Tuning intracellular homeostasis of human uroporphyrinogen III synthase by enzyme engineering at a single hotspot of congenital erythropoietic porphyria

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Congenital erythropoietic porphyria (CEP) results from a deficiency in uroporphyrinogen III synthase enzyme (UROIIIS) activity that ultimately stems from deleterious mutations in the uroS gene. C73 is a hotspot for these mutations and a C73R substitution, which drastically reduces the enzyme activity and stability, is found in almost one-third of all reported CEP cases. Here, we have studied the structural basis, by which mutations in this hotspot lead to UROIIIS destabilization. First, a strong interdependency is observed between the volume of the side chain at position 73 and the folded protein. Moreover, there is a correlation between the in vitro half-life of the mutated proteins and their expression levels in eukaryotic cell lines. Molecular modelling was used to rationalize the results, showing that the mutation site is coupled to the hinge region separating the two domains. Namely, mutations at position 73 modulate the inter-domain closure and ultimately affect protein stability. By incorporating residues capable of interacting with R73 to stabilize the hinge region, catalytic activity was fully restored and a moderate increase in the kinetic stability of the enzyme was observed. These results provide an unprecedented rationale for a destabilizing missense mutation and pave the way for the effective design of molecular chaperones as a therapy against CEP.

INTRODUCTION

Congenital erythropoietic porphyria (CEP) is a rare autosomal recessive disease caused by reduced catalytic activity in uroporphyrinogen III synthase (UROIIIS, OMIM: 606938), the fourth enzyme in the haem metabolic biosynthetic pathway (1,2). CEP presents with a plethora of different symptoms, with patients suffering from skin defects produced by the massive accumulation of porphyrins in the body (3). Efforts to cure the disease have focused on bone marrow transplantation, which has been performed successfully in a small number of CEP patients with severe manifestations (4,5), and gene therapy, which is under development and has not yet been tested on patients (6). Due to the severity of the pathology, alternative treatment options that increase the quality of life of most CEP patients are also required.

At the molecular level, almost 30 mutations located in the coding region of UROIIIS have been identified in CEP patients (7). Most corresponding to missense mutations that reduce the in vitro catalytic activity by, in most cases, lowering the enzyme’s thermodynamic stability (8). When analysed in cells, the low stability found for the mutated UROIIIS ultimately limits the enzyme’s homeostasis: experiments in both human cell lines (9) and with a murine model of the disease (10) have shown that the protein is rapidly eliminated, partially via the proteasome. The most aggressive mutations, like the hotspot C73R substitution which is present in almost one-third of all the reported CEP cases (11), dramatically accelerate intracellular UROIIIS degradation (9). The decreased stability produced by a mutation can be overcome if the concentration of the folded protein is successfully restored in the cellular medium (12). In this context, molecular chaperones are able to
upregulate the protein’s homeostasis by binding to the enzyme in order to stabilize the folded conformation (13). However, in practice this strategy is challenging since the interaction between the enzyme and the chaperone has to be energetically productive (i.e. stabilizing the enzyme). Therefore, in order to provide the most effective outcome in chaperone-based therapies, it is very important to understand the structural basis for UROIIIS destabilization by C73 mutation.

Here, we have investigated the mechanism by which C73R destabilizes UROIIIS with atomic resolution, as a first step towards a new therapy against CEP based on the rational design of effective molecular chaperones. Expanding on a previous study, we have evaluated the effect of different mutations at the position C73. Remarkably, changes in the volume of the side chain present at this position correlate with the cellular half-life of the protein. Molecular dynamics simulations revealed a coupling between the C72 hotspot and the hinge region that connects the two domains of the enzyme. Based on these results, we engineered C73R-UROIIIS to enhance its stability. Expansion of the pocket and the introduction of negatively charged residues at a distance that modulate the interaction between R73 and the hinge resulted in a stabilizing effect and fully recovered the in vitro catalytic activity. In the cellular environment, the engineered mutants showed a modest increase in catalytic activity.

Thus, our work clarifies the mechanism of intracellular protein degradation induced by the mutation and paves the way for the design of molecular chaperones to effectively treat CEP.

RESULTS

Residual catalytic activity in C73R-UROIIIS

It was recently shown that C73R-UROIIIS is kinetically unstable, with the substitution accelerating the protein’s unfolding ≈380 times when compared with wild-type (WT) UROIIIS (8). Nonetheless, several reports indicate that C73R-UROIIIS should retain a certain degree of catalytic activity, both in cells and in vitro (9,11,14). Figure 1 shows catalytic activity assays testing WT and C73R-UROIIIS, and the derived kinetic constants are listed in Table 1. The enzymatic assay is able to determine the amount of enzymatic product (uroporphyrin III, uroIII) and the concentration of the by-product uroporphyrin I (uroI), which is spontaneously generated and ultimately responsible for most of the symptoms observed in CEP patients (15). WT-UROIIIS follows a canonical Michaelis–Menten mechanism (Fig. 1A, blue) and the catalytic efficiency is high enough to prevent the appearance of uroI (Fig. 1B). Freshly purified C73R-UROIIIS shows residual activity that is best modelled when accounting for inhibition due to an excess of substrate (Fig. 1A, brown), and has a catalytic efficiency 15 000-fold weaker than WT-UROIIIS (Table 1) due to deterioration of the folded conformation during the enzymatic assay. Such low activity results in the generation of large amounts of uroI (Fig. 1C), consistent with the pathogenic character of this mutation.

The C73 hotspot regulates the enzyme’s homeostasis in the cell

C73 is not essential for catalysis (9) but is evolutionarily conserved: analysis of all the sequences available in the PUBMED gene repository shows that the vast majority of living organisms encode for a C (61%), S (22%) or A (14%) at this position (Supplementary Material, Table S1). Here, we have investigated the putative role of the cysteine located at the position 73 in the human sequence by studying the effect in protein stability of several amino acid substitutions (C73S, C73K, C73E, C73V and C73Q) on protein stability thus expanding on a previous study that already characterized five other mutants at this position (C73L, C73Y, C73N, C73D and C73A) (9). In agreement with the previous study, all new mutant proteins tested showed kinetic stabilization, with a spontaneous loss of secondary structure over time that could be monitored by circular dichroism (CD).

Figure 1. Catalytic activity for the engineered variants of UROIIIS. Left (A) Conversion rate as a function of the porphobilinogen concentration for WT (blue), C73R (brown), A69E/C73R-UROIIIS (green) and L43D/C73R (yellow). Solid lines correspond to the best fitting to a Michaelis–Menten equation (WT) or to a Michaelis–Menten with excess of substrate inhibition equation (mutants). The Michaelis constant (K_M), the catalytic rate (k_cat) and the inhibition constant (K_i) obtained from the fitting are listed in Table 1. Right (B–E) Chromatograms showing the HPLC separation between the enzymatic product (uroIII) and the by-product (uroI) from an enzymatic reaction mixture (obtained with 100 μM of substrate). Each plot has been obtained from a single protein variant, according to the left panel colour code.
The half-life reflecting the loss of the folded conformation \( k_{\text{app}} \) was used as a reporter for protein stability (see Materials & methods). As shown in Fig. 2A, \( k_{\text{app}} \) (in log scale) correlates remarkably well with the volume of the hydrophobic residue at position 73. According to the slope of the linear regression, the protein accelerates its rate of unfolding 10-fold for every 20 Å\(^3\) of aliphatic chains introduced at position 73, confirming that the incorporation of bulky side chains, like that of arginine, results in steric repulsion with nearby atoms in the domain and triggers the irreversible unfolding process.

To investigate if the alterations in the \textit{in vitro} stability modulation of the C73 mutants is also observed \textit{in cells}, the WT and several C73 variants of UROIIIS were analysed in human fibroblast-derived cell lines (named M1). M1 cells were transiently transfected with plasmids encoding C-terminal myc-tagged versions of WT or the mutant enzymes, and the levels of the ectopically expressed proteins were analysed by western blotting (Fig. 2B). Notably, intracellular enzyme levels were modulated by the amino acid carried at position 73: no expression was observed for C73R-, C73E- and C73Y-UROIIIS, while C73S-UROIIIS expressed protein up to WT levels and C73N-UROIIIS showed an intermediate expression level. The intracellular enzyme concentrations correlated with the \textit{in vitro} kinetic stability values, with no intracellular protein accumulation of mutants with \( \log(k_{\text{app}}) < 1.5 \) (equivalent to an \textit{in vitro} lifetime of 30 h). This result cannot be assigned to additional stress induced by the ectopic expression of different versions of UROIIIS protein, because no significant differences were detected when the endogenous stress-inducible chaperone Hsp70 was analysed (Fig. 2B) (16). The mammalian vector pCR3.1 utilized in this study also carries the gene encoding neomycin phosphotransferase II (NPTII), which was detected at similar levels in all the transfection assays (Fig. 2B). In addition, in the presence of the proteasomal inhibitor MG132, protein levels partially recovered (Supplementary Material, Fig. S1). Together, these results indicate that all of transfected cells contain functional vectors for expressing NPTII and UROIIIS, confirming that the lack of C73R-, C73E- and C73Y-UROIIIS protein mutants is due to degradation promoted by the mutations.

### Table 1. Enzymatic parameters for WT-UROIIIS and the mutants under consideration

<table>
<thead>
<tr>
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<th>( K_M ) (( \mu \text{M} ))</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( k_{\text{cat}}/K_M ) (M(^{-1}) s(^{-1}))</th>
<th>( K_i ) (( \mu \text{M} ))</th>
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<tr>
<td>WT(^a)</td>
<td>60 ± 20</td>
<td>890 ± 50</td>
<td>( 1.5 \times 10^7 )</td>
<td>n.a.</td>
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<tr>
<td>C73R(^b)</td>
<td>&gt;10 000</td>
<td>10 ± 9</td>
<td>&lt;10(^3)</td>
<td>120</td>
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<tr>
<td>C73R/A69E(^b)</td>
<td>50 ± 20</td>
<td>170 ± 70</td>
<td>3.7 ( \times 10^6 )</td>
<td></td>
</tr>
<tr>
<td>C73R/L43D(^b)</td>
<td>70 ± 20</td>
<td>1200 ± 90</td>
<td>1.6 ( \times 10^7 )</td>
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\(^a\)Values obtained from the fitting to a Michaelis–Menten model.

\(^b\)Values obtained from the fitting to a Michaelis–Menten model with inhibition by excess of substrate.

Figure 2. The hotspot C73. (A) Correlation between the half-life time of the protein \( k_{\text{app}} \) (in log scale) with the amino acid volume of the residue introduced at position 73. Residues are labelled with the one letter code. The solid line corresponds to the linear regression of the data, with a slope of 0.043 s\(^{-1}\) Å\(^{-3}\). (B) Western blot analysis of the unmodified M1 cell lines (control) or M1 cells expressing C73R, C73E, C73N, C73S or C73Y versions of C-terminal myc-tagged UROIIIS proteins. Hsp70, actin and tubulin proteins serve as protein loading controls. Specific antibodies against neomycin NPTII and the myc-epitope were used to detect proteins from transfected vectors. (C) Amino acid variability among species at position 73 (black bars, including all the organisms available in the PUBMED gene repository) as a function of the kinetic stability of the protein containing the given amino acid. The kinetic stability for all the UROIIIS variants tested in A are also shown in shaded grey bars. Relative stabilities for the WT protein and the pathogenic mutant (CEP) are indicated on top.
Considering the (highly conserved) position 73 of UROIIIS, there is a high correlation between the conservation of this residue among species and the resulting kinetic stability of the enzyme (Fig. 2C): 97% of the analysed species contain residues at position 73 that result in proteins with high kinetic stability (in vitro protein lifetime > 50 h), while not a single species with a destabilizing residue (in vitro protein lifetime < 1 h) at position 73 could be found. Thus, the extensive conservation of position 73 avoids protein destabilization and is crucial to the maintenance of proper protein homeostasis in the cell.

A structural model for the hotspot destabilization

The crystal structure of WT-UROIIIS revealed that the protein is composed of two domains, connected by a hinge region (17). In the absence of high resolution structures for the mutant proteins and the impossibility of obtaining them because of their modest stability, molecular dynamics (MD) simulations in explicit solvent were used to generate realistic structural models that account for alteration in C73 (see the Experimental section). The time trajectories for WT- and C73R-UROIIIS obtained from the molecular simulations were analysed to characterize the effect of the modifications on the folded conformation and compared them with the starting structure. WT-UROIIIS shows a high degree of conservation of the native structure during the simulation’s trajectory, only deviating from the 1JR2 structure (18) in the orientation between the two domains (Fig. 3B). This result is in full agreement with a previous crystallographic study showing that UROIIIS adopts multiple inter-domain orientations with a preference for extended structures (19). In WT-UROIIIS, C73 interacts (intra-domain) with the nearby residues N77 and A69 (Fig. 3A and E), but remains totally uncoupled from residues of the hinge region. In C73R-UROIIIS (Fig. 3B), R73 is repositioned in a conformation that avoids steric clash with L43, providing a structural model to explain the experimental correlation observed between the reduced stability and the side chain volume at position 73. Moreover, the introduced arginine creates two new interactions with residues from the hinge region (L41 and S42), while the interaction between R73 and N77 is weakened (Fig. 3E). C73R-UROIIIS shows a twisting motion of the two domains when compared with WT-UROIIIS (of ~32° on average, Fig. 3F), probably as a result of the interaction between R73 and L41-S42. We hypothesize that the introduction of bulky residues at position 73 (like arginine) forces the side chain to form non-covalent interactions with the hinge region, twisting the domains and ultimately destabilizing the protein.

Engineering the hotspot stability site

We have engineered the residues surrounding R73 and evaluated their capability to rescue the catalytic properties of the enzyme.
Considering that the C73R substitution introduces a positive charge, our engineering strategy aimed to alter the conformation of R73 through the addition of negatively charged residues. Molecular dynamics simulations were used to model the interaction pattern of R73 and the new pocket configuration. First, we considered the introduction of a glutamic acid at position 69 (A69E/C73R-UROIIIS) that, according to MD simulations, should shift the orientation of R73 by \( \sim 30^\circ \) (in chi3, Fig. 3C), abrogating the interaction with L41 (Fig. 3E) and reducing the inter-domain twist by \( \sim 11^\circ \) (Fig. 3F). As shown in Fig. 1A (green), A69E/C73R-UROIIIS is able to partially restore the catalytic activity of C73R-UROIIIS. Specifically, the A69E mutation enhanced the catalytic activity by a factor of 16, showing little effect on the formation of the Michaelis complex (Table 1), as expected, since it is far from the substrate-binding site located in the cleft between the two domains (20).

Next, we considered the introduction of a negative charge at position 43 (L43D/C73R-UROIIIS). This mutant results in electroneutralization of the pocket but, according to the structural model, no interactions are created between D43 and R73 (Fig. 3D). Instead, the pocket becomes sterically relaxed, largely reconstituting the interaction pattern of WT-UROIIIS. When assayed, L43D/C73R-UROIIIS showed a larger increase in \( k_{\text{cat}} \) than A69E/C73R-UROIIIS (Fig. 1A, yellow). Remarkably, this construct fully restores the catalytic activity and its catalytic efficiency is comparable (within experimental error) with that of WT-UROIIIS (Table 1).

The time dependence of the catalytic activity at physiological temperature is shown in Fig. 4A for all the enzyme variants under consideration (as a fraction of their activity at time zero). In the timeframe of the experiment (<25 h), the activity of WT-UROIIIS remains largely unaffected while that of

![Figure 4](image-url)
C73R-UROIIIS is lost completely in >1 h. For L43D/C73R-UROIIIS, a significant decay over time is observed in catalytic power, declining to 50% of activity in 12 h. A69E/C73R-UROIIIS shows a slower decay over time, reaching half-life activity in \( \approx 16 \) h. The drop in catalytic activity over time correlates with a loss of secondary structure, according to the CD data (Fig. 4B and D). As shown previously (8) and in this work, the WT and all the pathogenic mutants investigated evolve towards a final aggregated state (Fig. 4B), invisible in the CD spectrum but detectable by turbidimetric analysis of the sample (Fig. 4C). On the contrary, for the rescued A69E mutant protein, the change in ellipticity at 222 nm (monitoring the losses in \( \alpha \)-helical content) is coupled with the emergence of a new band at 208 nm (Fig. 4D). The well-defined isodichroic point that emerges from the manifold of CD spectra (at \( \approx 217 \) nm) suggests full interconversion between the two conformations. Thus, incorporation of this mutation neutralizes the charge at position 73 and not only rescues the enzyme activity but also modifies the kinetic destabilization pathway of the protein, abrogating the aggregated state (Fig. 4C and Supplementary Material, Fig. S2).

To investigate if the double mutants L43D/C73R- and A69E/C73R-UROIIIS would also compensate for the reduced stability and activity of the single C73R-UROIIIS mutant in the cellular environment, we expressed tagged versions of the two proteins in the human M1 cell lines. M1 cells were transiently transfected with C-terminal myc-tagged versions of WT-, C73R, L43D/C73R and A69E/C73R versions of C-terminal myc-tagged UROIIIS proteins incubated without or plus MG-132 in the media. Hsp70 protein serve as protein loading control and specific antibodies against neomycin phosphotransferase II (NPTII) and myc-epitope were used to detect proteins from transfected vectors. Molecular weights of detected proteins are indicated. (B) UROIIIS activity was determined in cells transiently expressing the indicated protein versions of UROIIIS that were incubated in the presence of MG132. Specific activity (left) is obtained after normalization by the micrograms of proteins and referred to the activity obtained for the WT version of the protein while the enhanced activity (right) has been compared with C73R-UROIIIS and normalized by the individual protein expression.

DISCUSSION

The investigation of second-site mutations that suppress aberrant alleles is a useful strategy for the unbiased identification of residues involved in quaternary assembly and of structural regions that might be manipulated to restore WT function. This approach can also represent an easy way to assess the extent, to which folding lesions can be corrected. Several studies performed in different diseases including McCune–Albright syndrome (21), familial hyperinsulinism (22) and cystic fibrosis (23,24) support this strategy. Because UROIIIS misfolding is accelerated upon mutation (8), we have applied the concept to the study of CEP.

After systematic analysis of the protein variants at position 73 it is now clear that the loss of stability found in the pathogenic C73R is produced by the steric clashing between the (bulky) arginine side chain and nearby atoms. The excellent correlation found
with the volume of the side chain at this position and the agreement with the phylogenetic conservation underlines the relevance of this site in maintaining protein integrity and its role in regulating the protein’s intracellular homeostasis. In silico calculations provide a plausible model for the rearrangement of the interaction network upon mutation and suggest a coupling between position 73 and the hinge residues. In bi-domain proteins, the composition and structure of the hinge is often pivotal to the stability and functionality of the protein (25). This hypothesis was subsequently tested with rescue mutations: second-site mutations designed to abrogate such coupling and to reconstitute the interaction network observed for WT-UROIIIS (Fig. 3) succeeded in rescuing the WT protein’s folding protein properties and the activity defects caused by the deleterious mutation in vitro.

The double mutants that successfully rescued the enzyme activity also showed a partial recovery of their kinetic stability, in vitro (Fig. 4A). Actually, for this enzyme, an increase in the protein’s (kinetic) stability is almost always accompanied by an increase in catalytic efficiency, consistent with the fact that the enzyme lacks a truly active site and a large number of residues are involved in the catalytic mechanism (17,20). Interestingly, one of these double mutants was also able to significantly enhance the enzymatic activity in eukaryotic cells, supporting the idea that a therapy employing molecular chaperones emulating the effect of A69E on the C73R-UROIIIS mutant protein could benefit CEP patients. However, in contrast to the protein expression found in bacterial systems, expression of the double mutants (L43D/C73R and A69E/C73R) in a mammalian cellular environment was not sufficient to slow down the intracellular degradation caused by the single C73R mutation. This could be due to the fact that mammalian cells display more complex mechanisms (e.g. ERAD) for protein quality control than bacterial systems. Consistently, in the case of UROIIIS the intracellular levels of the mutant proteins in the presence of MG132—a proteasome inhibitor—were not similar to the levels reached by the WT protein, indicating that additional degradation pathways are acting on the eukaryotic intracellular homeostasis of this enzyme. These findings highlight the need for further investigations to unravel the pathways involved in the UROIIIS’s intracellular degradation in order to increase the success rate of molecular chaperone-based therapies.

In summary, with this study we have unravelled the delicate interplay between C73 and the kinetic stability of UROIIIS, and have proven that non-covalent electrostatic interactions at this site are able to partially restore the enzyme’s stability and activity. In this regard, docking studies show that the interaction network surrounding R73 conforms to a ‘druggable’ and partially solvent accessible pocket. Therefore, with this report, we establish a proof of principle for the use of molecular chaperones against this destabilization site and provide some structural determinants required for the molecule to be functional, paving the way for the design of molecular chaperones that may stabilize the pathogenic enzyme, ultimately constituting a new line of therapeutic intervention against CEP.

**MATERIALS AND METHODS**

**Site-directed mutagenesis**

The UROIIIS mutants were produced by site-directed mutagenesis employing the commercial QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) with custom-made oligonucleotides as polymerase chain reaction primers (Invitrogen). Human porphobilinogen deaminase (PBGD), required for the UROIIIS specific activity assay (15), was previously cloned into a pETM-11 expression vector using the HindIII and NcoI restriction sites (8). All new gene constructs were checked by gene sequencing analysis.

**Protein sample preparation**

Freshly transformed *Escherichia coli* OverExpress C41(DE3) chemically competent cells (Lucigen) were used for protein expression. UROIIIS (MW 31.1 kDa) was purified according to the following protocol: the cell pellet originating from 1 l of culture was resuspended in 20 ml of buffer A (20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, pH 7.4) and thoroughly sonicated. The supernatant was mixed with 3 ml of His-tag resin (Ni-NTA; Invitrogen) and eluted with buffer A containing 500 mM imidazole. The eluate was further purified by size-exclusion chromatography (Superdex 75; GE Healthcare) under isocratic conditions (20 mM Tris, 150 mM NaCl, pH 8.0). PBGD (MW 42.6 kDa) was purified following the same protocol except that the size-exclusion buffer was 20 mM Tris–HCl, pH 8.0, and 150 mM NaCl. Both purifications were done at 4°C to minimize proteolysis, and the resulting proteins were concentrated to 5 μM and stored at 4°C. Protein concentration was quantified by UV spectroscopy, employing extinction coefficients of 25 410 and 15 970 M⁻¹ cm⁻¹ for UROIIIS and PBGD, respectively.

**UROIIIS enzymatic assay**

The determination of UROIIIS specific activity was based on the method developed by Jordan and modified by Hart and Battersby and Tsai *et al.* (26,27). For the determination of the enzyme kinetic constants, duplicate data were collected and subsequently fitted to a Michaelis–Menten equation. Monte Carlo analysis was used for the determination of the parameter’s uncertainties. When testing the activity in eukaryotic cells, an aliquot of 10 million MG132-treated cells (8) was lysed and the protein concentration quantified with a Bradford assay.

**Circular dichroism and fluorescence spectroscopy**

CD experiments were conducted on a JASCO J-810 spectropolarimeter and analysed as described previously (28). CD experiments were performed in a quartz cuvette with a 0.2 cm path length. The sample concentration was set to 5 μM. The interval scanning measurement was run at 37°C for up to 72 h, with freshly purified samples. Data analysis was performed using in-house built scripts in Matlab®.

**Computational modelling**

The initial coordinates for the simulations were taken from the X-ray structure of human UROIIIS (Protein Data Bank ID: 1JR2, chain A, resolution 1.84 A) (18). The mutants (C73R, C73R_L43D, C73R_A69E) were prepared using BioEdit program for introducing point mutations into the sequence and the CPHmodel online server for homology modelling of the tertiary structures of the mutated proteins (29). Visual molecular dynamics was used for the system preparation and result analysis. The MD simulations were performed using NAMD2 and the images were produced in...
PyMOL (30). The protein was solvated in a water sphere and the system was neutralized with NaCl. Using the CHARMM27 force field MD simulations were carried out at a constant temperature (300 K) and constant volume. To control the temperature, Langevin dynamics with a 5 ps$^{-1}$ coupling coefficient was applied to non-hydrogen atoms. The centre coordinates and the radius of the sphere defined the spherical harmonic boundary conditions with a force constant of 10 kcal mol$^{-1}$ Å$^{-1}$ keeping the system together. The conjugated gradient method of minimization was used to relax the structure before production. All MD runs were performed for 2000 ps (minimization not included) and all the systems became stable before reaching 200 ps; analysis of the energies and RMSDs between conformations stored during the sampling and the starting structure did not show significant folding changes.

**Cell culture and reagents**

Human fibroblastoid M1 cells were grown in complete DMEM medium [Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 0.1 mg/ml streptomycin and 100 units/ml penicillin]. When indicated, cells were cultured for 12 h in the presence of 10 μM MG132, purchased from Sigma-Aldrich. Monoclonal antibodies were purchased from the following vendors: anti-Myc tag (clone 4A6) from Upstate (Lake Placid, NY, USA), anti-neomycin NPTII (clone 4B4D1) from Abcam (Cambridge, UK), and anti-Hsp70 (clone BRM-22). Horseradish peroxidase-conjugated secondary antibody was purchased from GE Healthcare. All other reagents were of analytical grade and generally acquired from Sigma-Aldrich.

**Generation of transiently transfected mammalian cell lines**

For transient transfection, the M1 cell lines were grown to a confluence of 60–70%. Cells were transfected with plasmids encoding C-terminal myc-tagged WT-, C73R-, C73E-, C73N-, C73S-, C73Y-, L43D/C73R- or A69E/C73R-UROIIIS using FuGENE 6 (Roche Diagnostics, Manheim, Germany) and OptiMEM medium (Invitrogen) as described in the manufacturer’s instructions. The medium was changed to complete Dulbecco’s modified Eagle’s medium 5 h post-transfection and 12 h later the cells were collected for further analysis.

**Western blot analysis**

One million trypanosized M1 cells were lysed for 15 min on ice in the presence of 100 μl of lysis buffer (300 mM NaCl, 50 mM Tris, pH 7.4, 0.5% Triton X-100 and protease inhibitors). After clarification of the samples by centrifugation at 20,000 g, the supernatant was transferred to a fresh Eppendorf tube. The protein concentration of the cell lysates was determined with a Bradford protein assay (Bio-Rad) using bovine serum albumin (BSA) as the standard. Sodium dodecyl sulphate sample buffer was added and samples were incubated for 5 min at 37, 65 and 95°C and separated on 4–12% precast acrylamide gels (Invitrogen). After transferring to nitrocellulose membranes (Millipore, Bedford, MA, USA) and blocking overnight [5% milk and 0.05% Tween 20 in phosphate-buffered saline (PBS)], the primary antibody was added for 1 h, followed by a PBS wash and application of the secondary HRP-conjugated antibody. Chemiluminescent detection of bands was performed with ECL Plus reagent (GE Healthcare).

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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