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Identification of temperature-sensitive mutations and characterization of thermolabile RNase II variants

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The RNase II family of ribonucleases is ubiquitous and critical for RNA metabolism. The *rnb500* allele has been widely used for over 30 years; however, the underlying genetic changes which result in RNase II thermolabile activity remain unknown. Here, we combine molecular and biophysical studies to carry out an *in vivo* and *in vitro* investigation of RNase II mutation(s) that confer the *rnb500* phenotype. Our findings indicate that RNase II thermolability is due to the Cys284Tyr mutation within the RNB domain, which abolishes activity by increasing protein kinetic instability at the nonpermissive temperature. These findings have important implications for the design of temperature-sensitive variants of other RNase II enzymes, namely those with yet unknown functions.

Keywords: Dis3L2; exosome; ribonuclease; RNA degradation; RNase R; RNB protein domain

The continuous synthesis and degradation of RNA are fundamental for the control of gene expression. Therefore, it is crucial to investigate factors regulating RNA metabolism. Ribonucleases (RNases) are major players in this process since they process, degrade, and recycle all types of RNA [1,2]. *Escherichia coli* ribonuclease II (RNase II, encoded by the *rnb* gene) is the prototype of RNB family of enzymes [1–3], exoribonucleases which are widespread among the three domains of life. Members include prokaryotic RNase II and RNase R, as well as the eukaryotic Rrp44/Dis3, Dis3L1, catalytic subunits of the exosome, and Dis3L2 proteins [2]. Members of this family hydrolyze RNA in the 3′–5′ direction in a processive way and play very important functions in the cell. These enzymes are essential for cell growth [4], can be developmentally regulated [5], and mutations in its gene have been linked with abnormal chloroplast biogenesis [6], mitotic control, and cancer [7,8]. Namely patients with multiple myeloma have mutations in Dis3, and Perlman syndrome and Wilms tumor are related with mutations in Dis3L2 [8]. In prokaryotes, RNase II

Abbreviations
CSD, cold shock domain; OB fold, oligonucleotide-binding fold; PNPase, polynucleotide phosphorylase; RNase II, ribonuclease II; RNB, RNase II catalytic domain; ts, temperature-sensitive.

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family of enzymes were also shown to be very important for RNA metabolism. They were involved in RNA and protein quality control, stress responses, were required for virulence in several organisms, and play a role in bacterial motility, competence, and biofilm formation [9–20]. In *E. coli,* RNase II is responsible for 90% of the exoribonucleolytic activity in crude extracts [21], and recently, it was shown that RNase II is essential for the survival of *E. coli* during starvation and prolonged stationary phase [22]. Therefore, RNase II has been the subject of extensive genetic, biochemical, and structural studies aimed at understanding its precise biological role and mechanism of action.

A thermosensitive (ts) *rnb500* allele has been essential for the determination of the cellular function of RNase II, and also of other ribonucleases [23–26]. This mutant allele was generated *in vitro* by hydroxylamine mutagenesis of the cloned wild-type *rnb* gene, and is thermolabile *in vitro* and *in vivo* at 44 °C [24]. A strain with this temperature-sensitive (ts) mutant demonstrated that either polynucleotide phosphorylase (PNPase) or RNase II is required for cell viability in *E. coli*, suggesting the involvement of both ribonucleases in a common essential process in the cell, more specifically in RNA decay [24]. This pioneering work contributed substantially to major discoveries in the field of RNA degradation [1,27]. This allele was also important in the identification and characterization of 3′ polyadenylation in *E. coli* [26,28]. Despite the wide use of *rnb500* during the last 30 years, nothing is known about the mutation responsible for its phenotype. The work presented here describes the identification of the mutation responsible for the ts *rnb500* allele.

Materials and methods

Restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were purchased from Fermentas. Phusion DNA polymerase is from Finnzymes. Oligonucleotides were synthesized by STABVida, Portugal. The primers used for the construction of RNase II mutants are given in Table 1.

E. coli strains

The following *E. coli* strains were used: DH5α [fhuA2 Δ(argF-lacZ)U169 phoAglnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 [29]] for cloning experiments, strain CMA401 [*thy*A715, *pnp*200 *rnb*Δ::tet^R], an isogenic derivative of *E. coli* MG1693 strain was used for the genetic complementation assay, and BL21(DE3) [F$^-$ $r_B^- m_B^-$ gal ompT (int::P_{lacUV5} T7 gen1 imm21 nin5) [30]] was used for expression and purification of enzymes. The *pnp200* thermosensitive allele was previously described [31] and the thermosensitive *rnb500* allele was obtained from SK5689 *E. coli* strain [25].

Identification and cloning of *rnb*500 mutations

The *rnb*500 allele was amplified from the chromosomal DNA of *E. coli* SK5689 [25] using a standard PCR reaction with Pfu DNA polymerase. The primers used for the amplification matched perfectly 37 nucleotides upstream of the initiation codon (5′-CTGTCAGCCGCTCTAATGGCC-3′) and 33 nucleotides downstream of the stop codon (5′-GCCCATC-CATGAGGAATGGGC-3′) of RNase II, respectively. The DNA sequence of the 2005-bp PCR product was determined (STABvida, Oeiras, Portugal). Two point mutations were detected: a G → A substitution at position 376 (residue substitution D126N) and a G → A substitution at position 851 (residue substitution C284Y) of the *rnb* gene. These mutations were further inserted into the previously described pCMA01 [32] and pFCT6.9 plasmid [33], by site-directed mutagenesis. The nucleotide sequences of the mutagenic primers used to generate point mutations are shown in Table 1. The mutant constructs were screened by restriction digestion and verified by sequencing at STAB Vida.

Genetic complementation assay

Escherichia coli MG1693-derivative cells (strain CMA401) [*thy*A715, *pnp*200 *rnb*Δ::tet^R] were transformed with pBluescript SK+ (Stratagene, La jolla, CA, USA) or pCMA01 [32] encoding either wild-type RNase II protein or its mutant derivatives, and grown on agar plates supplemented with 100 μg·mL^{-1} ampicillin and 50 μg·mL^{-1} thymine. The genetic complementation ability of the various RNase II mutant derivatives was assessed by examining growth of

Table 1. Mutagenic primers used in this study. The mismatched nucleotide in each mutagenic primer is underlined and the restriction site is in lowercase letter.

Primer	Sequence	Amino acid change
oFRA3	5′-GAAGGCAACTGGGCGGTTGCCGAAATGCGaCGTCATCC-3′	D126N
oFRA4	5′-ATGACGtCGCATTTCGGCAACCGCCCAGTTGCCTTC-3′	
oFRA5	5′-TGGCATACCGCATGACGCTCTCaGCTGATG-3′	C284Y
oFRA6	5′-CAGCtGAGAGCGTCATGCGGTATGCCAGTA-3′	

colonies after 1 day incubation at the nonpermissive temperature (44 °C). In the complementation experiments, cells transformed with pBluescript SK+ (vector alone) and pCMA01 (i.e., wild-type RNase II protein) served as the negative and positive controls, respectively.

Overexpression and purification of RNase II proteins

The plasmid used for expression of wild-type *E. coli* histidine-tagged RNase II protein was pFCT6.9 plasmid [33]. This plasmid contains the *rnb* gene cloned into pET-15b vector (Novagen supplied by Merck, Darmstadt, Germany) under the control of φ10 promoter, allowing the expression of the (His) 6-tagged RNase II fusion protein or its mutant derivatives.

Escherichia coli BL21(DE3) containing the recombinant plasmid of interest was grown at 30 °C in 200 mL of TB medium supplemented with 200 $\mu g \cdot mL^{-1}$ ampicillin to an optical density at 600 nm (OD600) of 1.5. Protein expression was then induced by addition of 0.5 mM IPTG for 20 h at 26 °C. Cells were harvested by centrifugation and stored at −80 °C.

Purification was performed by histidine affinity chromatography using HisTrap Chelating HP columns (GE Healthcare Europe GMBH, Carnaxide, Portugal) and an ÄKTA HPLC system following the protocol previously described [34,35]. Briefly, frozen cells were thawed and resuspended on 6 mL of buffer A [20 mM Tris-HCl, 0.5 M NaCl, and 20 mM imidazole, pH 8]. Cell suspensions were lysed using a French press at 9000 psi in the presence of 1 mM PMSF. The crude extracts were treated with benzonase (Sigma-Aldrich, Lisboa, Portugal) to degrade the nucleic acids and clarified by a 60-min centrifugation at 10 000 ***g***. The clarified extracts were then loaded into a HisTrap Chelating Sepharose 1 mL column equilibrated in buffer A. Protein elution was achieved with a continuous imidazole gradient (from 20 to 500 mM) in buffer A. Eluted protein was buffer exchanged with buffer C [20 mM Tris-HCl, 250 mM NaCl, pH 8]. Protein concentrations were determined by spectrophotometry using a NanoDrop 1000 instrument (Alfagene, Carcavelos, Portugal), and 50% (v/v) glycerol and 5 mM DTT was added to the final fractions prior to storage at −20 °C.

Activity assays

Exoribonucleolytic activity was assayed using a 30-mer oligoribonucleotide (5′-CCCGACACCAACCACUAAAA AAAAAAAAAA-3′) as a substrate. The oligoribonucleotide was labeled at 5′-end with [γ-^{32}P]ATP with T4 polynucleotide kinase. Before the reaction, the RNA substrate was heated for 5 min and cooled to 30 °C temperature prior to determining activities. The exoribonucleolytic reactions were performed in a final volume of 12.5 μL containing 20 mM of Tris/HCl, pH 8, 1 mM of DTT, 100 mM of KCl, and 1 mM of $MgCl_2$ [34]. The *in vitro* thermolability of RNase II was measured by preincubating 1 nM of each protein in the reaction buffer at 44 °C for 30 min, to inactivate the enzymes, and cooled to 30 °C for 20 min [24]. Reactions were started by the addition of the substrate and mixtures were incubated at 30 °C. Samples were withdrawn at the times indicated in the figures, and the reaction was stopped by adding an equal volume of formamide-containing dye supplemented with 10 mM EDTA. Samples were loaded onto a 20% polyacrylamide/8 M urea gel for electrophoresis. Gels were imaged using a STORM 860 scanner.

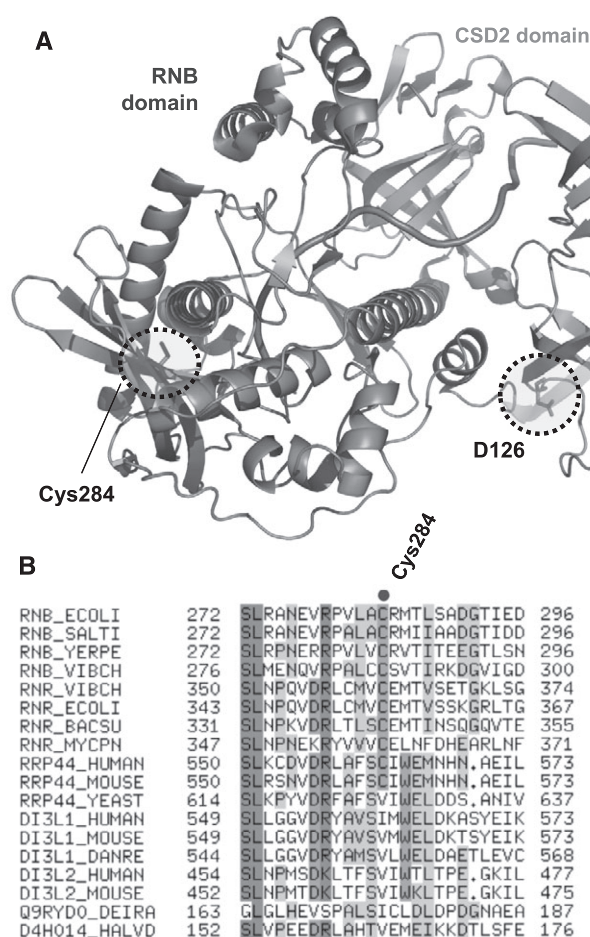

Fig. 1. Structural mapping and partial multiple sequence alignment of RNase II thermosensitive phenotype mutations. (A) The cartoon depicts part of the *E. coli* RNase structure representing the catalytic domain (RNB domain, dark gray) and the CSD2 domain (light gray). The C284 and D126 positions are highlighted for clarity. Made with Pymol using the PDB 2ix1 coordinates. (B) Partial multiple sequence alignment of representative RNase II, RNase R, Rrp44, and Dis3 proteins from different sources. Residues are highlighted according to sequence conservation.

Circular dichroism (CD) spectroscopy

CD experiments were performed in a Jasco J-815 spectropolarimeter equipped with a Peltier-controlled thermostated cell support. The far UV CD spectra were recorded for RNase II (wild-type and C284Y) at 0.25 mg·mL^{-1} in PBS buffer, pH 7.4.

Thermal denaturation and kinetic assays

Thermal denaturation experiments were carried out increasing the temperature from 20 to 90 °C at a heating rate of 1 °C·min^{-1}. Changes in the CD signal at 222 nm were plotted as a function of temperature and the apparent T_m was determined from fitting to a sigmoidal two-state transition. For the thermal kinetic assays, RNase II samples at a concentration of 0.25 mg·mL^{-1} were incubated at 44 °C for 30 min, while recording the CD signal at 222 nm. To access the relative structural variation, the far-UV CD spectra were recorded before and after the incubation, as described above.

Results and Discussion

Identification of RNase II mutations in the thermosensitive rnb500 allele

In order to identify the mutation(s) responsible for the ts phenotype, we sequenced the gene encoding the thermolabile RNase II protein. The chromosomal DNA of *E. coli* SK5689 strain [25] was used to amplify the *rnb500* gene by PCR, and the sequence of the gene was determined. To our surprise, two missense mutations were observed. One is the result of a G to A substitution at nucleotide 376 that resulted in an Asp to an Asn substitution at amino acid 126 (D126N). The other point mutation is a G to A transition at nucleotide 851, resulting in a Tyr residue in place of a Cys residue at amino acid 284 (C284Y). The RNase II enzyme family exhibits a common modular organization: a central nuclease domain (known as RNB) flanked by oligonucleotide-binding (OB) family folds at the N terminus [cold shock domains (CSD) 1 and 2] and at the C terminus a S1 domain [36]. The RNB domain is well conserved and is exclusive to RNase II family of proteins [3,36]. Based on the crystal structure of RNase II, D126 lies in CSD2 and C284 lies in the RNB catalytic domain (Fig. 1A). Although RNase II C284 has not been shown to be directly involved in substrate binding or catalysis, the conservation of the RNB domain within the multiple organisms that contains RNB-like enzymes suggests that this residue is important; perhaps it is involved in maintaining the structure and consequently the function of RNase II. Indeed, sequence alignments revealed that most of RNase II enzymes have a conserved cysteine in this position, including mouse and human Dis3 family members (Fig. 1B). A previous study involving a ts strain at 35 °C was described in *Saccharomyces cerevisiae* in which was demonstrated that a vector expressing human *DIS3* in the ts bacteria could rescue the growth defect at this temperature [37].

In vivo analysis of the identified thermosensitive RNase II mutations

Strains deficient for both PNPase and RNase II are not viable [38]. Therefore, to determine which mutant is responsible for the ts phenotype, we decided to use a double exonuclease mutant (strain CMA401) harboring the *pnp200* allele encoding a thermosensitive PNPase [31] in conjunction with a *rnb* deletion allele [39]. We chose pCMA01 [32] as the vector for genetic complementation because it contains the natural *rnb* promoter. To determine whether a single mutation or both are responsible for the thermolability of *rnb500* allele, we introduced each point mutation individually (single mutant) or the two substitutions (double mutant) in pCMA01, by site-directed mutagenesis. Transformation of the ts *pnp200* Δ*rnb* *E. coli* strain with a plasmid

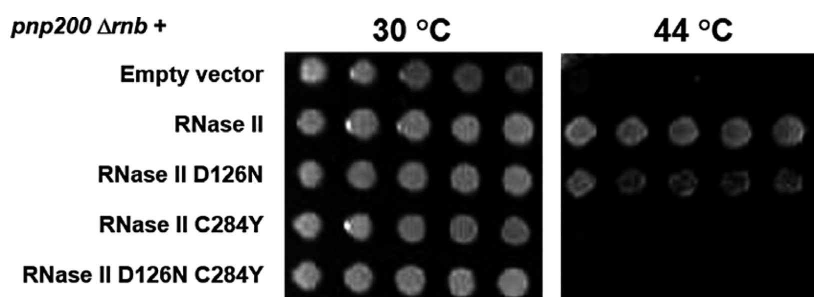

Fig. 2. The RNase II C284Y mutation confers thermosensitivity at 44 °C. Growth at the permissive (30 °C) and nonpermissive temperature (44 °C) of a thermosensitive *E. coli* strain, harboring empty vector or vectors encoding either wild-type RNase II or the indicated mutations. Bacterial cultures were grown on LA medium, supplemented with ampicillin (100 μg·mL^{-1}) and thymine (50 μg·mL^{-1}) for 1 day.

bearing the wild-type *rnb* gene can abolish the ts phenotype at 44 °C (Fig. 2). Consequently, it is easy to score which mutant supports RNase II function *in vivo*. Comparing to the wild-type, RNase II D126N exhibited a slightly decreased growth at 44 °C suggestion some thermosensitivity which however does not account for a major phenotype, indicating that this individual mutation is not *per se* detrimental for the function of RNase II *in vivo*. Strikingly, the expression of RNase II C284Y and the double mutant (D126N and C284Y) did not allow growth at the nonpermissive temperature (44 °C; Fig. 2). This result demonstrates that a single amino acid change (C284Y) is enough to generate the ts phenotype.

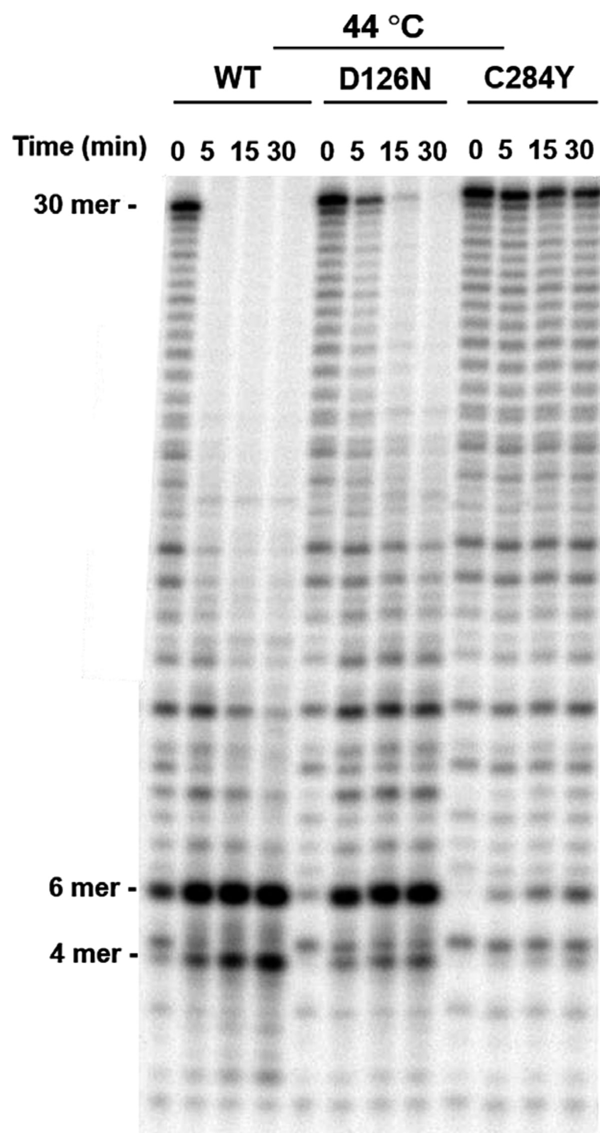

Fig. 3. Exoribonucleolytic activity of RNase II and mutant versions of RNase II. Activity assays were performed as described in Materials and Methods using 1 nM of enzymes and 4 nM of substrate. Samples were collected at the indicated time-points. Length of substrates and degradation products are indicated in the figure.

In vitro analysis of the thermolability of the RNase II C284Y variant activity

To determine whether any of these substitutions altered RNase II activity, *in vitro* enzyme activity assays were carried. For this purpose, single mutations were inserted by site-directed mutagenesis into the previously described inducible plasmid pFCT6.9 [33] with cloned wild-type RNase II, and the new plasmids were transferred to *E. coli* BL21(DE3), to overproduce the corresponding His_6-tagged RNase II variant proteins. The *in vitro* thermolability of RNase II was measured after preincubating the purified proteins at 44 °C for 30 min, and subsequent measurement of the RNase II activity at 30 °C [24]. In the activity assay, wild-type RNase II behaved as expected (Fig. 3), catalyzing the complete degradation of a 30 ss substrate to a final product with 4–6 nt, in < 5 min, using 1 nM of enzyme [35]. Not surprisingly, the RNase II D126N variant exhibited substantial catalytic activity confirming that this individual mutation is not the one responsible for the ts phenotype of *rnb500*. In contrast, the RNase II C284Y mutant enzyme was nearly inactive in respect to substrate degradation, even after 30 min of incubation (Fig. 3). The decreased activity relative to wild-type, after the shift to the nonpermissive temperature in the *in vitro* experiment, is in good agreement with what has been seen previously for enzyme activity in experiments with a strain carrying the *rnb500* allele [24]. Indeed, when lysates of a RNase II-deficient (*rnb296*) strain harboring a *rnb500* plasmid (pDK39) were incubated at 44 °C for 30 min, the lysates lost more than 95% of activity [24]. These results demonstrate that the thermolability of the *rnb500* proteins is due to the single amino acid substitution C248Y in the RNB domain.

Analysis of the thermal and kinetic stability of the RNase II C284Y mutant

Having established which mutation confers the *ts* phenotype to the *rnb500 allele*, we have carried out an investigation of the folding and stability properties of RNase II C284Y. Spectroscopic analysis of the purified mutant protein by far-UV circular dichroism (CD) has shown bands typical of an α/β fold, with minima at 208 and 222 nm (Fig. 4A). Also, the CD spectrum obtained for the RNase II C284Y mutant is superimposable to that of the wild-type protein, indicating that the point

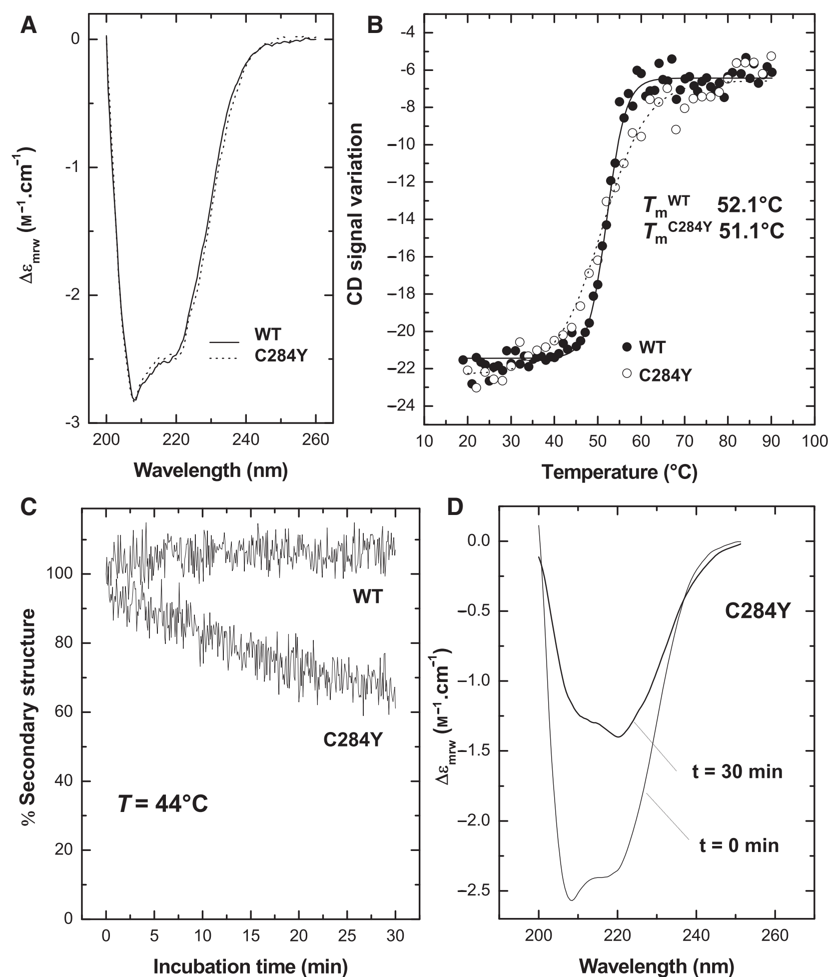

Fig. 4. Folding and thermal stability properties of the RNase II C284Y variant. The biophysical properties of the RNase II C284Y mutant were investigated in comparison to those of the wild-type enzyme. (A) Normalized far-UV CD spectrum; (B) Thermal denaturation (C); kinetics of thermal perturbation at 44 °C represented as % of secondary structure loss; (D) comparison of the far-UV CD spectra of RNase II C284Y before (t = 0) and after (t = 30 min) the incubation at 44 °C (panel C). See materials and methods for further details.

mutation is not affecting the folding of the expressed protein. We then evaluated the effect of the mutation on protein stability, by carrying out a thermal unfolding experiment during which the temperature was linearly increased from 20 to 90 °C, while the relative variation in secondary structure was followed by monitoring the CD signal at 222 nm. For both RNase II wild-type and C284Y mutant, protein denaturation led to aggregation, thus making the thermal unfolding reaction irreversible. Consequently, fitting of the observed transitions to a two-state model yields apparent, not thermodynamic, midpoint transitions or melting temperatures (T_m), which can nevertheless be used to compare relative stabilities of proteins that undergo irreversible denaturation [40–42]. This analysis evidenced a small difference between the apparent T_m of the wild-type (T_m = 52.1 ± 0.1 °C) and C284Y (T_m = 51.1 ± 0.1 °C) RNase II (Fig. 4B). However, there is a difference on the cooperativity of the thermal unfolding reaction, denoted by the less sharp transition of the C284Y RNase II variant, which indicates a larger ensemble of protein conformers during the thermal transition. Therefore, the observed thermosensitivity is not the result of a substantially decreased thermal stability of the mutant RNase II but may rather lie on its broader conformational dyanamics.

We then investigated if the *ts* phenotype could result from differences in protein kinetic stability. To address this issue, we have analyzed the time course of structural changes resulting from thermal perturbation of RNase II C284Y at 44 °C. The results showed that the RNase II C284Y variant is indeed kinetically unstable in comparison to the wild-type enzyme: upon a 30 min incubation at 44 °C results in a notorious loss of secondary structure (≈35%), whereas the wild-type enzyme remains unaffected (Fig. 4C). No aggregation was observed during this experiment. Analysis of the far-UV CD spectrum after incubation showed that RNase II C284Y underwent substantial conformational changes, as noted from the decrease in signal

intensity at 222 nm and changes in the shape of the spectral bands (Fig. 4D). These structural effects likely underpin important modifications at the protein active site that result in loss of function.

Conclusions

Alleles with thermosensitive phenotypes are extremely useful tools to investigate cellular functions of gene products. For over the last 30 years, the *rnb500* allele has been extensively used to study *E. coli* RNase II as well as other ribonucleases. However, the molecular origins for this phenotype have never been investigated. Here, we report the finding that the RNase II thermolabile phenotype conferred by the *rnb500* allele is due to the Cys284Tyr mutation within the RNB domain. Sequencing of the *rnb500* allele responsible for the thermolabile phenotype of RNase II at 44 °C elicited two point mutations, D126N and C2484Y, whose effect on catalytic activity was investigated both *in vivo* and *in vitro*. The results obtained pointed out to the importance of the C284Y residue in *rnb500* phenotype, as by itself this mutation is deleterious to RNase II activity at the nonpermissive temperature. The RNase D126N mutation corresponds to an allowed polymorphic change, as it marginally affects enzymatic activity; also, no synergistic effects are observed when the mutations are combined, thus clearly showing that the C284Y substitution alone fully recreates the *rnb500* ts phenotype. Biophysical analysis of the purified RNase II C284Y variant showed that this mutation does not affect protein thermal stability, as the melting midpoint transition of this variant is comparable to that of the wild-type ($T_m \approx 51$ °C). Rather, the mutation affects RNase II dynamics and consequently its thermal kinetic stability: indeed, a progressive loss of secondary structure and conformational changes are observed during incubation of the protein at the nonpermissive temperature. Structural mapping of this mutation on the available crystal structure shows that it locates at the RNB domain (Fig. 1A), in which the RNase II active site is buried in a pocket formed by four conserved sequence motifs [36]. It is very likely the C284Y change results in subtle conformational changes within the β-sheets in which this position is found to influence protein breathing dynamics and propagate to the nearby active site. Consequently, it may affect the Mg^{2+} coordination and substrate binding, thus resulting in catalytic inactivation. The identification and localization of this mutation can, thus, become a useful tool for engineering RNase II enzymes from other organisms, especially those for which ts proteins and functions still remain unknown. This is extremely relevant when taking into account the importance of the RNase II family of enzymes, which are widespread among the three domains of life.

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Author contributions

FPR designed experiments, performed experiments, analyzed the data and wrote the paper, CB contributed to the writing and revision of the paper, PGP performed modeling experiments wrote and revised the manuscript, CMA designed the study and coordinated the work and revised the manuscript, CMG co-designed the study with CMA, performed experiments, analysed the data and wrote the paper.

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