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PAPER

Replacing a single atom accelerates the folding of a protein and increases its thermostability†

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The conformational attributes of proline can have a substantial effect on the folding of polypeptide chains into a native structure and on the stability of that structure. Replacing the 4*S* hydrogen of a proline residue with fluorine is known to elicit stereoelectronic effects that favor a *cis* peptide bond. Here, semisynthesis is used to replace a *cis*-proline residue in ribonuclease A with (2*S*,4*S*)-4-fluoroproline. This subtle substitution accelerates the folding of the polypeptide chain into its three-dimensional structure and increases the thermostability of that structure without compromising its catalytic activity. Thus, an appropriately situated fluorine can serve as a prosthetic atom in the context of a protein.

Introduction

A long-term goal of biological chemists is to create new proteins with desirable properties.[1] The traditional approach has involved interrogation of extant proteins with the twenty natural amino acids.[2–4] Powerful new methods allow for the installation of a limitless variety of nonnatural modules into proteins and for the creation of proteins *de novo*.[5–8] Granted this freedom, biological chemists must now identify those synthetic components that are truly useful. The subtlest changes are the least likely to disrupt the structure of a protein or perturb its function. Subtle changes are also the most likely to provide information applicable to other architectures.[9]

Among the twenty canonical amino acids, proline (Pro) is unique.[10] Its ϕ dihedral angle (that is, C'_{i-1}–N_i–C_i^{α}–C'_i) lies within a pyrrolidine ring, which limits flexibility. This rigidity is apparent in proline oligomers, which can act as a molecular "scaffold"[11] or "ruler",[12] and taints proline as the amino-acid residue (besides tryptophan) that serves least well as a substitute for other residues.[13] Moreover, as a secondary amine, proline forms tertiary amides. Thus, proline residues cannot donate a hydrogen bond, and are rare in α-helices and β-sheets,[14] which are stabilized by hydrogen bonds between main-chain amides.[15,16] Proline residues are, though, common in turns.[17,18] There, prolyl peptide bonds reside as the *trans* or *cis* isomer (Fig. 1A), depending on context.[19,20]

The *trans* : *cis* ratio of a prolyl peptide bond is influenced by an $n\rightarrow\pi^*$ interaction.[21–24] This interaction entails the overlap of a lone pair (n) of the oxygen (O_{i-1}) of a peptide bond with the antibonding orbital (π^*) of the carbonyl group ($C'_i{=}O_i$) of the subsequent peptide bond. An $n\rightarrow\pi^*$ interaction can occur

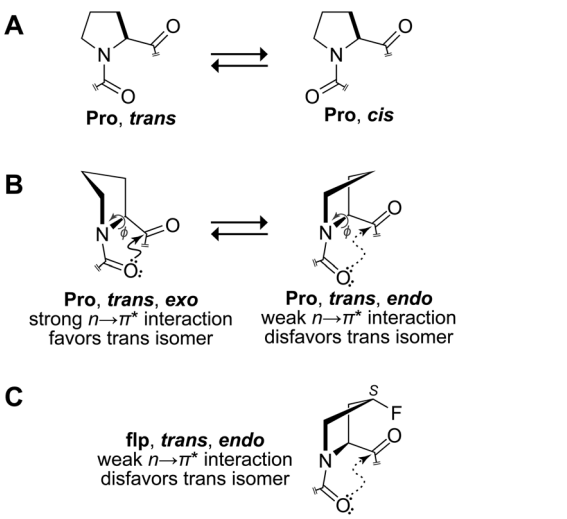

Fig. 1 Conformational attributes of proline residues. (A) Equilibrating *trans* and *cis* isomers of a prolyl peptide bond. (B) Equilibrating *exo* and *endo* pyrrolidine ring puckers of a Pro residue with a *trans* peptide bond. The ϕ dihedral angle is constrained by the ring but tends to be larger (*i.e.*, less negative) in the *exo* pucker than in the *endo* pucker.[25] The calculated C=O···C=O distance is 2.87 Å in the *exo* pucker and 3.06 Å in the *endo* pucker.[22] (C) *endo* Pyrrolidine ring pucker of a flp residue,[30] which is enforced by a *gauche* effect that restricts the ϕ dihedral angle.[29] The calculated C=O···C=O distance is 3.23 Å.[22]

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only in the *trans* isomer (Fig. 1B). Accordingly, strengthening the $n\rightarrow\pi^*$ interaction favors the *trans* isomer, and weakening that interaction favors the *cis* isomer.

The most subtle known means to influence the strength of the $n\rightarrow\pi^*$ interaction of a proline residue is to replace a hydrogen atom on C^γ with a fluorine atom.[26–28] The ensuing *gauche* effect alters the pyrrolidine ring pucker, which in turn changes the distance between main-chain carbonyl groups.[21,22,29] Specifically, a 4*S* fluoro group increases the C═O···C═O distance, which weakens the $n\rightarrow\pi^*$ interaction and disfavors the *trans* isomer (Fig. 1C). Indeed, a $(flp)_{10}$ peptide has a strong tendency to fold into a polyproline type I helix, in which each peptide bond is *cis*.[31] 4-Fluoroproline residues have been incorporated into other peptides, especially ones that mimic collagen,[32,33] as well as proteins. (For a recent review, see ref. 27.) In addition to eliciting a *gauche* effect, the through-bond inductive effect of a 4-fluoro group diminishes amidic resonance and thus accelerates the interconversion of the *trans* and *cis* isomers of a prolyl peptide bond,[29] a process that can limit the rate of protein folding.[34,35]

Here, we discern the effects of these principles on a model protein—ribonuclease (RNase) A, which has been the object of much seminal work in protein chemistry.[36,37] In folded RNase A, residues Gly112–Asn113–Pro114–Tyr115 of RNase A form a β-turn in which the Asn113–Pro114 peptide bond is *cis*. We used expressed protein ligation[38] to replace Pro114, which is one of four Pro residues in RNase A, with (2*S*,4*S*)-4-fluoroproline (flp), which differs from Pro only in its 4*S* fluoro group. We find that *replacing a single atom* accelerates the folding of RNase A into its native conformation and increases the thermostability of that conformation.

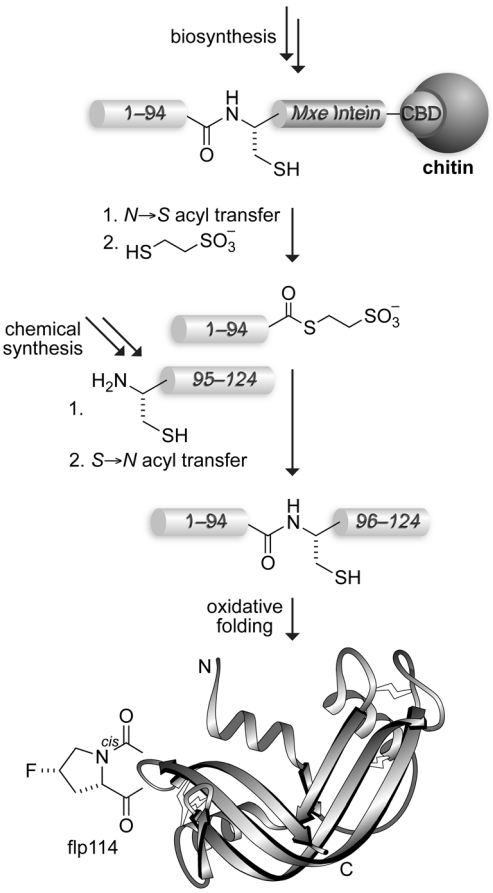

Fig. 2 Scheme for the semisynthesis of the Pro114 → flp variant of RNase A. This semisynthetic enzyme has a methionine residue at position −1, which is not consequential for catalytic activity or conformational stability.[56] CBD: chitin-binding domain.

Results and discussion

Protein semisynthesis

Wild-type RNase A and its Pro114flp variant were prepared by a semisynthetic route (Fig. 2). Circular dichroism (CD) spectroscopy in the near UV and far UV range suggested that the structure of RNase A was unaffected by the Pro114 → flp substitution (Fig. 3). Likewise, an assay of catalytic activity, which is a highly sensitive measure of the native structure of an enzyme,[9] revealed that Pro114flp RNase A had k_{cat}/K_M = $(1.3 \pm 0.3) \times 10^7$ M^{-1} s^{-1}, which is indistinguishable from that of either the semisynthetic wild-type enzyme, which had k_{cat}/K_M = $(1.4 \pm 0.5) \times 10^7$ M^{-1} s^{-1}, or commercial RNase A, which had k_{cat}/K_M = $(1.5 \pm 0.2) \times 10^7$ M^{-1} s^{-1}.

Temperature-induced conformational transition

The thermostability of wild-type RNase A and its Pro114flp variant was investigated by temperature-induced unfolding using the change in CD signal at 278 nm (Fig. 4A). Thermal unfolding proved to be reversible and followed a two-state transition model as judged from the fit of the data. The Pro114 → flp substitution results in an increase in the value of T_m by (1.3 ± 0.3) °C (Table 1). The increase in T_m corresponds to an increase in $\Delta G°$ (relative to $\Delta G°$ of RNase A at the T_m of the variant) of (2.2 ± 0.2) kJ mol^{-1}. Notably, replacing Pro114 of RNase A with a glycine or alanine residue *decreases* the value of T_m by >6 °C.[41,42] Replacing Pro114 with (2*S*)-5,5-dimethylproline does increase the T_m by 2.8 °C, but this nonnatural residue has six additional atoms in its side chain.[43] Other derivatives of proline that promote the *cis* conformation are also unsubtle.[44]

Gdn·HCl-induced conformational transition

The stability of RNase A and its Pro114flp variant against unfolding induced by guanidine hydrochloride (Gdn·HCl) was investigated by following the change in fluorescence at 303 nm as a function of Gdn·HCl concentration (Fig. 4B). Gdn·HCl-induced unfolding proved to be reversible and follows a two-state transition model as judged from the fit of the data. In contrast to the results of the thermal unfolding experiments, the Pro114 → flp substitution did not result in a discernible increase in conformational stability, as judged by the value of the transition midpoint: $[Gdn·HCl]_{1/2}$. This dichotomy is not unexpected, as thermal and chemical denaturation are distinct

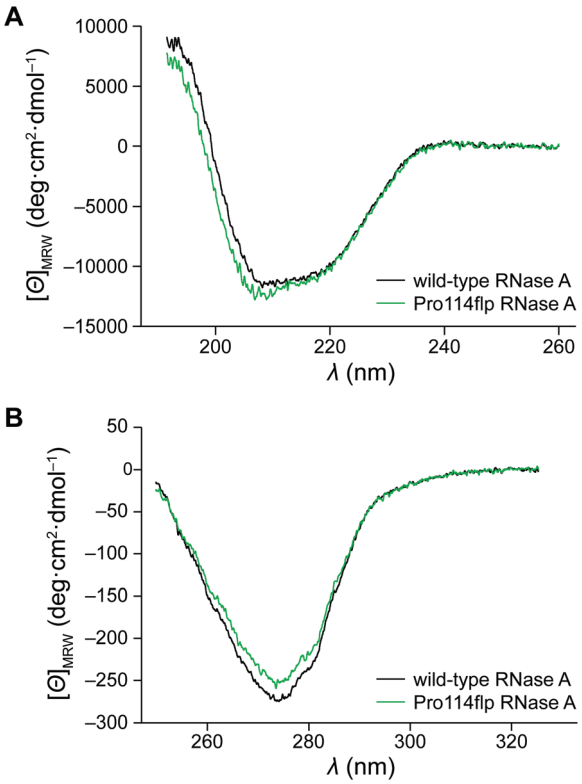

Fig. 3 Circular dichroism spectra of wild-type RNase A and its Pro114 → flp variant. (A) Far-UV region. (B) Near-UV region.

Table 1 Conformational parameters for wild-type RNase A and its Pro114flp variant

RNase A	T_m (°C)	$\Delta\Delta G^{\circ}$ [a] (kJ mol^{-1})	$[Gdn{\cdot}HCl]_{1/2}$ (M)	$\Delta\Delta G_U^{\ddagger}$ [b] (kJ mol^{-1})	$\Delta\Delta G_f^{\ddagger}$ [b] (kJ mol^{-1})
Wild-type	64.0 ± 0.1	—	2.8 ± 0.1	—	—
Pro114flp	65.3 ± 0.2	2.2 ± 0.2	2.8 ± 0.2	−0.1 ± 0.4	2.8 ± 0.5

[a] Calculated at 65.3 °C from the temperature-induced unfolding data by using the modified Gibbs–Helmholtz equation[39] with ΔH_m = (428 ± 20) kJ mol^{-1} and ΔC_p = (4.81 ± 0.09) kJ mol^{-1} M^{-1}.[40] [b] Average from the temperature range of the experiments. Values were calculated from values of k_U or k_f with the Eyring equation.

processes.[45] For example, compactness derived from the maintenance of a *cis* peptide bond by flp114 in the unfolded state could alter the interactions of the Pro114flp variant with solvent and solutes.[46] We note too that data from chemical denaturation experiments are typically less precise than are data from thermal denaturation experiments.

Unfolding rate constants

The rate of thermal unfolding was monitored by limited proteolysis with thermolysin. As is apparent in an Arrhenius plot (Fig. 5A), the rate of RNase A unfolding is not influenced by the Pro114 → flp substitution. This result is in accord with the generally accepted model for the unfolding pathway of RNase

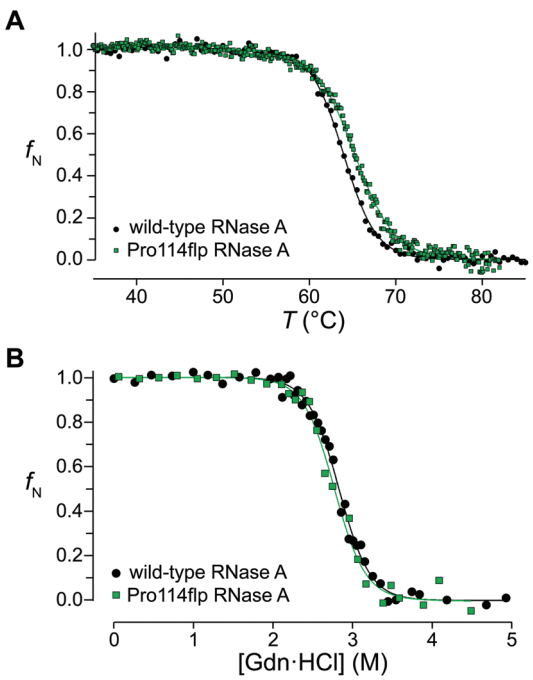

Fig. 4 Conformational stability of wild-type RNase A and its Pro114 → flp variant. The parameter f_N refers to the fraction of native protein. (A) Temperature-induced transition monitored by circular dichroism spectroscopy. (B) Gdn·HCl-induced transition monitored by fluorescence spectroscopy.

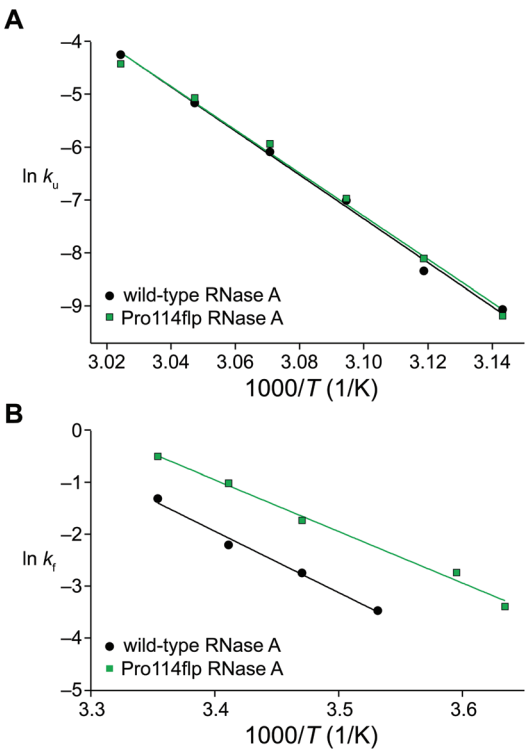

Fig. 5 Kinetics of the unfolding and folding of wild-type RNase A and its Pro114 → flp variant. (A) Arrhenius plot of unfolding as determined by limited proteolysis with thermolysin. (B) Arrhenius plot of folding as determined by the gain of enzymatic activity.

A.[47–50] The unfolding of RNase A is a one-step process, which is followed by the *cis*/*trans* isomerization of prolyl peptide bonds. Accordingly, effects on *cis*/*trans* isomerization are not reflected in the conformational unfolding reaction.

Refolding experiments monitored by stopped-flow fluorescence spectroscopy

In contrast to the unfolding process, the folding of RNase A is a multi-step process with rate-limiting steps such as prolyl peptide bond isomerization.[47,50] The refolding of RNase A and its Pro114flp variant was monitored by the reactivation from the Gdn·HCl-unfolded protein at 2.5–25.0 °C. The obtained fluorescence signals could be fitted to a single-exponential equation plus a steady-state term.[43] At all temperatures investigated, the reactivation of Pro114flp RNase A was faster than that of the wild-type enzyme (Fig. 5B). The $\Delta\Delta G_f^{\ddagger}$ values (Table 1) are comparable to the $\Delta\Delta G^{\circ}$ values determined from thermal unfolding, indicating that the stabilizing effect of the flp residue is due to its acceleration of protein folding.

Conclusions

Cram articulated the principle of preorganization by stating that "the more highly hosts and guests are organized for binding and low solvation prior to their complexation, the more stable will be their complexes".[51] We have employed this principle not for stabilizing a bimolecular complex, but for stabilizing the three-dimensional structure of a protein. A proline residue with a 4*S* fluoro group is more preorganized as the *cis* isomer than is a proline residue.[21,22,29] Moreover, the interconversion of the *trans* and *cis* isomers is more rapid.[29] We have found that these consequences of a 4*S* fluoro group are manifested in Pro114flp RNase A, leading to greater thermostability (Fig. 4A) and faster folding (Fig. 5B). These attributes likely arise from alleviation of the entropic penalty for the folding of the polypeptide chain. In essence, the unfolded state is destabilized because preorganization reduces its entropy.[33]

Both wild-type RNase A (here, Met(−1)RNase A) and its Pro114flp variant have 1870 atoms. The only difference between these two proteins is the replacement of the hydrogen atom in the 4*S* position of Pro114 with a fluorine atom. We conclude that this most subtle of changes can endow a protein with benefits that could be of practical utility.

Experimental section

Materials

Commercial RNase A was from Sigma Chemical (St Louis, MO). The fluorogenic RNase A substrate 6-carboxyfluorescein–dArU(dA)$_2$–6-carboxytetramethylrhodamine (FAM–ArUAA–TAMRA)[52] was from metabion international AG (Martinsried, Germany). FmocflpOH was a generous gift from Dr J. A. Hodges (University of Wisconsin–Madison), and was synthesized as described previously.[53] All other chemicals were of the purest grade commercially available.

Peptide synthesis

The sequence of the synthetic peptide was CAYKTTQANKHII-VACEGN–flp–YVPVHFDASV, which corresponds to residues 95–124 with flp as residue 114. This peptide and its wild-type congener were synthesized on a 100 μmol scale with a Pioneer automated peptide synthesizer (Applied Biosystems) at the University of Wisconsin Biotechnology Center.

A methylbenzhydrylamine polystyrene resin that had been functionalized with a 4-hydroxymethylphenoxy acid-labile linker and loaded with valine (which is the C-terminal residue of RNase A) was used for peptide synthesis. Appropriate Fmoc-protected monomers and Fmoc-flpOH were coupled by using cycles in which the carboxyl group was activated with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate/diisopropylethyl amine (HATU) and the Fmoc group was removed by treatment with piperidine. After cleavage from the resin and side-chain deprotection, peptides were purified by high-performance liquid chromatography on a C18 reverse-phase column. The mass of the purified peptide was determined with a Reflex mass spectrometry from Bruker–Franzen (Bremen, Germany) after desalting of the peptide solutions by using ZipTip pipet tips from Millipore (Schwalbach, Germany), and was in accord with the expected values. Wild-type peptide had *m*/*z* 3275.2 (expected: 3276.6); Pro114flp peptide had *m*/*z* 3295.0 (expected: 3294.7).

Production of thioester-tagged protein and its ligation

A plasmid that directs the expression of Met(−1)RNase A(1–94) fused to the *mxe*-intein was used to produce thioester-tagged Met(−1)RNase A(1–94), as described previously.[43,54,55] Ligation with the wild-type and Pro114flp 30-mer peptides, and refolding of the ensuing polypeptides were also performed as described previously.[55] The molecular mass of each ligation product was determined with a Biflex III mass spectrometer from Bruker (Billerica, MA) after desalting of the protein samples by using ZipTip pipet tips. The two proteins produced by this method have an additional methionine residue at their N terminus, Met(−1), which has no discernable effect on the catalytic activity or conformational stability of RNase A.[56] Semisynthetic Met(−1)RNase A had *m*/*z* 13 813 (expected: 13 813); semisynthetic Pro114flp Met(−1)RNase A had *m*/*z* 13 838 (expected: 13 831).

Catalytic activity

Values of k_{cat}/K_M for the enzymatic cleavage of the fluorogenic substrate FAM–ArUAA–TAMRA were determined as described previously.[57] Activity was measured at 20 °C in 100 mM 2-(*N*-morpholino)ethanesulfonic acid–NaOH buffer, pH 6.0, containing NaCl (100 mM).

CD spectroscopy

CD spectra of RNase A and its Pro114flp variant were recorded at 20 °C in 50 mM sodium phosphate buffer, pH 8.0, contain-

ing protein (1.0 or 0.5 mg mL^{-1}) and NaCl (25 mM) on a J-810 CD spectrometer from Jasco (Groß-Umstadt, Germany). Cuvettes of 1 cm and 0.01 cm path length were used for CD spectroscopy in the near-UV (250–350 nm) and far-UV (190–250 nm) region, respectively.

Temperature-induced transition

Values of T_m for wild-type RNase A and its Pro114flp variant were obtained with CD spectroscopy at 278 nm using a heating rate of 1 K min^{-1} with a J-810 CD spectrometer. Measurements were carried out in 50 mM sodium phosphate buffer, pH 8.0, containing protein (1.0 or 0.5 mg mL^{-1}) and NaCl (25 mM). The molar ellipticity was fitted as described by Pace and co-workers[39] to obtain values of T_m, which is the temperature at which 50% of the protein is unfolded.

Gdn·HCl-induced transition

Gdn·HCl-induced transition curves of wild-type RNase A and its Pro114flp variant were obtained with fluorescence spectroscopy. Fluorescence emission spectra of protein samples with increasing concentration of Gdn·HCl were recorded from 290–350 nm in 1.0 nm increments with a FluoroMax-2 spectrometer from Yvon-Spex (Kyoto, Japan). The slit width was 5 nm for excitation at 278 nm and 5 nm for emission, and the cuvette path-length was 1.0 cm. Measurements were carried out at 25 °C in 100 mM MES–NaOH buffer, pH 6.0, containing protein (0.10 mg mL^{-1}), NaCl (100 mM), and Gdn·HCl (0.0–6.0 M). To calculate values of $[Gdn{\cdot}HCl]_{1/2}$, which is the concentration of denaturant at which 50% of the protein is unfolded, signals from multiple experiments were combined and fitted by nonlinear regression as described previously.[58,59]

Determination of the unfolding rate constants (k_U)

Values of k_U for wild-type RNase A and its Pro114flp variant were determined by proteolysis with thermolysin as described previously.[49,60] In a typical experiment, a 20 μL solution of thermolysin (2 mg mL^{-1}) in 50 mM Tris–HCl buffer, pH 8.0, containing $CaCl_2$ (10 mM) was added to 160 μL of 50 mM Tris–HCl buffer, pH 8.0, which had been pre-incubated in a bath from LAUDA–Brinkmann (Delran, NJ) that maintained temperature with an accuracy of ±0.1 °C. The unfolding transition was initiated by the addition of wild-type RNase A or its Pro114flp variant (20 μL of a 1.0 mg mL^{-1} solution). At known time intervals, a 25 μL aliquot was removed and mixed rapidly with 8 μL of 50 mM EDTA. SDS-PAGE and densitometric evaluation of the RNase A bands were carried out as described previously. From the decrease in the peak areas of the intact RNase A band, which followed a first-order reaction, the rate constants of proteolysis were determined. Under the conditions applied these values correspond to the respective unfolding rate constants. The free energy of activation for the unfolding reaction ($\Delta\Delta G_U^{\ddagger}$) was determined with the Eyring equation.

Refolding monitored by stopped-flow fluorescence spectroscopy

The recovery of enzymatic activity was used as a measure for refolding of the enzymes as has been described previously.[43] Wild-type RNase A or its Pro114flp variant (500 ng mL^{-1}) were incubated at 5–25 °C in 4.5 M Gdn·HCl in 0.10 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M). Refolding was initiated by mixing one volume of RNase A solution with 25 volumes of 0.10 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M) and FAM–ArUAA–TAMRA (50 nM). The resulting Gdn·HCl concentration was 173 mM. Refolding was followed by fluorescence spectroscopy using an Applied Photophysics BioSequential DX.17 MV stopped-flow spectrometer. Emission was recorded as integral fluorescence using a 515 nm cut-off filter with excitation at 490 nm. Typically, data from 8–10 traces were averaged. Data were fitted to the equation:

$$\text{fluorescence}(t) = Ae^{-k_f t} + mt + n \quad (1)$$

where fluorescence(t) is the fluorescence signal at time t, A is the signal amplitude, k_f is the reactivation rate constant, m is the slope of the linear part, and n is the signal off-set.

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