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Generation of a Non-prolyl *cis* Peptide Bond in Ribonuclease T_1

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The *cis* conformation of the 38-39 peptide bond of ribonuclease T_1 is retained after the replacement of *cis* Pro39 by an alanine residue. This conformation is demonstrated by the presence of a NOESY cross-peak in the NMR spectrum between the C^{α} protons of Tyr38 and Ala39 in the Pro39→Ala variant. The presence of this non-prolyl *cis* peptide bond explains the retention of the catalytic activity, the strong decrease in stability and the changes in the folding mechanism that were observed after the Pro39→Ala mutation in ribonuclease T_1. We suggest that a *cis* peptide bond is retained in a protein after the substitution of a *cis* proline at positions, where a *trans* bond would destabilize the protein more strongly than a non-prolyl peptide bond in the energetically unfavourable *cis* conformation.

Keywords: protein folding; *cis* peptide bond; protein NMR; prolyl *cis/trans* isomerization

Peptide bonds are planar and can adopt either the *cis* or the *trans* conformation. The *cis* isomer is often found for peptide bonds that precede proline, and about 6% of all Xaa-Pro bonds are *cis* in native proteins (MacArthur *et al.*, 1991). For peptide bonds which do not involve proline, the *cis* isomer is extremely rare, and only 0·05% of the non-prolyl peptide bonds in folded proteins are found to be *cis* (Stewart *et al.*, 1990). Here we show by two-dimensional NMR spectroscopy that a Tyr-Ala *cis* peptide bond is generated in ribonuclease T_1 (RNase T_1§) by substituting *cis*-Pro39‖ with an alanine.

RNase T_1 contains two *cis* prolyl peptide bonds; Ser54-Pro55 is in a loop, exposed to solvent, and Tyr38-Pro39 is in the interior of the protein (Heinemann & Saenger, 1982; Martinez-Oyanedel *et al.*, 1991). The main-chain around Pro39 is extensively hydrogen-bonded and the two flanking amino acid residues Tyr38 and His40 are catalytic residues (Grunert *et al.*, 1991; Steyaert *et al.*, 1990; Steyaert & Wyns, 1993). Regarding these structural constraints we decided to replace *cis*-Pro39 by an alanine residue to possibly generate a non-prolyl *cis* peptide bond in RNase T_1. This mutation led to a decrease in the free energy of stabilization by about 20 kJ/mol (Mayr & Schmid, 1993a; Mayr *et al.*, 1993), and to a novel very slow step in refolding (Mayr & Schmid, 1993b). In contrast, the catalytic activity was almost unchanged (Mayr *et al.*, 1993), in spite of the close proximity of position 39 to the active site (Zegers *et al.*, 1992).

To determine the isomeric state of the Tyr38-Ala39 peptide bond after the Pro39→Ala mutation we decided to use methods of two-dimensional NMR. The *cis* and the *trans* conformation of a peptide bond can be distinguished in solution by NMR spectroscopy (Wüthrich *et al.*, 1984; Wüthrich, 1986). The distance between two C^{α} protons is 4·2 Å or larger when they are connected by a *trans* peptide bond, and sequential NOESY cross-peaks between $C^{\alpha}H(i)$ and $C^{\alpha}H(i+1)$ are not observed in this case. In *cis* peptide bonds this distance is only 2·5 Å to 3·8 Å, depending on the torsion angles ψ_i and φ_{i+1} and the observation of a NOESY cross-peak between two successive $C^{\alpha}H$ provides clear evidence for a *cis* peptide bond. We therefore assigned the $C^{\alpha}H$ and the NH resonances of both wild-type RNase T1 and the Pro39Ala variant independently under our experimental conditions of pH 5·0, 25°C by a combination of COSY, NOESY and TOCSY spectra using standard procedures (Wüthrich *et al.*, 1984; Wüthrich, 1986).

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§ Abbreviations used: RNase T_1, ribonuclease T_1; Pro39Ala-RNase T_1, variant of RNase T_1 with *cis* Pro39 substituted by an alanine; COSY, 2D correlation spectroscopy; TOCSY, 2D total correlation spectroscopy; NOESY, 2D nuclear Overhauser effect spectroscopy.

‖ We use the expression *cis*-proline for a *cis* peptide bond in the native protein between an amino acid Xaa and a proline residue.

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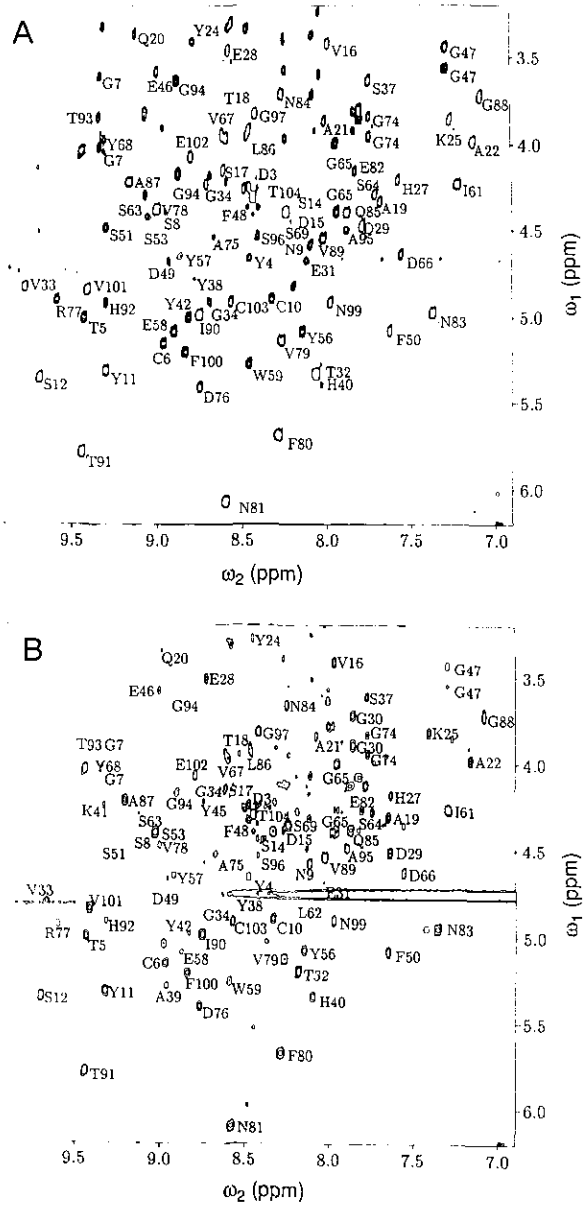

Figure 1. The 600 MHz ^{1}H-NMR TOCSY spectra of A, RNase T_1 wild-type and B, the Pro39Ala-variant in the fingerprint region. Indicated are the NH-C$^\alpha$H cross-peaks of the individual amino acid residues (one-letter code). Solvent conditions were 0·02 M sodium oxalate, (pH 5·0), 25 °C. In each case the protein concentration was 2 mM in $^1H_2O/^2H_2O$-solution (9 : 1, v/v) and the mixing time τ_m was 65 ms. Clean-TOCSY spectra (Griesinger *et al.*, 1988) were obtained on a Bruker AMX600 spectrometer in the phase-sensitive mode with quadrature detection in both dimensions using the TPPI technique (Marion & Wüthrich, 1983). The frequency width was 7215 Hz in both dimensions and water suppression was achieved by continuous coherent irradiation prior to the first excitation pulse: 512 t_1 values of 4096 t_2 data points were recorded. Data were processed with a square sine-bell window function shifted by $\pi/6$ in both dimensions and additional zero-filling was used to obtain a spectral data matrix of 1K × 1K. Base-line corrections were carried out with a 6th-order polynomal function. Data were processed on Unix workstations using the NDEE software package (F. Hermann, unpublished results). The proton ppm values were calibrated using the water resonance, with a chemical shift of 4·75 ppm at 25 °C relative to 3-(trimethylsilyl)-1-propane-sulphonic acid. The individual C$^\alpha$H and NH resonances of wild-type RNase T_1 and of the Pro39Ala variant were identified by the sequential assignment procedures developed by Wüthrich and co-workers (Wüthrich *et al.*, 1984, Wüthrich, 1986). The lower signal-to-noise ratio in Figure 1B is probably caused by the marginal stability of the Pro39Ala variant.

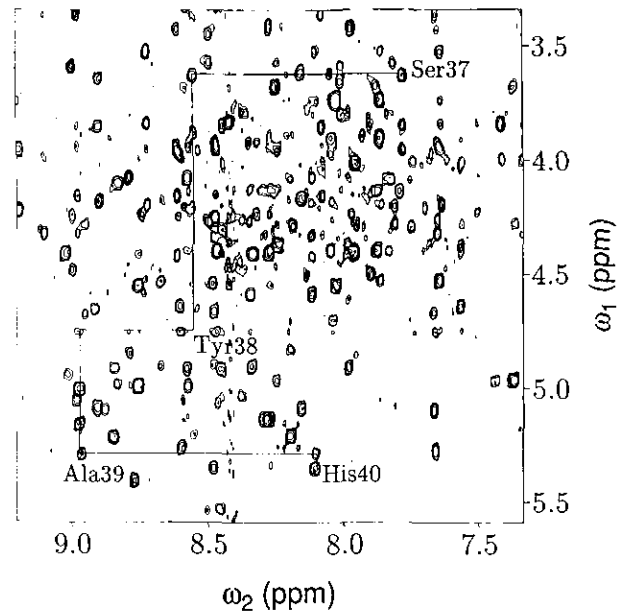

Figure 2. Superposition of the fingerprint regions of the NOESY and the TOCSY spectra of Pro39Ala-RNase T1. The Figure shows the sequential assignments *via* NH(i) to C$^\alpha$H($i+1$) from Ser37 to His40. The TOCSY spectrum was recorded as described in the legend to Figure 1. The NOESY spectrum (Kumar *et al.*, 1980; Macura & Ernst, 1980) was taken in the same solvent system, and the mixing time τ_m was 150 ms. It was measured on a Bruker AMX600 spectrometer with parameters essentially as for Figure 1. After processing, the spectral data matrix was 2K × 1K. NOESY cross-peaks, caused by exchange between amide protons and water protons, can be seen near the H_2O resonance.

These solvent conditions had been used previously for investigating the folding and stability of Pro39Ala-RNase T_1. The assignments for the wild-type protein at pH 5·3, 40 °C obtained by Hoffmann & Rüterjans (1988) could not be used for comparison directly, because the Pro39Ala variant is partially unfolded at 40 °C. The TOCSY spectra of the two forms of RNase T_1 in the "fingerprint" region (Figure 1) are nearly identical, indicating that the backbone conformation of the protein is almost unaffected by this mutation. The C$^\alpha$H resonance of Tyr38 lies very close to the water signal. The chain tracing between residues 37 and 40 to locate this resonance in the spectrum of the Pro39Ala variant is shown in Figure 2. The validity of this assignment is corroborated by the identification of the spin system of Tyr38 in NOESY and COSY

Table 1

Short distances between protons in the 38-39 region in the crystal structure of wild-type RNase T_1 and NOE cross-peaks observed in the NOESY spectrum of the Pro39Ala variant

Short inter-proton distances	Distance (Å)	NOESY cross-peak
$C^{\alpha}H$(Tyr38)-$C^{\alpha}H$(Ala39)	3·0	Yes
$C^{\alpha}H$(Tyr38)-$C^{\beta}H$(Ala39)	4·2	Yes
$C^{\beta}H$(Tyr38)-$C^{\alpha}H$(Ala39)	3·7	Yes
$C^{\beta}H$(Tyr38)-$C^{\beta}H$(Ala39)	4·3	Yes
$C^{\beta}H$(Tyr38)-NH(His40)	3·3	Yes
$C^{2,4}H$(Tyr38)-NH(His40)	4·6	Yes
$C^{\alpha}H$(Ala39)-NH(His40)	2·4	Yes
$C^{\alpha}H$(Ala39)-$C^{2,6}$(Tyr38)	4·4	Yes
$C^{\beta}H$(Ala39)-$C^{2,6}$(Tyr38)	4·8	Yes
$C^{\beta}H$(Ala39)-NH(His40)	3·2	Yes
$C^{\beta}H$(Ala39)-$C^{\alpha}H$(His40)	4·5	Weak
$C^{\beta}H$(Ala39)-$C^{\beta}H$(Tyr57)	3·5; 4·8	Yes
$C^{\beta}H$(Ala39)-$C^{2,4}$(Tyr57)	4·6	Yes
$C^{\beta}H$(Ala39)-$C^{\alpha}H$(Trp59)	4·3	Yes
$C^{\beta}H$(Ala39)-$C^{\beta}H$(Trp59)	3·5	Yes
$C^{\beta}H$(Ala39)-$C^{4}H$(Trp59)	3·3	Yes
$C^{\beta}H$(Ala39)-$C^{4}H$(Phe80)	3·2	Yes
$C^{\beta}H$(Ala39)-$C^{\beta}H$(His27)	4·2	No

Proton-proton distances shorter than 5 Å are given in column 2. They are derived from the co-ordinates of free RNase T_1 (Martinez-Oyanedel *et al.*, 1991). The positions of the H atoms were generated with the program SYBYL (Tripos. Assoc., St. Louis, U.S.A.). The conditions for the NMR experiments were as described for Figure 4.

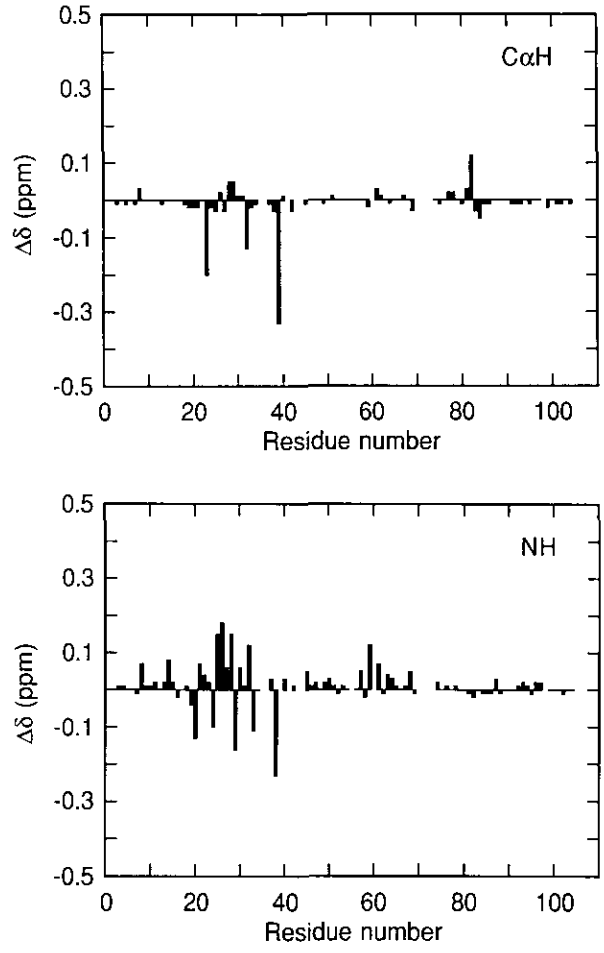

Figure 3. Plots of the differences in chemical shift positions ($\Delta\delta$ in ppm) of Pro39Ala-RNase T_1 minus the wild-type protein at pH 5·0, 25 °C, for the $C^{\alpha}H$ (top) and the NH protons (bottom) plotted as a function of the amino acid sequence. The chemical shift values were obtained from the spectra shown in Figure 1A and B, respectively. No differences are given when the NH is absent (Pro residues) or when either the NH or the $C^{\alpha}H$ resonances could not be assigned unambiguously for both proteins.

spectra in ^2H_2O (data not shown) and by several NOESY cross-peaks to neighbouring protons (see Table 1).

The differences in chemical shifts of the $C^{\alpha}H$ and the NH resonances between the two forms are generally smaller than 0·2 ppm (Figure 3). Significant differences in the C^{α} region are observed only for position 39, where the Pro→Ala substitution occurred and for Gly23, Thr32 and Glu82, which are close to Pro39 in the folded wild-type protein (Heinemann & Saenger, 1982; Martinez-Oyanedel *et al.*, 1991). The mutation at position 39 does not affect the chemical shifts of the $C^{\alpha}H$ of the two neighbouring residues, Tyr38 and His40 (Figure 3). For the chemical shifts of the NH resonances (Figure 3) small differences between the wild-type protein and the Pro39Ala variant are found for Tyr38, for the carboxy-terminal part of the 13-29 helix and for Trp59, all of which are close to Pro39 in the wild-type protein. In all other chain regions the differences in the chemical shifts are smaller than 0·1 ppm (Figure 3). Together with the virtually unchanged CD spectra in the amide and in the aromatic regions, this indicates that the main chain conformation of RNase T_1 is almost unaffected by the replacement of Pro39 by Ala. A list of the assigned $C^{\alpha}H$, NH, $C^{\beta}H$, and side-chain resonances for the wild-type protein and the Pro39Ala variant was deposited at the BioMagResBank.

For wild-type RNase T1 a NOESY cross-peak between the C^{α} protons of Tyr38 and *cis*Pro39 is indeed observed (Figure 4A). By using the cross-peak intensities of the ring protons of Tyr4 for calibration, the distance between $C^{\alpha}H$(Tyr38) and $C^{\alpha}H$(Pro39) in solution was estimated to be 2·9 Å. This value corresponds well with the distance of 3·0 Å between these two protons in the crystal structure of wild-type RNase T_1 (Martinez-Oyanedel *et al.*, 1991) and indicates that Pro39 is *cis* in solution as well. A NOESY cross-peak between $C^{\alpha}H$(Tyr38) and $C^{\alpha}H$(Ala39) is also observed after the substitution of Pro39 by Ala (Figure 4B), and using again the cross-peak intensities of the ring protons of Tyr4 for calibration an almost identical value of 2·8 Å is obtained for the distance between the two C^{α} protons in the Pro39Ala variant. The resonances of all protons located within a shell of 6 Å around the $C^{\alpha}H$ of residue 39 in the crystal structure could be identified unambiguously in the

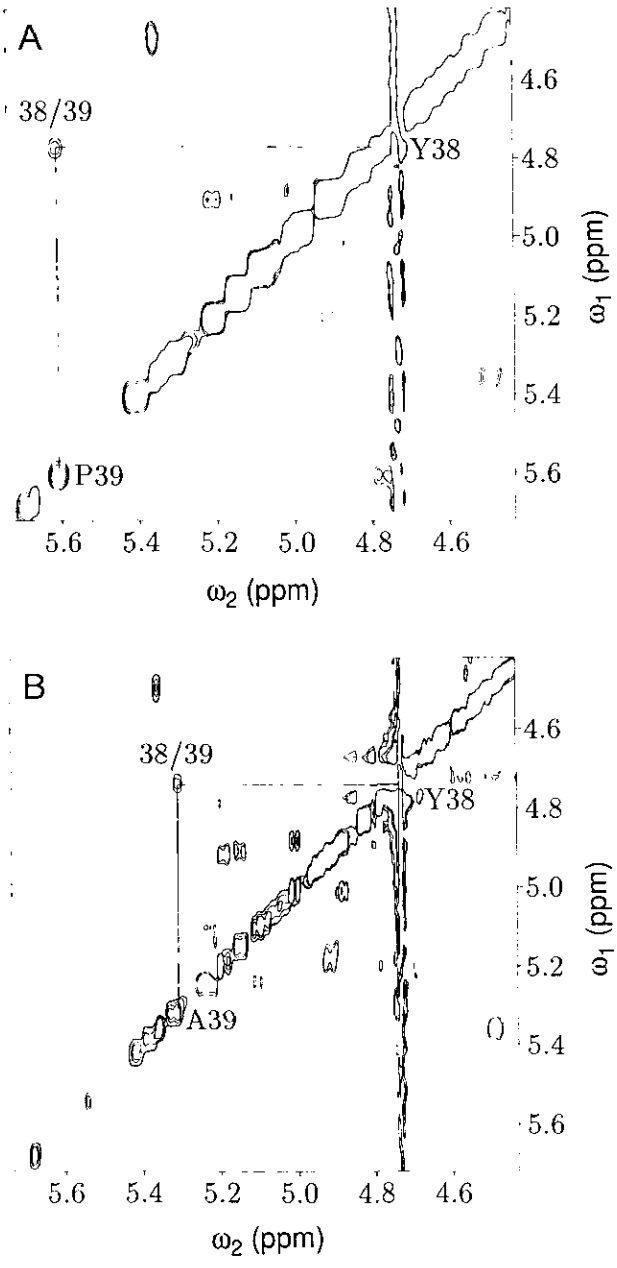

Figure 4. Part of the 600 MHz ^{1}H-NMR NOESY spectra of: A, wild-type RNase T_1; and B, the Pro39Ala-variant. Indicated are the $C^\alpha H$-$C^\alpha H$ cross-peak between Tyr38 and Pro39 for wild-type RNase T1 and the cross-peak between Tyr38 and Ala39 of the Pro39Ala-variant, respectively. Solvent conditions were 0·02 M sodium oxalate (pH 5·0), 25°C. In each case the protein concentration was 2 mM in ^2H_2O-solution (99·99%), and the mixing time τ_m was 150 ms. NOESY spectra (Kumar *et al.*, 1980; Macura & Ernst, 1980) were measured on a Bruker AMX600 spectrometer with parameters essentially as for Figure 1. After processing, the spectral data matrix was 2K × 1K.

NMR spectrum of the Pro39Ala variant. None of them maps near the $C^\alpha H$ of Tyr38 and so could not give rise to the observed NOESY cross-peak. This supports our conclusion that this cross-peak indeed reflects the interaction between the C^α protons of Tyr38 and Ala39 connected by a *cis* peptide bond. To gain further insight into the local structure around this non-prolyl *cis* peptide bond we searched for short H-H distances (≤ 5 Å) involving the $C^\alpha H$ and $C^\beta H$ of Tyr38 and residue 39 (Pro in the wild-type protein and Ala in the Pro39Ala variant) in the crystal structure of the wild-type protein (Martinez-Oyanedel *et al.*, 1991). Then we examined whether NOE cross-peaks could be observed between the resonances of these protons in the NOESY spectrum of the Pro39Ala variant. The results in Table 1 indicate that in almost all cases NOESY cross-peaks are indeed observed. The long-range NOEs of $C^\beta H$(Ala39) to Tyr57, Trp59 and Phe80 and the NOE between the C^βHs of Tyr38 and Ala39 suggest that the position of the $C^\beta H$ of Ala39 in the structure of the Pro39Ala variant is very similar to the position of the $C^\beta H$ of Pro39 in the wild-type protein. Usually, the distance between two successive C^β protons is larger than 5 Å when they are linked by a *trans* peptide bond, and a NOE cross-peak between them is not observed.

Together, these results indicate (1) that the resonances of the C^α protons of Tyr38 and Ala39 were correctly assigned and (2) that the local structure around these two C^α protons is largely identical in the wild-type protein and the Pro39Ala variant as expected, if the 38-39 peptide bond is *cis* in both forms of RNase T_1. Sequential $C^\alpha H(i)-C^\alpha H(i+1)$ cross-peaks in the NOESY spectra were also observed for the *cis* prolyl bonds 38-39 and 54-55 in the homologous RNase F1 (Nakai *et al.*, 1992) and for Ser54-Pro55 in Pro39Ala-RNase T_1 (our data, not shown). Other $C^\alpha H(i)-C^\alpha H(i+1)$ cross-peaks could not be found.

We conclude from these results that, after the substitution of Pro39 by Ala, the conformation of the Tyr38-Ala39 peptide bond is not changed to *trans*, but rather the *cis* state is maintained and the spatial orientation of the peptide backbone is conserved. Additional support for this conclusion comes from the high enzymatic activity of the Pro39Ala variant (96% of the activity of the wild-type protein towards guanylyl(3′-5′)cytidine; Mayr *et al.*, 1993). Both Tyr38 and His40 are catalytic residues and contribute to the stabilization of the transition state in RNase T_1-mediated hydrolysis of dinucleotides (Steyaert & Wyns, 1993). A change in the backbone structure caused by a *trans* Tyr38-Ala39 peptide bond would almost certainly disrupt the structure of the active site and lead to a severe decrease in the catalytic activity. Interestingly, this artificial generation of a *cis* peptide bond by the replacement of a proline residue has a natural counterpart. A *cis* peptide bond near the active site of β-lactamase is followed by proline in the enzyme from *Escherichia coli* (Strydnadka *et al.*, 1992), but by isoleucine in the homologous enzyme from *Staphylococcus aureus* (Herzberg, 1991). In carbonic anhydrase II a *cis* peptide bond was also retained after the *cis* Pro202→Ala mutation, as shown by a recent X-ray

crystallographic analysis (Tweedy *et al.*, 1993). Again, this position is located close to the active site of the protein.

The strong destabilization of RNase T_1 by 21·4 kJ/mol (at pH 5·0, 25°C) as a consequence of the Pro39→Ala mutation (Mayr *et al.*, 1993) can now be largely understood. A *cis* peptide bond destabilizes a folded protein because the *cis* isomer of a non-prolyl peptide bond is intrinsically unstable. Model compound data (Drakenberg *et al.*, 1972; Radzicka *et al.*, 1988) and theoretical considerations (Ramachandran & Mitra, 1976; Jorgensen & Gao, 1988) suggest that in the absence of ordered structure the *cis*/*trans* ratio is between 0·01 and 0·001. Thus, a *cis* peptide bond should destabilize a folded protein by 15 (±5) kJ/mol, as observed for Pro39Ala-RNase T_1 (Mayr *et al.*, 1993). It should be noted that carbonic anhydrase is also destabilized by about 20 kJ/mol by the *cis* Pro202→Ala substitution (Tweedy *et al.*, 1993).

The retention of the 38-39 *cis* peptide bond also explains the altered folding mechanism of Pro39Ala-RNase T_1. Based on the evidence for a non-prolyl *cis* peptide bond we propose that, after unfolding, the *cis* Tyr38-Ala39 bond isomerizes to *trans* in virtually all molecules. As a consequence, during refolding a *trans*→*cis* re-isomerization has to occur in all molecules. This explains why a fast refolding reaction could not be observed for the Pro39Ala variant, and why the slowest step of refolding is no longer a prolyl isomerization (Mayr & Schmid, 1993*b*). We propose that the new rate-determining step of refolding is indeed the *trans*→*cis* isomerization of the Tyr38-Ala39 peptide bond.

The function of *cis* peptide bonds in folded proteins is not clear (Herzberg & Moult, 1991). They are certainly suited to induce tight turns and thereby allow unusual main-chain conformations. The energetic cost for incorporating a *cis* peptide bond is small for Xaa-Pro, but high for non-prolyl peptide bonds. As a consequence, *cis* prolyl bonds are frequently found, and non-prolyl *cis* bonds are extremely rare (Stewart *et al.*, 1990; MacArthur & Thornton, 1991; Herzberg & Moult, 1991). Here we have shown that a *cis* prolyl peptide bond can be converted into a non-prolyl *cis* peptide bond by replacing proline with another amino acid. Such a *cis* bond can form only if sufficient folding energy is available to compensate for the energetic cost of maintaining the *cis* conformation. Presumably, this is possible only at positions where the structural rearrangements that are coupled to the *cis*→*trans* isomerization would destabilize the protein more strongly than a non-prolyl *cis* amide bond.

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