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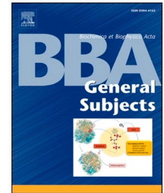

Pyridoxine non-responsive p.R336C mutation alters the molecular properties of cystathionine beta-synthase leading to severe homocystinuria phenotype

Duaa W. Al-Sadeq [a,b], Angelos Thanassoulas [b], Zeyaul Islam [c], Prasanna Kolatkar [c], Nader Al-Dewik [d], Bared Safieh-Garabedian [b], Gheyath K. Nasrallah [a,e], Michail Nomikos [b,*]

[a] Biomedical Research Center, Qatar University, Doha 2713, Qatar
[b] College of Medicine, QU Health, Qatar University, P.O. Box 2713, Doha, Qatar
[c] Diabetes Research Center (DRC), Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Doha, Qatar
[d] Clinical and Metabolic Genetics Section, Pediatrics Department, Hamad General Hospital (HGH), Women's Wellness and Research Center (WWRC), Interim Translational Research Institute (iTRI), Hamad Medical Corporation (HMC), College of Health and Life Science (CHLS), Hamad Bin Khalifa University (HBKU), Doha, Qatar, P.O. Box 3050, Doha, Qatar
[e] Department of Biomedical Science, College of Health Sciences, QU Health, Qatar University, P.O. Box 2713, Doha, Qatar

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A B S T R A C T

- The prevalence of homocystinuria in Qatar is 1:1800, mainly due to a founder missense mutation p. R336C.
- The cystathionine beta-synthase (CBS) R336C mutant was bacterially expressed, purified and its molecular properties were compared to CBS wild type (WT) recombinant protein.
- Our data revealed that p.R336C mutation results in a dramatic reduction (~86%) of CBS enzymatic activity.
- Circular Dichroism experiments suggested that the p.R336C mutation does not significantly alter the secondary structure of the CBS protein.
- CD spectra also revealed distinct differences in the thermal unfolding mechanisms of CBS WT and R336C mutant protein species.
- Chemical denaturation experiments indicated that the WT CBS protein is thermodynamically more stable than the R336C mutant, suggesting a destabilizing effect of the p.R336C mutation.
- This study provides mechanistic insight into the pathogenicity of the p.R336C mutation that leads to a severe homocystinuria phenotype.

1. Introduction

Classical homocystinuria is an inborn error of metabolism due to cystathionine beta-synthase (CBS) deficiency [1]. It is a condition characterized by non-physiological high levels of plasma total homocysteine. Subsequently, this build-up can lead to several complications, such as intellectual disability and behavioral disorders [2,3]. The prevalence of homocystinuria is around 1/200,000 births worldwide [4]. Thus, it is generally considered a rare genetic disease. However, its prevalence in the Gulf region, particularly in Qatar, is exceptionally high, reaching 1:1800 births [5,6]. This high incident number was reported to be due to a founder mutation in the Qatari population c.1006C > T in the *CBS* gene (p.R336C). Approximately 6% of the Qatari population has a heterozygous p.R336C mutation with an allele frequency of 1% [7].

Treating homocystinuria patients with the traditional therapeutic approaches is challenging, and some CBS mutations, such as p.R336C, are pyridoxine non-responsive [8]. To gain an insight into the molecular

* Corresponding author at: College of Medicine, QU Health, Qatar University, Doha 2713, Qatar.
E-mail address: mnomikos@qu.edu.qa (M. Nomikos).

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properties of this pathogenic CBS variant, we employed a bacterial expression system to generate the human wild-type CBS and p.R336C mutant as recombinant proteins and directly compare their biochemical and biophysical properties. This is the first study to report the molecular impact of variant-specific p.R336C on CBS enzymatic activity, secondary structure and thermodynamic stability, contributing to a better understanding of homocystinuria pyridoxine non-responsive mutations.

2. Materials and methods

Human CBS (NCBI Reference Sequence NM_000071.2) was amplified by PCR from pLW2:hCBS. CBS WT and R336C mutant were then cloned in the pET28a vector between *Nde*I and *Xho*I restriction sites. The primers used for the amplification of the CBS constructs were: 5'-CAGTCATATGCCTTCTGAGACCCCCCA-3′ (forward) and 5′- GATACTCGAGTCACTTCTGGTCCCGCTC-3′ (reverse).

Human CBS WT and p.R336C constructs were transformed in competent *E. coli* [BL21- CodonPlus (DE3)-RILP; Stratagene] cells by heat shock. Cells were cultured at 37 °C until the optical density at 600 nm reached 0.6. Recombinant CBS proteins were then extracted and purified. The recombinant proteins present N-terminally a 6xHis tag with a high affinity for Ni^{2+} ions, allowing protein purification by immobilized metal affinity chromatography using a Ni^{2+} – chelating resin (QIAGEN). Purified recombinant proteins were stored at −80 °C.

The enzymatic activities of the purified recombinant WT and p. R336C proteins were determined in a final reaction volume of 200 μL and as previously described [9], with some minor modifications. The assay reaction contained 30 μL of different protein concentrations (0.1 μg, 0.5 μg, 1 μg, 2.5 μg, 5 μg) into the well plus 170 μL of master mix. The fluorescence was measured immediately at excitation/emission 368/460 nm in kinetic mode for 40–60 min at 37 °C. Specific enzyme activities were expressed as U/mL (the amount of enzyme that generates 1 nmol of 7-amino-4-methylcoumarin per minute at pH 8.0 at 37 °C).

Circular dichroism (CD) measurements were conducted using a JASCO-1100 spectropolarimeter with a Peltier type cell holder, allowing temperature control. Wavelength scans in the far (195 to 260 nm) UV region was performed using Quartz SUPRASIL (HELLMA) precision cells of 0.1 cm path length. For each measurement, 250 μL of a 5 μM solution of protein sample in PBS buffer were loaded onto the cuvette. Each spectrum was obtained by averaging eight successive accumulations with a wavelength step of 0.2 nm at a rate of 20 nm min^{-1}, response time 1 s and bandwidth 1 nm. Buffer spectra were accumulated and subtracted from the sample scans. The results in all experiments have been expressed as molar ellipticity [θ] (deg cm^2 $dmol^{-1}$).

For thermal denaturation experiments, the CD signal at 222 nm was followed as the sample chamber temperature was increased from 20 to 95 °C using a step size of 0.5 °C and at a rate of 1.5 °C/min. For each measurement, 250 μL of a 5 μM solution of protein sample in PBS buffer were loaded onto the cuvette. Buffer blank spectra, obtained in identical conditions, were subtracted from the raw data. The results in all experiments have been expressed as molar ellipticity change $\Delta[\theta]^{222\ nm}$ (deg cm^2 $dmol^{-1}$). The final spectra were analyzed using the CDNN program [10,11].

Fluorescence measurements were performed in a Horiba Fluoromax 4 Spectrofluorometer (HORIBA Advanced Techno - Kyoto, Japan) with a xenon short-arc lamp (Ushio), in a 4 mL quartz cuvette (Roth, Germany). The chemical denaturation profiles of CBS recombinant proteins were recorded by gradually injecting small aliquots of an 8 M guanidinium chloride (GuHCl) solution in a 1 cm path length cuvette, containing 0.05 mg/mL of sample in PBS buffer. After each injection, the mixture was stirred and incubated at 25 °C until a new equilibrium was reached. Emission spectra were then collected from 300 to 450 nm with excitation at 280 nm. Buffer background emissions at each GuHCl concentration were collected in a separate experiment and were subtracted from the final data. The protein concentration was kept constant by adding the appropriate amount of a dense protein solution to the cuvette before each measurement. The titration was carried on up to 4.5 M GuHCl. Chemical denaturation data were fitted to a two-state model describing the induced unfolding as a single transition between the native (N) and denatured (D) state.

3. Results and discussion

Human CBS p.R336C has been previously reported to be a B6 non-responsive mutation [6,8]. Therefore, we aimed to assess the enzymatic activity of human CBS R336C mutant at different concentrations with the addition of pyridoxal and heme cofactors and directly compare it with the enzymatic activity of the CBS WT protein. The activity was determined by measuring the fluorescence emission at 460 nm (excitation at 368 nm). Our assays revealed that the activity of CBS R336C mutant was dramatically reduced (~86%; Fig. 1A) compared to CBS WT. Our findings are in good agreement with previous reports that examined using different assays the activity of CBS R336C mutant and reported a residual enzymatic activity of ~6% of the WT [12] or a complete loss of activity [13]. A similar enzymatic pattern was observed with other CBS mutations in a previous study that characterized 14 mutations found in the human CBS gene trying to investigate potential pathogenic nature. Eleven of these mutations exhibited an activity lower than 4% of the wild-type protein, supporting the pathogenicity of these variants [13].

Structure based protein stability analysis due to mutation was carried out by PremPS (Predicting the Effects of Mutations on Protein Stability). Results revealed that CBS R336C is located at the interface between the catalytic core and the first alpha helix of the interdomain peptide linker connecting the core with the Bateman module of the same subunit and in the proximities of the core from a complementary subunit [14]. A comparative interaction analysis of mutant structure with WT reveals the loss of a critical charge-based (ionic) interaction between R336 and D388, located at the second alpha helix of the interdomain linker, due to mutation at position 336 (Fig. 1B). This lack of mutant interaction may destabilize the human CBS overall structure (ΔΔG value of 0.69 kCal/mol) and associated reduction in activity.

Circular dichroism was employed to assess the secondary structure of the recombinant CBS proteins. No significant changes were observed, indicating that the CBS WT and mutant proteins retain a similar overall structural conformation (Fig. 1C). Analysis of the spectra revealed that at 25 °C, the α-helix content was 0.16 and 0.19, while the β-sheet 0.31 and 0.27 for CBS WT and R336C mutant, respectively.

To explore whether the dramatic loss of the enzymatic activity of the R336C mutant is directly linked to an altered thermostability of the molecule, we performed a series of thermal unfolding experiments to directly compare the thermostabilities of the WT and mutant proteins. The far-UV CD spectra revealed important differences in both the thermal behavior and the final structure of the protein species. Both protein species exhibit two independent two-state irreversible thermal transitions between 20 and 95 °C. The first transition (T_m = 41.2 °C and T_m = 45.0 °C for the WT and mutant, respectively) can be attributed to the unfolding of the regulatory domain, while the second transition (T_m = 71.5 °C and T_m = 83.5 °C for the WT and mutant respectively) can be associated catalytic domain [15,16]. Although the first transition is similar for the two protein forms (comparable T_ms and molar ellipticity changes), the catalytic domain of R336C displays a much higher thermal stability (ΔT_m = 12 °C). In addition, the thermal unfolding of WT is characterized by significant loss of secondary structure ($\Delta[\theta]^{222\ nm} = \sim$ 6000 deg.·cm^2·$dmol^{-1}$), while R336C retains most of its secondary structure up to 95 °C ($\Delta[\theta]^{222\ nm} = \sim$ 2400 deg.·cm^2·$dmol^{-1}$). This can be attributed to a different population distribution among the oligomeric states and to structural rearrangements that lead to a more rigid and thermostable architecture in the case of R336C (molten globule state).

Furthermore, chemical denaturation assays were employed to analyze the conformational stability of the CBS recombinant proteins.

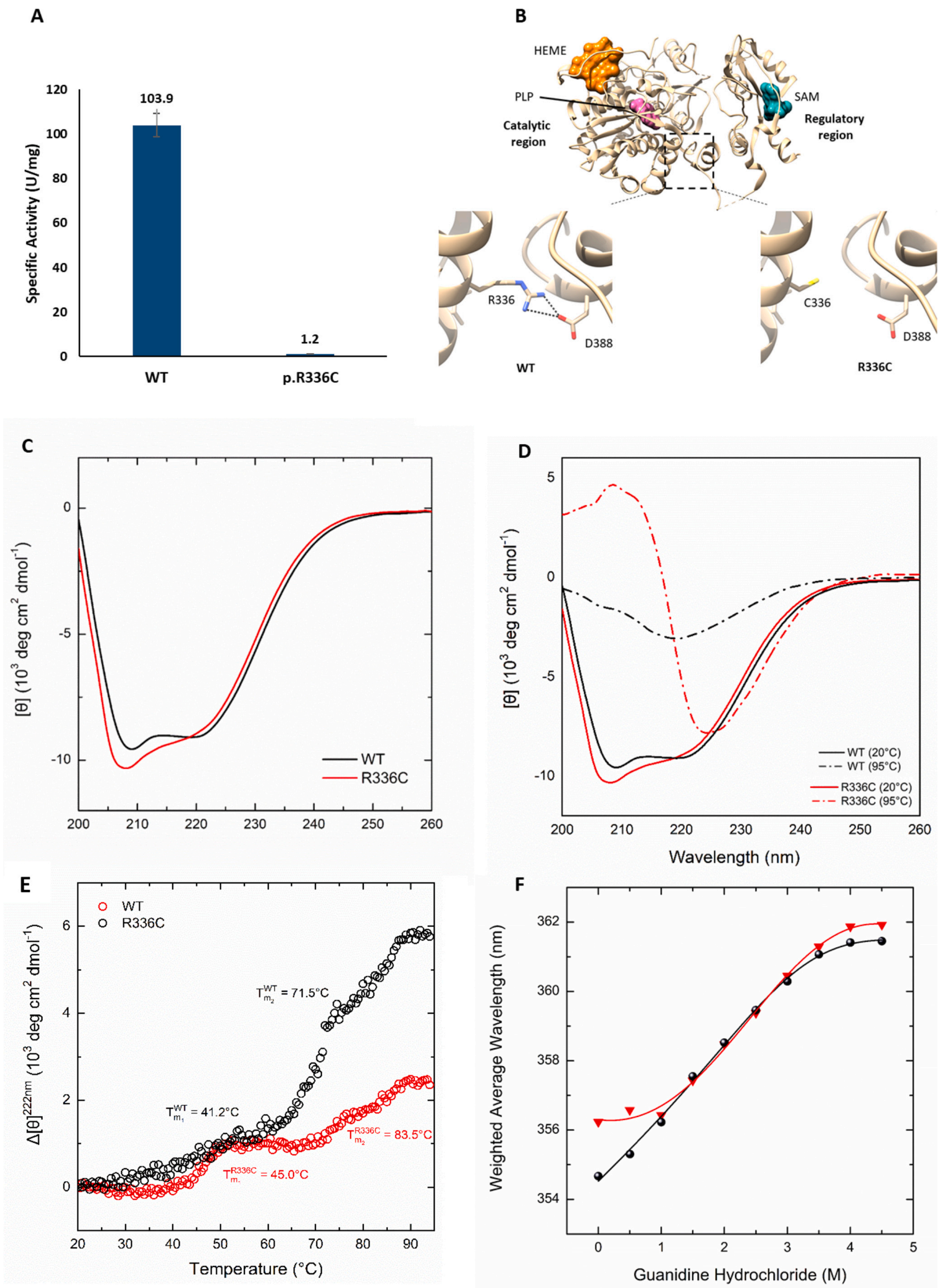

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Fig. 1. Biophysical and biochemical assessment of CBS WT and p.R336C proteins. A) CBS WT and p.R336C enzymatic activity. B) Structure based protein stability analysis. C) Normalized far-UV CD spectra for WT (black) and R336C (red) CBS at 20 °C. D) Normalized far-UV CD spectra for CBS WT (black) and CBS R336C (red) at 20 °C and 95 °C. E) CD melting profile of CBS WT (black) and CBS R336C (red) monitored at 222 nm and F) Chemical denaturation profiles of CBS WT (black) and CBS R336C (red), plotted as weighted average emission wavelength versus solution GuHCl concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This was achieved by monitoring the protein spectra changes through fluorescence emission spectroscopy. GuHCl was used as a denaturant due to its ability to weaken or break the noncovalent interactions, including salt bridges and hydrogen bonds. Results showed that the free energy change difference ($\Delta\Delta G_{D\text{-}N}$) between WT and mutant proteins was 0.72 kcal/mol (Fig. 1F). This indicates that the CBS WT protein is thermodynamically more stable than the R336C mutant. Therefore, our data strongly suggest that p.R336C mutation has a destabilizing effect on the CBS protein.

In conclusion, although CBS protein has been extensively studied, the exact molecular mechanism by which the p.R336C missense variant leads to severe phenotypes remains elusive. This is mainly due to the lack of a comprehensive understanding of the impact of this mutation on the molecular properties of this protein. Therefore, this study investigated the effects of p.R336C missense CBS mutation on both biochemical and biophysical characteristics. As a result, we demonstrated that the p. R336C mutation significantly alters the stability of human CBS protein resulting in a dramatic decrease of the specific enzyme activity, which explains the severe phenotype of the respective homozygous patients. Other potential mechanisms can also be involved, such as disrupting other important protein-protein or protein-cofactor interactions.

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Credit author statement

Conceptualization: GKN and MN; Methodology: BSG, GKN, PK, MN; Formal Analysis: DWA, AT, PK, MN; Validation: GKN, DWA, MN; Investigation: DWA, AT, ZI; Resources: GKN, MN; Data Curation: AT, MN; Writing - Original Draft Preparation: DWA, MN; Writing - Review & Editing: DWA, AT, ZI, PK, NA, BSG, GKN, MN; Supervision: BSG, GKN, MN; All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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