-organizing feature maps (22). After this classification, homogeneous populations were obtained and averaged.

To generate an initial model, either for the nucleotide-free or the ATP-bound samples, selected particles were subjected to a common lines procedure implemented in EMAN software package (23) imposing C7 symmetry. Working with a total of 11,011 and 8,303 images (for nucleotide-free and ATP-bound samples respectively) these initial rough models were refined imposing 7-fold or no symmetry using EMAN (23) and SPIDER software subsequently (24). After reaching the convergence of these refinements, the reconstructions yielded resolutions of 23 Å and 24 Å for non symmetrized nucleotide-free and ATP-bound structures and of 27 Å for the non symmetrized native structure (Fourier Shell Correlation of 0.3 criterion). These models obtained from the negatively stained preparations were scaled and input to a two independent refinement using 10,312 CrioEM particles with or without imposing C7 symmetry, using SPIDER software (24). The resolution of the final maps were estimated to 12 Å and 18 Å for the non symmetrized and symmetrized structures. These values were used to low-pass filter the volume in the final model (Fig. 3A and S2).

Docking experiments were carried out using SITUS software (25). Volume handling was carried out using XMIPP software (20) and general visualization was performed using Chimera (26) and PyMol (27).

RESULTS AND DISCUSSION

The coexpression of the GroEL/GroES and AGXT-LTM increases the solubility of the mutant protein and results in the formation of a GroEL:AGXT-LTM stable complex. We first measured the AGXT activity from the soluble extracts when AGXT-LTM was expressed in E. coli either in the absence or presence of the GroEL-GroES. Our data show that the AGXT activity of E.coli lysates increases when mutant AGXT-LTM is expressed in presence of the GroEL-GroES (Fig. 1A). Western blot analysis shows that this increase in activity is related with the amount of soluble AGXT-LTM mutant protein (Fig. 1B). This result indicates that GroEL-GroES co-expression improves, in vivo, the yield of soluble and active AGXT-LTM mutant protein.

Lysates overexpressing His-tagged AGXT-LTM and GroEL-GroES were subjected to nickel-affinity chromatography. The SDS-PAGE electrophoresis of the eluted fractions showed two bands corresponding to the molecular weight of AGXT-LTM protein and to GroEL protomers. A final purification step by gel filtration chromatography revealed that the largest proportion of AGXT-LTM copurifies as a complex with GroEL (Fig. 1C). Docking experiments results suggest that GroEL captures partially folded copies of AGXT-LTM mutant protein for a folding reaction cycle. Once the cell lysis occurs and the ATP is consumed, the folding reaction is blocked, trapping a stable GroEL:AGXT-LTM complex. The overexpression of the wild-type AGXT together with the GroEL-GroES system produced similar complexes, but the amount of stable and folded AGXT protein predominates (Fig. 1C).

The purified complex was then incubated in the presence of a 10 molar excess ATP (ATP-bound) for comparison purposes. The new sample was subjected to the same purification steps and the
analysis of the SDS-PAGE electrophoresis confirms that the GroEL:AGXT-LTM complex is largely maintained in presence of ATP.

Electron microscopy (EM) confirms two conformational steps of GroEL trapping AGXT-LTM mutant. The resulting GroEL:AGXT-LTM complex was stained for observation in the electron microscope to elucidate the nature of the interaction between both proteins before and after the conformational change undergone by GroEL upon ATP binding (see Experimental procedures section and Supp. Mat. Fig. 1). 11,011 and 8,303 particles of the nucleotide-free and ATP-bound GroEL:AGXT-LTM complex were selected respectively, and two-dimensional (2D) averages were generated using a Maximum Likelihood 2D reference-free alignment process (Fig. 2A). The analysis revealed several classes which encompass the two typical views of GroEL, the end-on view revealing its heptameric nature, and the side view showing its two ring structure. The analysis of these classes confirms the formation of GroEL:AGXT-LTM complexes as the end-on view averages clearly show some extra density at the central folding cavity. The comparison of the equivalent 2D classes corresponding to the nucleotide-free and ATP-bound samples revealed structural changes (Supp. Mat. Fig. 1). We analyzed these differences by comparing their low-resolution three-dimensional (3D) structures generated after angular refinement. Seven-fold symmetrized volumes of the two samples were first generated. The comparison of the two 3D reconstructions showed differences both in the conformation of the GroEL chaperonin and in the relative position of the substrate with respect to the chaperonin (Fig. 2B and 2C). The nucleotide-free structure showed around 10% increased in height and width of the GroEL barrel with respect to the size of the ATP-bound sample, being the latter similar to the size observed for the substrate-free, nucleotide-free GroEL (10). This enlargement results in an expansion of the folding cavity that has already been reported for other substrate-bound GroEL complexes (28, 29) and has been attributed to the plasticity of the GroEL ring to accommodate large protein substrates. Both complexes display extra density occluding the GroEL cavity that can be uniquely attributed to the presence of the protein substrate. However, this density protrudes from the chaperonin in the nucleotide-free complex and is internalized upon ATP binding.

In order to further characterize the interactions between GroEL and the bound, unfolded protein, a three-dimensional reconstruction of the two different complexes was attempted without imposing any symmetry. It was not possible to obtain a stable asymmetric model for the ATP-bound preparation. This could be a reflection of heterogeneity in our data set due to substrate structural conformational variability. Indeed, the substrate density in the ATP-bound symmetrical model is considerably smaller than that observed in the nucleotide-free complex. This effect cannot be solely attributed to the application of 7-fold symmetry but also to the variable conformational distribution of the substrate in the GroEL cavity in the ATP-bound complex. Further evidence of this heterogeneity can be found in the analysis of the representative averaged end-on views obtained in the 2D analysis of the samples. While the nucleotide-free sample appears to be quite homogeneous, the ATP-bound sample is clearly heterogeneous, with the substrate adopting several conformations (see Fig. 2A). This is in agreement with the data presented by Elad et al. 2008 (30) in which unfolded malate dehydrogenase internalized into GroEL cavity adopts several conformations. On the other hand, the analysis of the low resolution nucleotide-free structure provides information of the nature of the GroEL:AGXT-LTM interaction. The level of resolution of this complex (23 Å) allows the identification of the density corresponding the AGXT-LTM mutant protein. It crosses the GroEL central cavity and makes contacts with distant GroEL apical domains.

Cryo-EM confirms that AGXT-LTM is partially folded and provides the visualization of an early step of the folding reaction cycle. To define a higher resolution description of the native complex, the sample was subjected to Cryo-EM techniques (see Experimental procedures section and Supp. Mat. Fig. 2). More than 10,000 particles were selected and prepared for angular refinement methods. The models from the negative stained samples were the input for further refinement. Two independent refinement processes were carried out, with and without imposing 7-fold symmetry. The three-dimensional reconstruction obtained without imposing symmetry allowed to obtain a model that contains details on the interaction between GroEL and AGXT-LTM mutant but the resolution obtained was significantly lower. On the contrary, the refinement imposing 7-fold symmetry lacked this detailed information but could achieve better
resolution for the chaperone moiety (Supp. Mat. Fig. 2). Thus, the final structure shown in Fig. 3A represents a composite map formed by the GroEL moiety derived from the structure refined with symmetry and the substrate mass derived from the volume refined without symmetry. Therefore, the resolution for either section of the model is different: 12 Å for the chaperone and 18 Å for the substrate complex.

The atomic coordinates of the GroEL crystal structure (PDB code 1OEL, 31) were fitted into the EM density map as described in the Experimental procedures. Considering each GroEL protomer as a rigid body, the analysis of the fitting clearly showed that GroEL apical domains lay outside the map. Then, we proceeded to readjust the position of the domain individually. This rendered an expanded GroEL “open” barrel in which the connections between the two ring structures are maintained but the apical domains are twisted by approximately 20 degrees (Fig. 3B). We did not attempt to carry out any finer adjustments of the atomic coordinates to the Cryo-EM map.

The extra density located in the cavity can be unequivocally assigned to AGXT-LTM and it is structured in two domains. These are clearly asymmetric, each one interacting with the apical domains of opposite GroEL subunits. AGXT is a homodimer formed by two 43 KDa subunits, whose atomic structure is known (PDB code 1H0C, 16). Each protomer is folded into a large N-terminal domain (residues 22–282) and a smaller C-terminal domain (283–392). Besides, a long unstructured N-terminal tail (residues 1-21) grabs the subunits within the dimer (Supp. Mat. Fig. 3). The analysis of the size and shape of the AGXT-LTM density suggests that the protein adopts an almost folded but open conformation in which the two domains of AGXT interact with distant apical domains of GroEL. The AGXT atomic coordinates were used to define its two topological domains and fit them into the CryoEM density map. Each domain fits well in the CryoEM map. Remarkably, the unfolded AGXT binding. Remarkably, the unfolded AGXT interacts solely with the apical domains of GroEL. In this model, the surface patches responsible of the AGXT dimerization face either the hydrophobic patches of the apical domains or the folding cavity of the chaperone. This situation displays a striking similarity to that observed for the chaperonin-containing TCP1 (CCT) interaction with actin and tubulin (32, 33) in which CCT stabilizes both cytoskeletal proteins in open and quasi-folded conformations through interactions between distant subunits of the chaperonin ring.

The combined biochemical and structural analysis on the GroEL:AGXT-LTM complexes indicates that we have been able to trap a functional folding intermediate of AGXT-LTM mutant protein that will proceed to its final native structure with the assistance of GroEL. Binding of unfolded AGXT to GroEL involves distant sides of the chaperonin folding cavity so that the substrate displays an expanded conformation. Single molecule FRET experiments have demonstrated that subsequent ATP-induced domain movements in GroEL cause the stretching of the substrate and further unfolding and compactation of the substrate on the GroEL surface (13, 14, 15). Indeed, our structural data support this model since we observed that the incubation of the purified GroEL:AGXT-LTM complex with ATP induces the internalization of the density corresponding the AGXT-LTM substrate. Our data also support the model for a chaperone folding reaction cycle in which binding of a quasi-native AGXT to GroEL proceeds in an ATP-independent manner. This conformation could be reached with or without the help of other cofactors, or it could result from an unsuccessful GroEL folding reaction cycle.

Nowadays, the most effective treatment for PH1 consists of either kidney and liver double transplantation or preemptive liver transplantation. Among the new pharmacological therapies under study, the use of chemical chaperones to bind and stabilize a native AGXT protein conformation is very attractive. With early intervention, such chemoprophylactic approach would minimize renal damage and potentially avoid the significant morbi-mortality of liver transplantation and the adverse effects of immunosuppression. The effectiveness of the treatment would depend on the effect of the pathogenic mutations and subsequent protein conformation changes. Our data show that the aggregation-prone AGXT-LTM mutant protein retains partial function, that is also able to form folding intermediates and that the GroEL:GroES...
system improves the yield of active and soluble protein. Consequently it represents a promising target for pharmacological chaperones.

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REFERENCES

FIGURE LEGENDS

Fig. 1. GroEL/ES system facilitates the folding of AGXT-LTM. (A) Comparison of the AGXT activity assays performed on cell lysates when expressing either the GroEL/ES complex alone, AGXT-LTM mutant protein alone, or both GroEL/ES and AGXT-LTM. The mean values are indicated and the standard deviations are represented as error bars. Calculations have been performed using 10, 14 and 26 independent observations respectively. (B) AGXT-LTM protein present in 1 μg of the soluble fraction from E.coli pellets with or without coexpression of the GroEL/ES complex. Soluble wild type AGXT was included as a control. AGXT was detected by Western blotting with anti-AGXT antibody (1:5000). (C) Purification of GroEL:AGXT-LTM and GroEL:AGXT (thick and fine lines, respectively). The elution profile of the gel filtration chromatography and the SDS-PAGE of the sample loaded (S) and from each of the two peaks (labelled accordingly) are shown. The column was calibrated using Gel Filtration Standards from BioRad.

Fig. 2. Electron microscopy of GroEL:AGXT-LTM complexes. (A) A representative subset of 2D average images from a maximum likelihood reference-free alignment of negatively stained native (top) and ATP-bound (bottom) complexes. End-on and side views of the 3D structure of the GroEL complexes: (B) 7-fold symmetrized and non symmetrized reconstructions of the nucleotide-free sample (C) 7-fold symmetrized reconstruction of the ATP-bound sample.

Fig. 3. (A) Two views of the Cryo-EM 3D structure of the GroEL:AGXT-LTM complex. Docking of the atomic model of GroEL and AGXT onto the 3D reconstruction of the complex. The atomic models corresponding to the N-terminal and C-terminal domains of AGXT are depicted as yellow and red ribbons, respectively. Some of the GroEL protomers are omitted for clarity of the figure. (B) Details of the fitting of GroEL and AGXT crystal structures into the Cryo-EM map. Left, the superposition of the fitting of a single GroEL subunit (orange) and the conformational changes required to adapt the GroEL apical domain of the subunit into the experimental Cryo-EM map (green). Right, docking of the N-term (yellow) and C-term (red) domains of AGXT and the conformational changes required adapting the C-term domain for the original structure (purple). The straight arrow indicates a region of the AGXT mass that could be filled by the 22 residues of the N-terminal sequence of AGXT omitted from the AGXT atomic model. The curved arrow indicates the region of AGXT faced towards the GroEL folding cavity that is involved in the AGXT dimer formation.
FIGURE 1
FIGURE 3
The structure of GroEL in complex with an early folding intermediate of alanine glyoxylate aminotransferase
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