# Increasing Protein Conformational Stability by Optimizing β-turn Sequence

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## SUMMARY

Protein conformational stability is an important concern in many fields. Here, we describe a strategy for significantly increasing conformational stability by optimizing β-turn sequence. Proline and glycine residues are statistically preferred at several β-turn positions, presumably because their unique side chains contribute favorably to conformational stability in certain β-turn positions. However, β-turn sequences often deviate from preferred proline or preferred glycine. Therefore, our strategy involves replacing non-proline and non-glycine β-turn residues with preferred proline or preferred glycine residues. Here, we develop guidelines for selecting appropriate mutations, and present results for five mutations (S31P, S42G, S48P, T76P, Q77G) that significantly increase the conformational stability of Ribonuclease (RNase) Sa. The increases in stability ranged from 0.7 kcal/mol to 1.3 kcal/mol. The strategy was successful in overlapping or isolated β-turns, at buried (up to 50%) or completely exposed sites, and at relatively flexible or inflexible sites. Considering the significant number of β-turn residues in every globular protein and the frequent deviation of β-turn sequences from preferred proline and preferred glycine residues, this simple, efficient strategy will be useful for increasing the conformational stability of proteins.

### Keywords

protein conformational stability; β-turn; proline; glycine

## Introduction

Proteins carry out most of the important tasks in living cells, and they play an increasingly important role in the biotechnology and pharmaceutical industries. For proteins to perform these important tasks, they must fold to their proper, unique three-dimensional structure. This folding process depends on the protein having sufficient conformational stability. The conformational stability of a protein is defined as the free energy change ($\Delta G°$) for the reaction: folded (or native, N) $\Leftrightarrow$ unfolded (or denatured, D)[1]. This free energy change for most proteins

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is marginal – on the order of 5 to 15 kcal/mol. The marginal stability of proteins makes the use of certain proteins in industrial or pharmaceutical applications difficult especially when the protein is subjected to extremes of temperature and pH, or when the protein is subjected to destabilizing solvents such as organic solvents. Insufficient protein stability is also a common problem in general biochemical studies when an unstable recombinant protein of interest cannot be expressed at desirable levels due to aggregation and formation of inclusion bodies during expression.

Consequently, there is great interest in methods that can be used to increase the conformational stability of proteins. Many methods have been introduced[2–10], but it is still difficult to efficiently and effectively stabilize proteins. Here, we have developed a strategy for significantly increasing protein stability by optimizing β-turn sequence. β-turns are the smallest type of secondary structure. They join other elements of secondary structure such as α-helices and β-strands and abruptly change the direction of the polypeptide chain. Due to their polar nature, β-turns occur most often at protein surfaces, but sometimes they do occur in the protein interior[11]. β-turns usually contain four residues designated as $i$ (or −B1), $i+1$ (or L1), $i+2$ (or L2), $i+3$ (or +B1), and the distance between the α-carbons of the $i$ and $i+3$ residues in a β-turn is less than 7 Å. They are sometimes, but not always, stabilized by a hydrogen bond. Two common types of β-turns are type I and type II β-turns, but there are several other β-turn types determined by the φ, ψ angles of the $i+1$ and $i+2$ residues[12].

In statistical analyses of residues found in β-turns[12; 13], protein structures display preferences for proline or glycine at certain β-turn positions. For example, in a type II turn, the O atom of the $i+1$ residue crowds the Cβ atom of the $i+2$ residue. Therefore, the $i+2$ residue of type II turns is usually glycine. Glycine is also often found at other positions of different β-turn types since its lack of a β-carbon sterically allows a wider range of φ, ψ angles than other amino acids. Proline is a residue that is also found often in turns presumably due to its unique restricted φ angle, which is entropically favorable at certain turn positions. For example, the pyrrolidene ring of proline residues restricts the φ angle of this residue to −60°. Furthermore, the φ angle of the $i+1$ residue of type I and II turns is −60° (see Hutchinson and Thornton[12]). Therefore, proline probably assumes the −60° φ angle that is required at certain β-turn positions with a reduced entropic cost relative to other, more intrinsically flexible amino acids.

Though proline and glycine are preferred at certain β-turn positions, turn regions in proteins show large sequence variation[14–19]. Therefore, our strategy involves replacing non-proline and non-glycine residues at β-turn positions where proline or glycine are preferred based on β-turn sequence statistics[12; 13]. Stabilization strategies based on sequence statistics are the easiest and most straightforward to use[20], but it is not completely understood how these non-optimal β-turn sequences might be exploited to increase protein stability. Ohage et al.[18] and others[16; 21–23] have described approaches for optimizing β-turns. However, a strategy that focuses on both proline and glycine that tests different positions of different β-turn types has not been developed. Here, we present guidelines for using this strategy and results for several mutations that significantly increase the conformational stability of Ribonuclease (RNase) Sa.

## Results and Discussion

### Design strategy

To investigate whether stabilizing mutations to proline or glycine could be introduced at β-turn positions in RNase Sa, we used the program PROMOTIF[24] and the 1.2 Å crystal structure of RNase Sa (PDB code: 1RGG[25]) to identify residues in β-turns. Then, we used β-turn sequence statistics collected by Guruprasad and Rajkumar[13] to identify non-proline and non-glycine residues in positions where proline or glycine are preferred. The positional potentials of the wild-type residues and preferred proline or glycine residues are shown in Table 1. As

an example, position 31 in RNase Sa is the $i$+1 position of a type I β-turn. A serine residue is found at position 31 in wild-type RNase Sa, but sequence statistics[13] indicate that proline is preferred at the $i$+1 position of a type I β-turn (Table 1). Therefore, S31P is an example of a candidate for mutation. This process identified fourteen possible mutations out of thirty-two type I, II, I′, or II′ β-turn positions in RNase Sa. Mutations in type IV and VIII turns of RNase Sa were not made because the residues in these turns were mostly buried and therefore, probably not good targets for mutation.

Of the fourteen possible mutations, candidates were eliminated where the wild-type side chain was in the active site of the protein as determined by Yakovlev et al.[26] or where the side chain was involved in hydrogen bonding as determined from analysis using the program pfis[27] and the crystal structure of RNase Sa[25]. Also, for mutations to proline, candidates were eliminated where the amide nitrogen of the residue was a hydrogen bond donor since proline residues lack an amide hydrogen. Mutation of ionizable residues was also not considered. This is because the effect of mutating ionizable residues on overall stability has been shown to be difficult to predict in terms of electrostatic interaction energy[28].

Additional guidelines for candidate selection were discovered based on results for the Y49P, Y86G, and Q94P variants. The stability changes associated with these three variants as determined by thermal denaturations monitored by circular dichroism (CD) spectroscopy are shown in Table 2. The Y86G mutation increased the conformational stability of RNase Sa by only 0.4 kcal/mol. The Y49P mutation did not change the stability of the protein significantly as shown by its near-zero ΔΔG value, and the Q94P mutation destabilized the protein significantly by 0.8 kcal/mol.

Substitution of aromatic tyrosine residues in the Y49P and Y86G variants did not significantly increase the stability of RNase Sa. This is probably because these aromatic residues provide stabilizing interactions in β-turns. A study by Gibbs et al.[29] suggested that aromatic residues provide stabilizing interactions in a type II′ β-turn. There is also evidence in RNase Sa that suggests that aromatic residues in β-turns provide stabilizing interactions. For example, the Y49N mutation (position 49 is the $i$+1 position of a type II β-turn) in RNase Sa decreases the stability of the protein by 1 kcal/mol (S.T., unpublished observations). The hydroxyl group of this tyrosine is not involved in hydrogen bonding interactions, so it seems that the decrease in stability in the Y49N variant is caused by the loss of stabilizing interactions provided by the aromatic portion of the tyrosine. This loss of 1 kcal/mol is similar in magnitude to the stability gained in the T76P variant (position 76 is also the $i$+1 position of a type II β-turn) (Table 2). Therefore, in the Y49P variant, the gain in stability upon introduction of preferred proline is probably counterbalanced by the loss of stabilizing interactions provided by the aromatic tyrosine resulting in a near-zero change in conformational stability. Further evidence that aromatic residues can provide stabilizing interactions in β-turns is shown in our results from a previous study where we compared solubility values of 20 variants of RNase Sa at position 76 ($i$+1 position of a type II β-turn). We also measured the stability for each of these variants, and the results showed that the T76Y, T76F, and T76W variants all result in stability increases similar in magnitude to the T76P variant suggesting that the stabilizing contribution of aromatic residues in this case is similar to that of a preferred proline in a β-turn. Combining these RNase Sa results with the results of Gibbs et al.[29] suggests that significant stability cannot be gained by replacing aromatic residues in turns with glycine or proline. Indeed, this is what is observed for the Y49P and Y86G variants (Table 2).

The Q94P mutation destabilized RNase Sa despite mutation to a preferred proline. One reason might be that Gln94 is close in sequence to Cys96, which forms a disulfide bond with Cys7 in RNase Sa. A mutation to proline close to this bond might strain the bond and destabilize the protein – especially since this disulfide bond contributes dramatically to the stability of RNase

Sa (~ 7 kcal/mol)[30]. Another possible reason the Q94P variant destabilizes RNase Sa is that Gln94 is close in space to Asp79 which is a buried carboxyl group that does not form any intramolecular hydrogen bonds and hence, contributes unfavorably to the conformational stability of RNase Sa[31]. Gln94 is the only polar residue near Asp79, and mutating it to the more hydrophobic proline might make Asp79 contribute even more unfavorably to the conformational stability of RNase Sa.

Therefore, based on the results for the Y49P, Y86G, and Q94P variants, the following additional guidelines for using the strategy were established: mutation of aromatic residues and mutation of residues that were close in sequence to residues involved in the disulfide bond (Cys7 and Cys96) were avoided. Overall, this elimination process resulted in seven candidates for mutation. Five of these variants (S31P, S42G, S48P, T76P, and Q77G) were made. The other two (Q32P and T59P) were not made, because they are similar to the S48P mutation, i.e., a mutation at the *i* position of a type II β-turn. Note that Ser31 is at position *i*+1 in a type I β-turn, but Gln32 is at position *i* in a type II β-turn. This is a consequence of overlapping turns as discussed further below. Figure 1 shows the five candidates for mutation in RNase Sa (Ser31, Ser42, Ser48, Thr76, and Gln77).

### None of the mutations significantly affected the protein structure

To confirm that these mutations in RNase Sa did not change the solution structure of the protein, CD spectra of the wild-type protein and several of the β-turn variants were compared (data not shown). The results show similar CD spectra for wild-type and the β-turn variants suggesting that the variants retained structure similar to wild-type RNase Sa. In addition, all of the variants retained enzymatic activity (data not shown). In general, mutations to proline or glycine do not appear to significantly affect protein structure as determined by X-ray crystallography. For example, several studies have reported crystal structures of mutants involving proline and glycine substitutions in β-turns or other locations in protein structures[16; 17; 23; 32], and each study found no significant change in structure upon mutation to proline or glycine. Therefore, results from X-ray crystallography and our results suggest that these types of substitutions do not cause significant structural changes relative to the wild-type proteins.

### Stability increases for the β-turn variants in RNase Sa

To determine the conformational stability changes caused by the β-turn mutations, thermal denaturations monitored by circular dichroism spectroscopy at pH 7 were performed. Previously, our lab has characterized the folding and stability of RNase Sa and several variants[26; 28; 30; 31; 33–39]. The unfolding of RNase Sa and its variants is reversible and follows a 2-state mechanism. The thermodynamic parameters characterizing the thermal unfolding of these variants are shown in Table 2. The S31P, S42G, S48P, T76P, and Q77G mutations resulted in significant increases in conformational stability as shown by the positive ΔΔG values greater than 0.5 kcal/mol and the increased $T_m$ values. The double mutant, T76P-Q77G, containing two mutations in the same turn, stabilized the protein by 1.6 kcal/mol. A triple mutant, S31P-S42G-Q77G, was also constructed, and it stabilized RNase Sa by 1.9 kcal/mol.

### Thermodynamic effects of mutations to proline

In general, mutations to proline are expected to stabilize proteins by decreasing conformational entropy in the denatured state. Matthews et al.[40] estimated this stabilization to be on the order of 0.8 kcal/mol based on their A82P mutation in T4 lysozyme. The change in conformational entropy, or ΔΔS value, for A82P was negative (−10 cal mol$^{-1}$ K$^{-1}$) suggesting a decrease in conformational entropy in the denatured state, but did not appear significant based on the error[40]. Prajapati et al.[32] did another study of general mutations to proline that involved thirteen mutations to proline in four different proteins. They did observe significant ΔΔS

values, but the $\Delta\Delta S$ values varied widely including negative and positive values ($-55$ to $47$ cal mol$^{-1}$ K$^{-1}$). For our stabilizing mutations to proline in β-turns (S31P, S48P, and T76P), we also observed both positive and negative $\Delta\Delta S$ values ($-16$ to $12$ cal mol$^{-1}$ K$^{-1}$), but these values were not significant based on the error associated with the measurements (Table 2).

Other studies have investigated the effects of mutations to proline in β-turns. Suzuki and co-workers[41; 42] have investigated how mutations to proline at second sites (i.e., $i+1$ residues) of type I and II β-turns increase the thermal stability of oligo-1,6-glucosidase from *Bacillus cereus*, though they did not measure $T_m$ and $\Delta\Delta G°$ values. Furthermore, even though Choi and Mayo[43] introduced prolines at second sites of β-turns in protein G (K10P and A48P), they observed slight decreases in stability presumably because Lys10 formed favorable electrostatic interactions in the folded state and because the amide nitrogen of Ala48 was a hydrogen bond donor. In agreement with the results of Suzuki and co-workers[41; 42], the mutations to proline at second sites of type I and type II β-turns in RNase Sa (S31P and T76P) significantly increased protein stability (Table 2). In addition, the mutation to preferred proline at the first site of a type II turn in RNase Sa (S48P), resulted in the largest increase in conformational stability (1.3 kcal/mol) of all the mutations to preferred proline or preferred glycine (Table 2).

## Thermodynamic effects of mutations to glycine

Mutations to glycine in certain positions (not necessarily in β-turns) can relieve steric strain in the folded state caused by residues that contain a β-carbon. For example, certain residues are located in the left-handed α-helical (L-α) region of the Ramachandran plot ($0°<\varphi<90°$, $0°<\psi<90°$)[44]. Non-glycine residues in this region may cause steric strain involving the β-carbon of the residue and the peptide backbone. Indeed, Takano et al.[45] found that Gly to Ala substitutions at rigid L-α positions led to significant destabilization (1–2 kcal/mol) of human lysozyme. Masumoto et al.[46] found similar results in hen egg white lysozyme. Consequently, there have been several studies involving non-Gly to Gly mutations in L-α positions resulting in stability increases of 1 to 2 kcal/mol[21; 22; 47]. In contrast, Nicholson et al.[48] found that this strategy worked neither for the N55G variant nor the K124G variant in T4 lysozyme. However, Asn55 in T4 lysozyme is at the $i+1$ position of a type I′ β-turn, and Asn is highly preferred even relative to Gly at this position (the positional potentials are 6.07 and 1.73 for Asn and Gly, respectively[13]). Therefore, this might be a possible reason for the slight decrease in stability observed for the N55G mutation in T4 lysozyme. As for the K124G variant, Nicholson et al.[48] noted that Lys124 is involved in favorable electrostatic interactions in wild-type T4 lysozyme. The stabilizing results at positions 42 and 77 in RNase Sa (Table 2), whose $\varphi$, $\psi$ angles are in the L-α region as determined by the program pfis[27], agree with those of Kimura et al.[21], Stites et al.[47], and Kim et al.[22], and they suggest that non-glycine residues in the L-α region do cause strain which, by mutation to glycine, can be relieved to significantly increase protein stability. However, care must be taken to avoid mutating highly preferred Asn residues at $i+1$ positions of type I′ β-turns or residues that participate in favorable electrostatic interactions. In terms of changes in conformational entropy, mutations to glycine might be expected to have positive $\Delta\Delta S$ values[40]. However, our two stabilizing mutations to glycine (S42G and Q77G) did not have significant $\Delta\Delta S$ values (Table 2).

## Stabilizing mutations occurred in both isolated and overlapping β-turns

Some of the stabilizing mutations occurred in isolated β-turns while others were in β-turns that shared one or more residues with other β-turns. Such overlapping turns appear to be very common in proteins. In their survey of 205 proteins, Hutchinson and Thornton[12] found that 58% of β-turns share one or more residues with other β-turns. In RNase Sa, 75% of the β-turns overlap with other β-turns as determined by PROMOTIF[24]. Therefore, it is not surprising that some of the candidates for mutation in RNase Sa occurred in overlapping β-turns. According to the PROMOTIF[24] analysis of RNase Sa structure, position 48 is in a type II turn that overlaps

with a type VIII turn. Positions 31 and 42 are also in turns that overlap with other turns. On the other hand, positions 76 and 77 are in an isolated turn. All of these mutations significantly increased the stability of RNase Sa, so it seems that significant increases in stability can be obtained by optimizing either isolated or overlapping β-turns.

## Stability correlation with side chain burial and backbone flexibility

The extent to which side chains are buried has been suggested to play a role in the stability changes observed for β-turn mutations. Takano et al.[16] investigated the role of turn residues in the structure and stability of human lysozyme. They observed that the increase in stability upon mutations to preferred glycine was dependent upon whether the side chain was buried or exposed to solvent. For example, substitutions on the surface hardly changed the conformational stability whereas mutation at a buried site (Gln58) significantly increased the stability of human lysozyme. Their other mutations to glycine in β-turns (R50G and H78G) did not stabilize the protein, but this could have been due to the disruption of favorable electrostatic interactions. The stabilizing mutations to glycine in our study (S42G and Q77G) were at partially buried sites (52% and 39%, respectively) (Table 3). In contrast, the stabilizing mutations to proline in this study (S31P, S48P, and T76P) were at completely exposed sites, yet they yielded significant stability increases. Therefore, our results suggest that this strategy works at buried sites (up to 50%) as well as completely exposed sites.

The flexibility of the protein structure at the site of mutation has also been suggested to correlate with stability changes of mutations involving glycine and proline. Takano et al.[45] replaced glycine residues with alanine in human lysozyme to gauge the strain caused by having non-glycine residues in sites with left-handed helical $\varphi$, $\psi$ angles (i.e., sites where a β-carbon might cause steric strain). They showed that mutations at relatively flexible positions of the protein (B-factors $\geq 13$ Å$^2$) did not show large stability changes. Similarly, Yutani et al[49] replaced conserved proline residues in the tryptophan synthase alpha subunit with Ala or Gly and only saw significant stability changes in less mobile regions. Matthews and co-workers have also shown that mutations that significantly affect protein stability tend to be at rigid sites[50; 51]. In contrast, the S42G, S48P, T76P, and Q77G mutations in this study were at sites with B-factors $> 15$ Å$^2$ (Table 3), yet they still yielded significant increases in stability. Therefore, it seems that significant increases in stability can be achieved by optimization of relatively flexible or inflexible β-turn sites.

## Additivity of multiple β-turn mutations

To investigate the possibility of cooperative stability increases in multiple β-turn variants, a double mutant containing mutations in the same turn (T76P-Q77G) and a triple mutant containing mutations in three different turns (S31P-S42G-Q77G) were made. The results in Table 2 show that the stability increases for these multiple mutants were essentially additive. For example, the stability increase for the T76P-Q77G variant was 1.6 kcal/mol while the sum for the individual mutations is 1.8 kcal/mol. Therefore, it appears that multiple β-turn mutants yield increases in stability that are additive even when multiple mutations are made in the same turn.

# Conclusions and general guidelines

β-turn sequence statistics for large sets of proteins have been collected and show that proline and glycine residues are preferred at several positions of several β-turn types[12; 13]. However, β-turn sequences in RNase Sa and other proteins[12–19] often deviate from preferred proline or preferred glycine residues. Here we show that mutating non-proline and non-glycine residues to preferred proline or preferred glycine residues in β-turns results in significant increases in the conformational stability of RNase Sa. Also, there were a large number of candidates (seven)

for stabilizing β-turn mutations in the relatively small RNase Sa, and five of these mutations representing a wide range of β-turn positions result in significant increases in conformational stability of RNase Sa. Other studies have also shown that optimizing β-turn sequence can yield large increases in protein stability[16; 18; 21–23]. A large percentage of protein residues are found in β-turns. For example, it has been estimated that a quarter of all protein residues are in β-turns[52], and in RNase Sa, about 31% of the residues are in β-turns (not including residues in type IV or type VIII turns). Therefore, the variability of β-turn sequences (i.e., deviation from preferred residues) and the large percentage of turn residues found in proteins should provide ample opportunities for using this strategy to stabilize any protein of interest.

Based on this study as well as others, the following guidelines should be useful when attempting to increase protein stability by mutating non-proline and non-glycine residues to preferred proline or preferred glycine: 1) The wild-type residue should not be involved in stabilizing interactions such as hydrogen bonds or favorable electrostatic interactions. Also, since proline lacks an amide hydrogen, mutations to proline should be avoided at positions where the amide nitrogen is a hydrogen bond donor. 2) Bulky hydrophobic or aromatic residues such as Val, Leu, Ile, Phe, Tyr, and Trp should not be targeted as they might be partially buried and provide favorable hydrophobic interactions. 3) Mutations to proline near in sequence to Cys residues involved in disulfide bonds should be avoided. 4) Asparagine residues in the $i+1$ position of type I′ β-turns should not be targeted as they are highly preferred in these positions[13]. 5) Mutations in type IV or type VIII turns are less desirable as these turns are more often buried than other β-turn types. Following these guidelines, we have been successful in increasing the stability of RNase Sa by 0.7 to 1.9 kcal/mol. The strategy was effective in overlapping or isolated β-turns, at buried (up to 50%) or completely exposed sites, and at relatively flexible or inflexible sites. Also, the method is simple to use in that it doesn't require complex calculations or sequence/structural information of homologous proteins. Further development of this strategy is necessary as data for several positions in type I (the $i$ position), type I′ (the $i+2$ position), and type II′ (the $i+1$ position) β-turns is still lacking. The strategy presented here has not been included in lists of popularly considered strategies for increasing protein stability[2; 7; 10], but our results demonstrate the efficiency and effectiveness of increasing protein stability by optimizing β-turn sequence and should be useful for those interested in increasing protein stability.

## Materials and Methods

### Preparation of mutant plasmids and proteins

Primers were ordered from Integrated DNA Technologies at www.idtdna.com. Site-directed mutagenesis was performed using the QuikChange® Site-Directed Mutagenesis Kit from Stratagene. Mini-preps of the mutant plasmids were performed using the QIAprep® Spin Miniprep Kit from Qiagen. Sequencing of the mutant plasmids was done at the Gene Technologies Laboratory, Department of Biology, Texas A&M University. All proteins were expressed and purified as described previously[53].

### Thermal denaturation experiments

Mops buffer at 30 mM concentration was used at pH 7.0. Thermal denaturation experiments were done using an AVIV Circular Dichroism Spectrometer Model 62DS as previously described[27; 30]. The data were analyzed using KaleidaGraph version 3.6.4 (Synergy Software). The analysis of thermal denaturation curves has been previously described[54].

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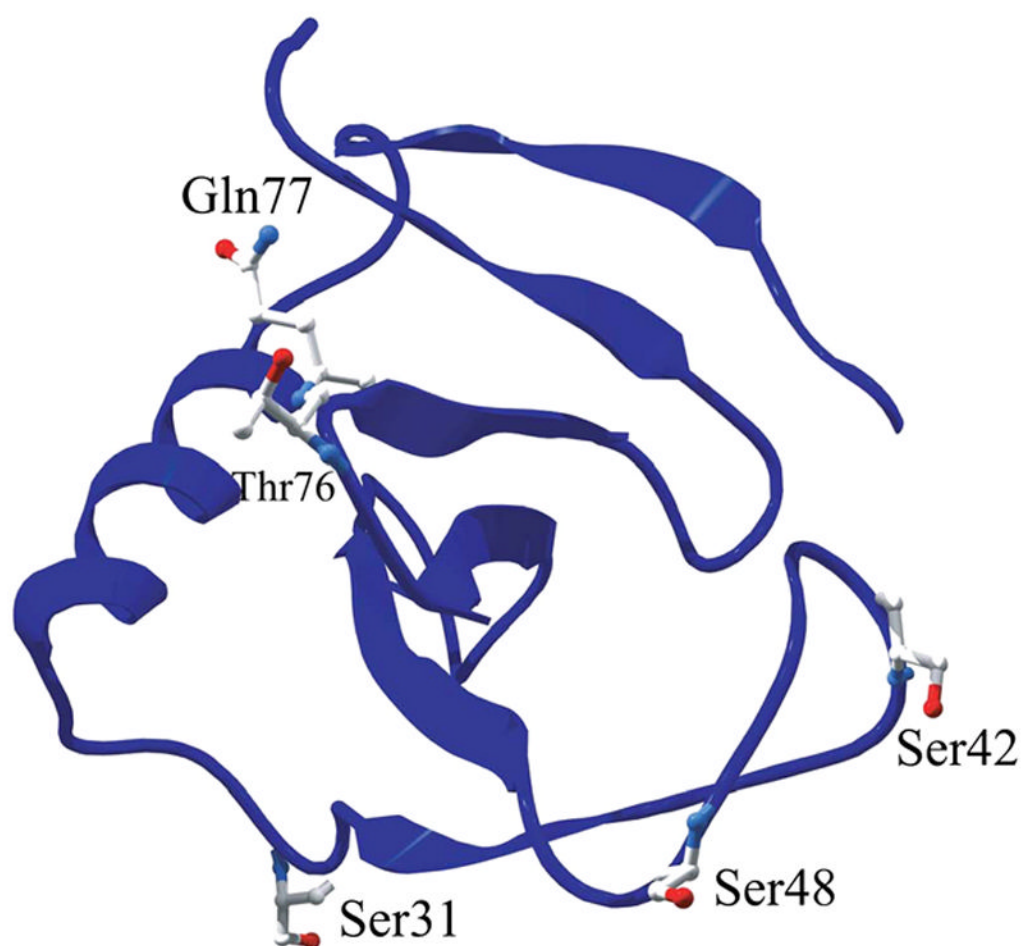
**Figure 1.**
Ribbon diagram showing candidates for optimizing β-turn sequence in RNase Sa. Ser31, Ser42, and Gln77 are the candidates shown. The figure was generated using the Swiss-Pdb Viewer program[55] and the 1.2 Å crystal structure of RNase Sa (PDB code: 1RGG[25]).

**Table 1**

Positional potentials of wild-type and preferred proline or glycine residues[a].

| Residue Number | Position | Turn Type | Positional Potential of Wild-Type Residue | Positional Potential of Preferred Glycine or Proline[b] |
|---|---|---|---|---|
| 31 | $i$+1 | I | Ser (1.61) | Pro (4.29) |
| 42 | $i$+3 | I | Ser (0.94) | Gly (2.57) |
| 48 | $i$ | II | Ser (0.65) | Pro (1.91) |
| 76 | $i$+1 | II | Thr (0.71) | Pro (4.92) |
| 77 | $i$+2 | II | Gln (0.17) | Gly (9.39) |

[a]Positional potentials as determined by Guruprasad and Rajkumar[13].

[b]"Preferred" refers to glycine or proline potentials that are statistically significant as determined by Guruprasad and Rajkumar[13].

**Table 2**

Parameters characterizing the thermal unfolding of RNase Sa and β-turn variants[a].

| Protein | Position | Turn Type | $\Delta H_m$ [b] | $\Delta\Delta H_m$ [c] | $\Delta S_m$ [d] | $\Delta\Delta S_m$ [e] | $T_m$ [f] | $\Delta T_m$ [g] | $\Delta\Delta G^{\circ}$ [h] |
|---|---|---|---|---|---|---|---|---|---|
| Wild-type | | | 91 | | 283 | | 48.4 | | |
| S31P | i+1 | I | 92 | 1 | 284 | 1 | 50.9 | 2.5 | 0.7 |
| S42G | i+3 | I | 91 | 0 | 281 | −2 | 51.0 | 2.6 | 0.7 |
| S48P | i | II | 87 | −4 | 267 | −16 | 52.9 | 4.5 | 1.3 |
| Y49P | i+1 | II | 85 | −6 | 265 | −18 | 47.7 | −0.7 | −0.2 |
| T76P | i+1 | II | 96 | 5 | 295 | 12 | 51.9 | 3.5 | 1.0 |
| Q77G | i+2 | I' | 88 | −3 | 271 | −12 | 51.3 | 2.9 | 0.8 |
| Y86G | i+2 | | 90 | −1 | 279 | −4 | 49.9 | 1.5 | 0.4 |
| Q94P | i+1 | I | 84 | −7 | 263 | −20 | 45.7 | −2.7 | −0.8 |
| T76P-Q77G | | | 84 | −7 | 257 | −26 | 54.2 | 5.8 | 1.6 |
| S31P-S42G-Q77G | | | 81 | −10 | 247 | −36 | 55.2 | 6.8 | 1.9 |

[a]The experiments were done in 30 mM Mops at pH 7.0.

[b]$\Delta H_m$, enthalpy of unfolding at $T_m$ (in kcal mol$^{-1}$). The error is ± 5 kcal mol$^{-1}$.

[c]$\Delta\Delta H_m = \Delta H_m$(mutant) − $\Delta H_m$(wild-type). The error is ± 7 kcal mol$^{-1}$.

[d]$\Delta S_m = \Delta H_m/T_m$ (in cal mol$^{-1}$ K$^{-1}$). The error is ± 15 cal mol$^{-1}$ K$^{-1}$.

[e]$\Delta\Delta S_m = \Delta S_m$(mutant) − $\Delta S_m$(wild-type). The error is ± 21 cal mol$^{-1}$ K$^{-1}$.

[f]$T_m$ = T (in °C), where $\Delta G^{\circ}$ = 0. The error is ± 0.3 °C.

[g]$\Delta T_m = T_m$(mutant) − $T_m$(wild-type) (in °C). The error is 0.4 °C.

[h]$\Delta\Delta G^{\circ} = 0.283$ ($\Delta S_m$ for wild-type in kcal mol$^{-1}$ K$^{-1}$) * $\Delta$:$T_m$. The error is ≤ 0.2 kcal mol$^{-1}$. A positive value indicates an increase in stability.

**Table 3**

Percent side chain burial and B-factors for the stabilizing β-turn variants in RNase Sa.

| Protein | % Side-chain burial[a] | B-factor ($Å^2$)[b] | ΔΔG° (kcal/mol) |
|---------|------------------------|---------------------|------------------|
| S31P | 0.7 | 12 | 0.7 |
| S42G | 52.0 | 27 | 0.7 |
| S48P | 2.1 | 16 | 1.3 |
| T76P | −2.5 | 30 | 1.0 |
| Q77G | 39.4 | 24 | 0.8 |

[a]Values for the wild-type residue.

[b]Average B-factors for backbone atoms obtained from the 1.2Å RNase Sa crystal structure (PDB code: 1RGG[25]).