Introducing a Third Disulfide Bond to Ribonuclease T1 and Expression of the Mutant Protein Using the Fusion Protein Method

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Abstrak

Perhitungan dinamika molekular meramalkan bahwa pembentukan ikatan disulfida yang ketiga pada RNase T1, yaitu antara Tyr 24 dan Asn 84, mungkin terjadi, tanpa menyebabkan perubahan pada catalytic site nya. Gen untuk RNase T1 mutan (Tyr 24 → Cys 24, Asn 84 → Cys 84) dibuat dengan cara cassette mutagenesis dengan menggunakan gen yang dibuat secara kimia. Untuk mengurangi aktivitas nukleolitik enzim pada keadaan in vivo, gen dièkspresikan dalam E. coli sebagai protein gabungan yang dihubungkan pada protein lain oleh metionin baik pada ujung -N maupun -C. Sesudah dibebaskan dari protein gabungan karena pemotongan pada batas metionin oleh sianogen bromida, RNase T1 mutan dimurnikan dengan menggunakan kromatografi kolom dan HPLC. Aktivitas nukleolitik terhadap pGpC kurang lebih sama dengan RNase T1, tetapi ketahanan RNase T1 mutan terhadap panas kurang lebih 10°C lebih tinggi.

Abstract

Molecular dynamic calculation has predicted that it is possible to form a third disulfide bond in RNase T1, between Tyr 24 and Asn 84, without major changes at the catalytic site. The gene for the mutant RNase T1 (Tyr24 ---> Cys24, Asn84 ---> Cys84), was constructed by the cassette mutagenesis method using a chemically synthesized gene. In order to reduce the nucleolytic activity of the enzyme in vivo, this gene was expressed in E. coli as a fused protein connected through methionin residues to an other protein at both the N- and C- termini. After liberation from the fused protein by cleavage with cyanogen bromide at the methionin junctions, the mutant RNase T1 was purified by column chromatography and HPLC. The nucleolytic activity with regard to pGpC was about the same as with RNase T1, but the mutant's heat resistance was about 10°C higher.

Keywords : Protein engineering, Chemically synthesized gene, Phosphotriester method, Mutant enzyme, Heat resistance.

INTRODUCTION

Ribonuclease T1 (RNase T1) from *Aspergillus oryzae* is an important enzyme in RNA research because it specifically hydrolyzes the phosphodiester linkages of guanosine 3'-phosphate residues in single stranded RNA.[1,2] It is a small (104 a.a.) acidic and globular protein containing two disulfide bonds (between Cys2 and Cys10, Cys6 and Cys103). This enzyme's optimal temperature is 37°C, and at 50°C its activity has dropped considerably, while in RNA research sometimes we have to use higher temperatures to hydrolyze compact structured RNA. Therefore, we aimed to engineer a thermally more stable enzyme.

Among fungal ribonucleases, there are at least two disulfide bonds. In ribonuclease F1, there is no disulfide bond between Cys2 and Cys10 as in ribonuclease T1, but, instead, it has a disulfide bond between Cys24 and Cys84 (fig.1).

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In RNase T1, residues number 24 and 84 are far from the catalytic site, and molecular dynamics calculation has hypothesized the possibility of the formation of a disulfide bond between these residues without a reliable change at the catalytic site.

Disulfide bonds have been succesfully introduced into proteins by site directed mutagenesis,[3,4,5] and some of these mutant proteins are thermally more stable than their respective wild type proteins.[3,4]

Therefore, by protein engineering techniques we have changed Tyr24 to Cys24 and Asn84 to Cys84 and investigated the activity and structural stability of the mutant enzyme (RNase T1S).

MATERIALS AND METHOD

Materials

T4 DNA ligase, polynucleotide kinase and various restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan) or New England Biolabs (Beverly, MA, U.S.A). The RNase T1 was a gift from Sankyo (Tokyo, Japan).

All materials were of reagent grade and were obtained from commercial sources as described previously.[1] Oligodeoxyribonucleotides were synthesized by the phosphotriester method[6] in an Apllied Biosystem synthesizer.

Construction of a mutant gene for RNase T1S and its expression plasmid.

The construction of the gene for the RNase T1S was performed in the same way as reported previously by Ikehara et al.' Segments II' and IVc' were used instead of segments II and IV as shown in fig.2. Segment II', encoding Cys24 instead of Tyr24 was synthesized from oligonucleotide MU5 (24C), d(CAGGCTGCTGG*TGT*) and oligonucleotide ML6 (24C), d (GCAGCTG*ACA*GCCAG) (italics indicate the changed codons). Segment IVc', encoding Cys84 instead of Asn84 and used for the insertion of the mutant gene (Tye24 ---> Cys 24, Asn 84 ---> Cys84) into the BglII site of pIGF8, was synthesized from oligo-nucleotide MU17 (84C), d (*TGT*CAGCTCGCTGGC), ML 17 (84C), d (GAAGCTG*ACA*GTTTTCGTTG), U21C, d(ACCATGCA) and L21C, d (GATCTGCATGGTGCATTC) instead of oligonucleotides U17, L17, U21 and L21. Other oligonucleotides and segments used were the same as those synthesized and described by Ikehara et al.[6] Plasmid pIGF8 (5 μg) was digested by BglII (30 units) at 37°C for 24 hours. After ethanol precipitation, linear plasmid DNA were treated with calf intestine alkaline phosphatase (0.25U) to remove the 5'- terminal phosphates, phenol extracted, ethanol precipitated and ligated with the synthetic mutant gene. This was designed to be expressed as a fused protein with a portion of hGH and IGF-I at the N- and C-terminal, under the control of Trp promoter as described by Nishikawa.[7] Desired plasmid was obtained from transformant of *E.coli* Hb 101 by standard procedures. The position and the nucleotide sequence of the RNase T1S gene (Tyr 24 ---> Cys24, Asn 84 ---> Cys84) was confirrmed by restriction analysis and the dideoxy method.[8]

Expression of the mutant gene, refolding and purification of RNase T1S.

The recombinant which harbored the plasmid pT1S was induced by the addition of 3-indoleacrylic acid, and the monitoring of the expression of the mutant gene was performed as described by Ikehara et al.[1] We started the purification using 51 culture grown to latelog phase. Harvested cells were suspended in 50mM Tris/HCl (pH 8.0), 30 mM NaCl/1mM EDTA at a concentration of 0.1 g cell/ml. The suspension of cells was treated with lysozyme (final concentration, 1mg/ml) at 0°C for 60 min, and then the mixture was treated with DNase (final concentration 1.5 μg/μl) at 0°C for 30 min and centrifuged at 28,000xg for 40 min. The precipitate was dissolved in the standard buffer (20 mM Tris/HCl pH 7.5, 10 mM 2-mercapto ethanol), which contained 7M urea and was left at 4°C overnight until all precipitates had dissolved.

The subsequent procedures : DEAE - cellulose column chromatography (DE 52, 3 x 6 cm) and cyanogen bromide treatment were basically the same as reported previously.[9,10] Cyanogen bromide treatment was performed without 10mM 2-mercaptoethanol.[7] After separation from cyanogen bromide, the mixture was subjected to refolding procedure, in 10mM (20mM - 10mM), then in 50mM Tris HCl (pH 7.5) and 100mM NaCl. The refolded enzymes were purified subsequently by Q sepharose column chromatography (2.4 x 13.5 cm), anion-exchange HPLC (mono Q,FPLC, Pharmacia), with a linear gradient of NaCl (0.15-0.45M) in 20mM Tris/HCl (pH 7.5) and finally subjected to sephadex G25 column chronatography (0.5 x 20 cm) to remove salts. The purity of the mutant enzyme was checked by SDS-polyacrylamide gel electrophoresis and the protein concentrations from each step were determined by Lowry's method or, in the case of the purified mutant enzyme, by using a modified molar absorption coefficient ($\sum$ 278= 19.830$M^{-1}cm^{-1}$), which was derived from those of

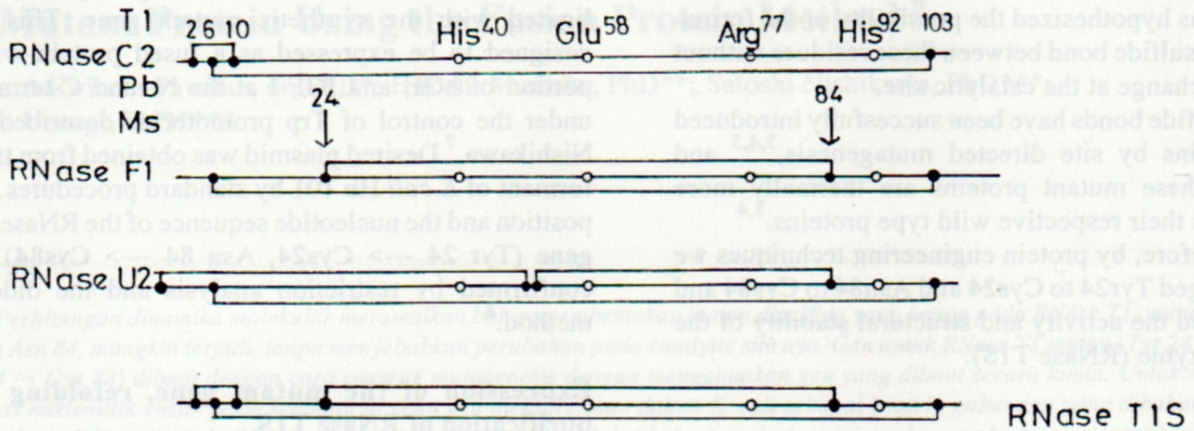

Figure 1. Schematic alignment of disulfide bonds in fungal ribonucleases. Closed circles indicate cystein, and open circles indicate active site residues : His 40, Glu58, Arg77 and His92.

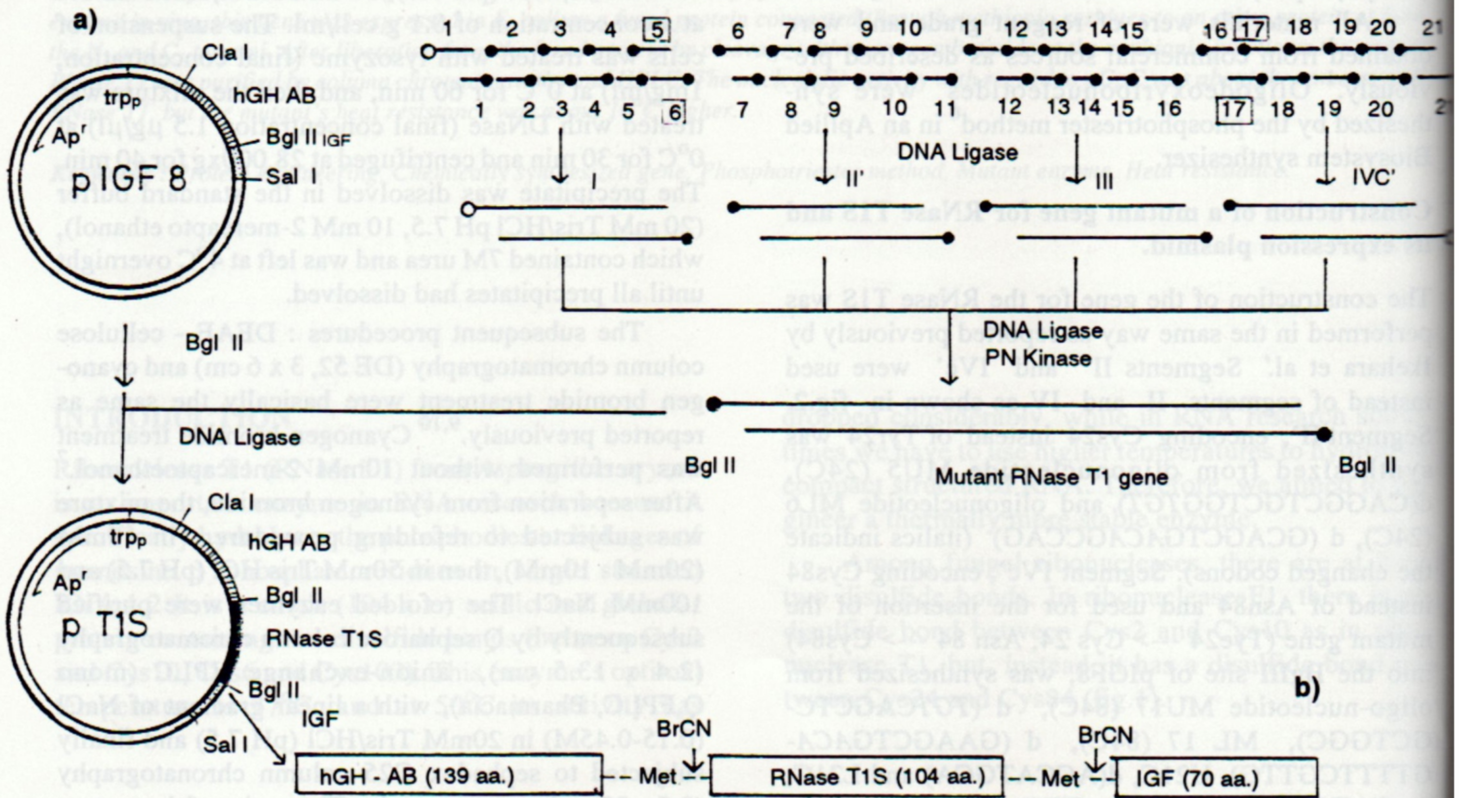

Figure 2. Synthesis of RNase T1S gene and construction of pT1S.

(a). Closed circles indicate 5'-phosphate, and open circles indicate 5'-hydroxyl groups. The gene for RNaseT1S was inserted at the BglII site of pIGF8.

(b). Schematic representation of the structure of the fused protein. The sites of cleavage by treatment with cyanogen bromide are marked by arrows.

RNase T1 (∑ 278=21.170$M^{-1}cm^{-1}$) and tyrosine (∑ 278=1.340 M^{-1} cm^{-1}).

Analyis of the nucleolytic activity

Nucleolytic activity was measured by analysis of the cleavage of [5'-32P] GpC (200 cpm/pmol).[1,10]

The standard reaction mixture contained 150 mM [5'-32P] GpC, 50 mM Tris/HCl (pH 7.5), 1mM EDTA and 5pg/μl RNase T1 or the mutant enzyme in a volume of 20 μl at 37°C. The reaction was stopped by 0.2 N HCl at 2,5,10 and 15 min. The product of the reaction, [5'- 32P]Gp, was separated from [5'-32P]GpC by DEAE-cellulose thin layer (CEL 300 DEAE/HR-2/15, Macherey-Nagel) chromatography by using a partially hydrolized RNA solution (homo-mixture VI) as the solvent system and detected by autoradiogram[11]. The radioactivity of each spot was measured in a liquid scintillation counter. To get the activity-temperature profile, the reaction was performed at 37°C, 45°C, 50°C, 55°C, 60°C and 70°C, and repeated 4 times.

Circular dichroism spectroscopy

CD spectra were recorded on a Jasco J-500 spectropolarimeter. To get the CD-temperature profile, measurements were taken at 5°C, 25°C, 30°C,35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C and 70°C.

Confirmation of the third disulfide bond formation

Confirmation of the third disulfide bond formation was obtained from non reducing SDS-polyacrylamide gel electrophoresis and free SH residue measurement,[12,13] in denaturing condition (7M guanidine HCl, 43 mM Tris/HCl pH 8.0 and 0.43 mg/ml EDTA), with L-Cysteine as standard

RESULTS AND DISCUSSION

Expression, refolding and purification of RNase T1S

Expression of RNase T1S, starting from 5l of culture broth, was performed in almost the same way as reported by Nishikawa et al,[9,10] yielding subsequently 5.5g of wet cells, and 247mg of protein mixture after DE 52 chromotography. After cyanogen bromide treatment, a considerable amount of precipitate appeared, indicating that a large portion of the enzyme was not in correct folding. It is clear that the additional disulfide bond in RNase T1S inhibited the establishment of the correct conformation after the urea was removed, perhaps due to random disulfide pairing. In RNaseT1, the conformation of the native protein molecule is the most stable, but under the influence of urea, random pairing of disulfide bonds will occur.[14] RNase T1S has 6 cysteines compared to 4 in native RNase T1, so the possibility of incorrect pairing is much greater. Therefore, we used 2-mercaptoethanol to open the disulfide bonds and allowed the molecule to regain its correct conformation by hydrogen bonding, and then we used 100mM NaCl in 50mM Tris/HCl pH 7.5 to restore the disulfide bonds, as the reactivation of reduced RNase-T1 is fast at neutral pH and in the presence of sodium chloride.[14] After the refolding procedure, almost all of the precipitate dissapeared and yielded 177.5 mg of protein mixture, which was subjected to Q sepharose column chromatography and resulted in 2 major peaks. Both peaks (T1SI = 13.6 mg, TISII = 6.7 mg) were collected and subsequently purified by mono Q HPLC (yielding 9.5 mg of T1SI and 5.9 mg of T1SII) and sephadex G25 column (yielding 5.1 mg of T1SI and 4.2 mg of T1SII). Both were checked by sds-polyacrilamide gel electrophoresis and shown to have been purified to homogeneity (fig.3).

Confirmation of the third disulfide bond formation

Free SH residue measurement showed that both RNase T1SI and RNase T1SII had no free SH residue, suggesting that RNaseT1SI and RNaseT1SII are conformers, both containing 3 disulfide linkages.

Non reducing SDS-polyacrylamide gel electrophoresis (fig.3) showed that both RNase T1SI and RNase T1SII migrated faster than RNaseT1. This finding suggested that a third disulfide bond had been formed, as the disulfide linkage caused the protein to have a smaller radius of gyration and therefore migrated further down the gel[3]. The difference between RNaseT1SII is thought to be due to the difference in the SDS-denatured state of the two conformers.

Another evidence of the third disulfide bond formation came from peptide mapping experiments using 16 μg of chymotrypsin in 100 μl of 0.2 M N-ethylmorpholine acetate buffer pH 8.0 to digest 400 μg heat denatured RNaseT1, RNaseT1SII (data not shown).

Circular dichroism spectroscopy

Circular dichroism measurements were performed at various temperatures. At 25°C, in the 200-260 nm region, both RNase T1SI and RNase T1SII showed a more negative extrema at about 209 nm (fig.4). This fact is thought to be due to the change of tyrosine 24 residing in the middle of the α helix to cysteine 24. This change would have stabilized the helix, as

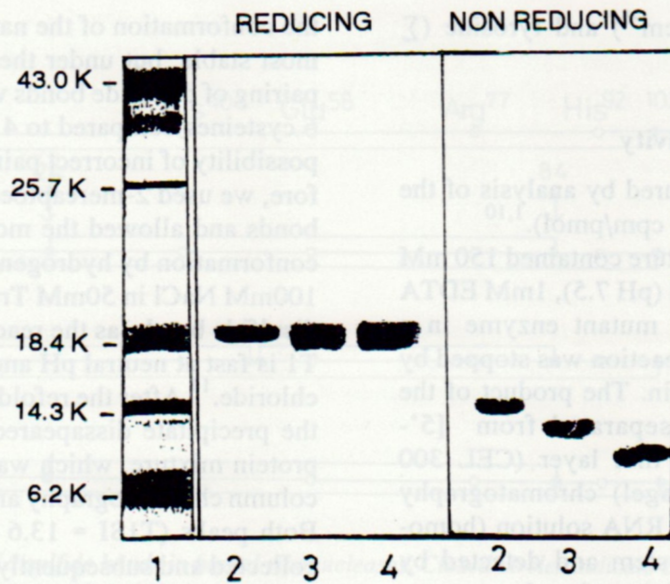

Figure 3. SDS-polyacrileamide gel electrophoresis (15% SDS-PAGE) of purified R-NaseT1. lane 1; molecular mass standard (values in KDa at side), lane 2; native R-NaseT1, lane 3; RNaseT1SI, lane 4; RNaseT1SII

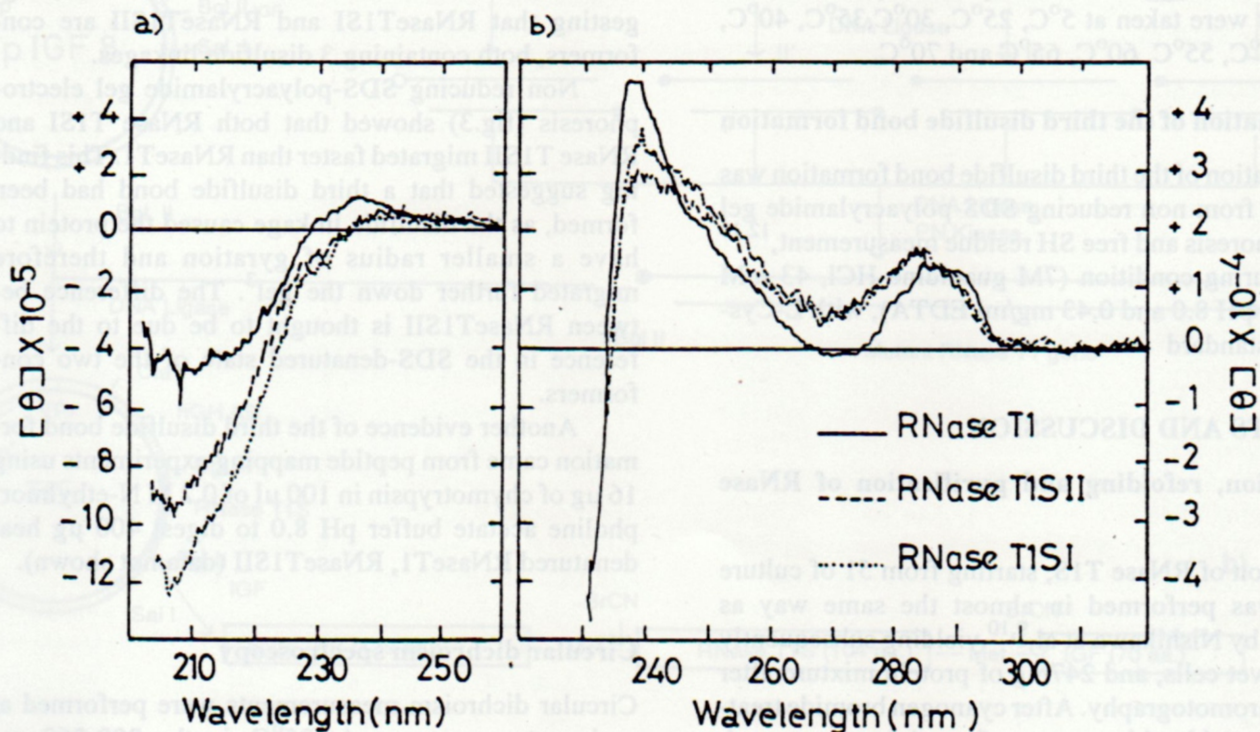

Figure 4. CD spectra of RNaseT1 and its mutants.

(a) CD spectra of wild type RNaseT1 (——), RNaseT1SI, (------) and RNaseT1SII (— — —) at 0.05 μg protein/μl, in H2O, at 25°C.

(b) At 0.2 μg protein/μl, in 10mM potassium phosphate, pH 7.5, at 25°C.

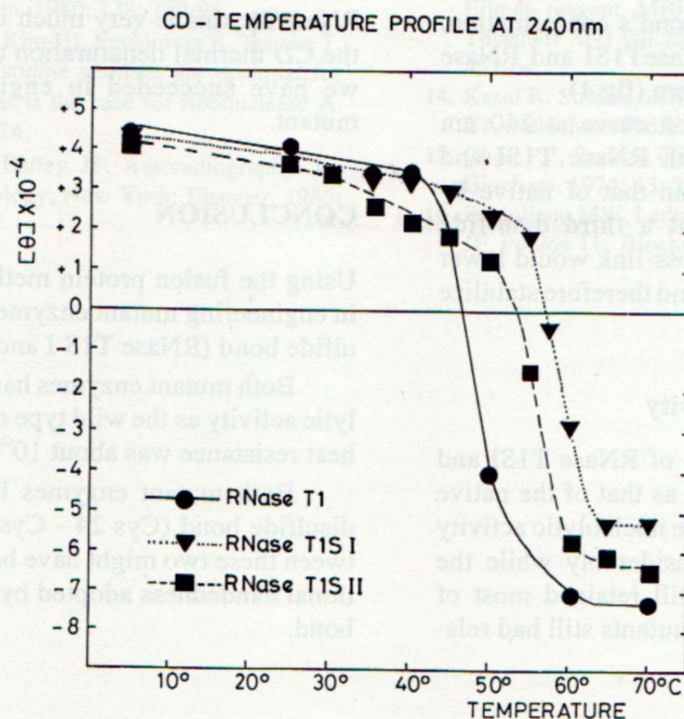

Figure 5. *Thermal denaturation curves monitored by CD ellipticity at 240 nm, for wild type RNaseT1, RNaseT1SI and RNaseT1SII. The spectra was measured at 0.2 μg protein/ml, in 10 mM potassium phosphate, pH 7.5, at 5°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C and 70°C, after a 15 min equilibration at each temperature.*

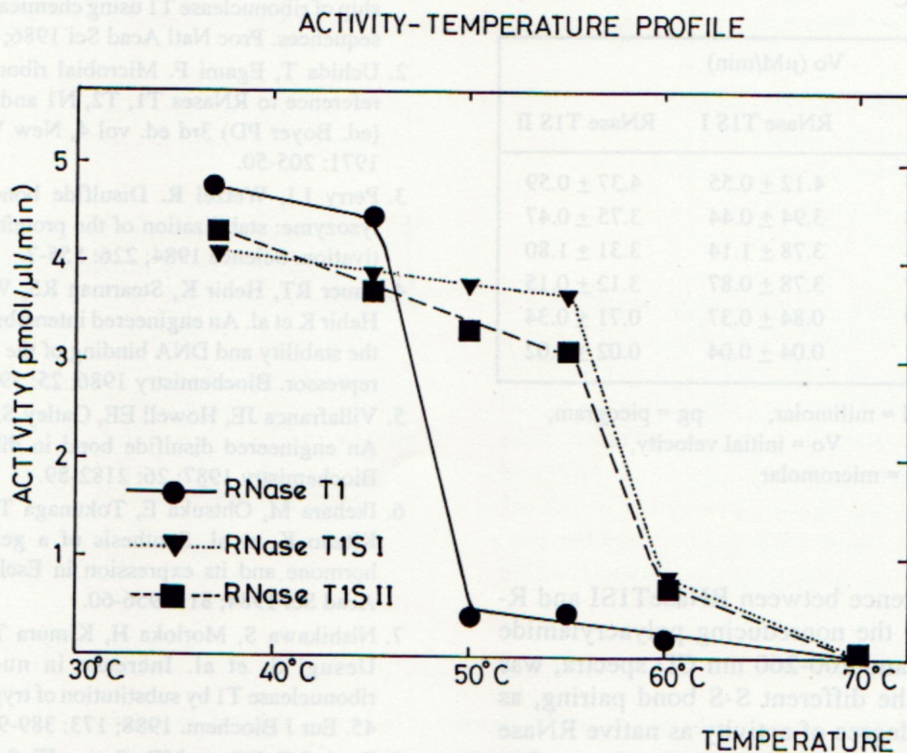

Figure 6. *Thermal denaturation curves monitored by the initial velocity of pGpC cleavage by RNaseT1, RNaseT1SI and RNaseT1SII. The initial velocities were measured at 37°C, 45°C, 50°C, 55°C, 60°C, and 70°C.*

tyrosine is an α helix breaker, while cystein is an α helix indifference residue.[15] The difference between RNaseT1SI and RNaseT1SII seems to be due to the difference of the third disulfide bond's conformation, as in the 230-320 nm region RNaseT1SI and RNase T1SII showed a very similar pattern (fig.4).

The CD thermal denaturation curve at 240 nm showed that the structure of both RNase T1SI and RNaseT1SII was more stable than that of native R-NaseT1 (fig.5), suggesting that a third disulfide linkage had been formed, as a cross-link would lower the entropy of the unfolded form and therefore stabilize the conformation.[16]

Analysis of the nucleolytic activity

At 37°C, the nucleolytic activity of RNase T1SI and RNaseT1SII was about the same as that of the native RNaseT1 (table 1), but at 50°C the nucleolytic activity of native RNaseT1 dropped considerably while the RNase T1SI and RNaseT1SII still retained most of their activity. Even at 55°C, the mutants still had relatively high activity (table 1).

Table 1. Initial velocity of pGpC cleavage by RNase T1 and its mutants. Reaction mixture: substrate 150 mM, enzyme 5 pg/µl, PH 7.5 Measured at 37°, 45°C, 50°C, 55°C, 60°C and 70°C

	Vo (µM/min)		
Temp	RNase T1	RNase T1S I	RNase T1S II
37°C	4.78 ± 0.95	4.12 ± 0.55	4.37 ± 0.59
45°C	4.45 ± 1.38	3.94 ± 0.44	3.75 ± 0.47
50°C	0.40 ± 0.03	3.78 ± 1.14	3.31 ± 1.80
55°C	0.47 ± 0.27	3.78 ± 0.87	3.12 ± 0.15
60°C	0.21 ± 0.19	0.84 ± 0.37	0.71 ± 0.34
70°C	0	0.04 ± 0.04	0.02 ± 0.02

min = minute, mM = milimolar, pg = picogram,
RNase = ribonuclease Vo = initial velocity,
µl = microliter, µM = micromolar

Here the difference between RNaseT1SI and R-NaseT1SII, seen by the nonreducing polyacrylamide gel electrophoresis and 200-260 nm CD spectra, was clearly not due to the different S-S bond pairing, as both had the same degree of activity as native RNase T1, so the difference is very likely due to the conformational handedness adopted by the third disulfide bond.

The activity-temperature profile (fig.6) showed that the heat resistance of both RNaseT1SI and RNase-T1SII was about 10°C higher than that of the native RNaseT1; this is very much in line with the result of the CD thermal denaturation curve and in this respect we have succeeded in engineering a more stable mutant.

CONCLUSION

Using the fusion protein method, we have succeeded in engineering mutant enzymes with an additional disulfide bond (RNase T1S I and RNase T1S II).

Both mutant enzymes had about the same nucleolytic activity as the wild type enzyme, but the mutant's heat resistance was about 10°C higher.

Both mutant enzymes had the same additional disulfide bond (Cys 24 - Cys 84). The difference between these two might have been due to the conformational handedness adopted by this additional disulfide bond.

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