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Proline isomerization during refolding of ribonuclease A is accelerated by the presence of folding intermediates

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The *trans*→*cis* isomerization of Pro 93 was measured during refolding of bovine ribonuclease A. This isomerization is slow (τ = 500 s) under marginally stable folding conditions of 2.0 M GdmCl, pH 6, at 10°C. However, it is strongly accelerated (τ = 100 s) in samples which, prior to isomerization, had been converted to a folding intermediate by a 15 s refolding pulse under strongly native conditions (0.8 M ammonium sulfate, 0°C). The results demonstrate that extensive folding is possible before Pro 93 isomerizes to its native *cis* state and that the presence of structural folding intermediates leads to a marked increase in the rate of subsequent proline isomerization.

Protein folding *Ribonuclease A* *Folding intermediate* *Proline isomerization*

1. INTRODUCTION

The role of proline isomerization in protein folding has remained an important issue since the first demonstration of fast- and slow-refolding species in RNase A by Garel and Baldwin [1] and the proposition of the proline hypothesis by Brandts et al. [2]. The involvement of proline isomerization in the unfolding pathway appears to be rather well understood for several proteins. In the $N \longrightarrow U_F$ unfolding step the ordered structure collapses and U_F species are produced initially, which have their prolines still in the correct configuration (eqn 1). Unfolding is followed by slow proline isomerization, leading to an equilibrium mixture of U_F and U_S species in the unfolded protein. Several U_S species may exist in proteins with more than one proline [2–8].

$$N \longrightarrow U_F \rightleftharpoons U_S \qquad (1)$$

Abbreviations: RNase A, bovine pancreatic ribonuclease A (EC 3.1.27.5); N, native protein; U_F and U_S species, fast- and slow-refolding species of unfolded RNase, respectively; I_1,I_N, folding intermediates; GdmCl, guanidinium chloride

In refolding, the role of proline isomerization in the slow $U_S \longrightarrow N$ reactions is much less clear. Contrasting models have been proposed for it. The simple model of Brandts and co-workers [2,9,10] assumes that under any conditions refolding occurs by a strict reversal of the unfolding mechanism (eqn 1), i.e. the obligatory first and rate-limiting step of refolding of U_S species is the re-isomerization of the incorrect proline(s) in the unfolded state ($U_S \longrightarrow U_F$) which is then followed by rapid refolding ($U_F \longrightarrow N$). Alternatively, the 'folding intermediates model' suggests that the role of proline re-isomerization for slow refolding depends on the folding conditions and on the location of the proline(s) in the amino acid sequence. Under conditions which effectively stabilize folded proteins ('strongly native' conditions) folding intermediates with incorrect isomers can be formed. Proline isomerization can occur as the last step of the folding pathway and its rate can be increased by the presence of a folded structure [4,11–19].

RNase A contains 4 proline residues, two of which (Pro 93 and Pro 114) are *cis* in the native state. There is a major slow-folding species in RNase A, U_S^{II}, which refolds via a native-like in-

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termediate, I_N, under strongly native conditions (eqn 2).

$$U_S^{II} \longrightarrow I_1 \longrightarrow I_N \longrightarrow N \quad (2)$$

I_N is compactly folded and enzymatically active, however, it still retains at least one incorrect isomer. I_N is populated only under strongly native conditions; under unfavourable conditions folding is very slow and intermediates are not detectable [11,14,18]. By using a new specific assay for Pro 93 isomerization (see below) Schmid et al. [19] showed that during refolding Pro 93 *trans* ⟶ *cis* re-isomerization is slower than the formation of I_N. They proposed that folding of the major U_S^{II} species is explained by the folding intermediates model, i.e. that the U_S^{II} molecules have an incorrect *trans* Pro 93 and that they can fold rapidly to I_N, before Pro 93 *trans* ⟶ *cis* isomerization occurs in the final $I_N \longrightarrow N$ step.

Based on isomer-specific proteolysis experiments Lin and Brandts [9], however, concluded that refolding of U_S molecules with an incorrect *trans* Pro 93 obeys the simple model, i.e. folding would be blocked until the *trans* ⟶ *cis* isomerization of Pro 93 occurs in the unfolded chain. Initial isomerization would then be followed by rapid folding to N.

2. MATERIALS AND METHODS

RNase A (type XII A) was purchased from Sigma (St. Louis, MO) and GdmCl (ultrapure) from Schwarz-Mann (Orangeburg, NY). Other chemicals were from Merck (Darmstadt).

Measurements of absorbance and fluorescence were carried out with a Cary 118C spectrophotometer and a Hitachi–Perkin-Elmer MPF-44 fluorescence spectrophotometer, respectively. Direct refolding was carried out by 20-fold dilution of 45 μl unfolded RNase (0.6 mM in 4.0 M GdmCl, 0.1 M glycine, pH 2.0, at 10°C) to final 2.0 M GdmCl, 0.16 M $(NH_4)_2SO_4$, pH 6, at 10°C in the spectrophotometer cell. Refolding was monitored by the increase in absorbance at 287 nm. Fluorescence unfolding assays for *trans* ⟶ *cis* isomerization of Pro 93 were carried out as described [19].

3. RESULTS AND DISCUSSION

To test the models for refolding, we determined whether *trans* ⟶ *cis* isomerization of Pro 93 could be accelerated by prior formation of native-like folded structure. In our approach we compared the kinetics of Pro 93 *trans* ⟶ *cis* isomerization of two samples under the same (marginally stable) folding conditions. The first sample of unfolded RNase was diluted directly into refolding conditions of 2.0 M GdmCl and 0.16 M $(NH_4)_2SO_4$, pH 6, 10°C. The second unfolded RNase sample was first exposed to a short refolding pulse under strongly native conditions (15 s at 0.8 M $(NH_4)_2SO_4$, 0.4 M GdmCl, pH 6, at 0°C) to produce a mixture of about 30% N, 55% I_N, and 15% U_S molecules [20–22] which was then transferred to the same final conditions (2.0 M GdmCl, 0.16 M $(NH_4)_2SO_4$, pH 6, 10°C). In both samples the kinetics of Pro 93 *trans* ⟶ *cis* isomerization were then measured by a specific unfolding assay. This assay is based on the observation that the fluorescence of Tyr 92 is affected by the isomerization of the adjacent Tyr 92–Pro 93 peptide bond in the unfolded state [7,19]. To measure the kinetics of Pro 93 *trans* ⟶ *cis* isomerization, aliquots were taken out of the refolding solution after varying times, unfolded rapidly, and the amplitudes of the subsequent slow fluorescence changes determined. The amplitudes are proportional to the fraction of U_S molecules which had already undergone Pro 93 *trans* ⟶ *cis* isomerization during the refolding step and consequently reverted back to the equilibrium *cis/trans* distribution of the unfolded state in the assay [19].

The results are shown in fig.1. The kinetics of Pro 93 *trans* ⟶ *cis* isomerization are slow (τ = 500 s) during direct refolding at 2.0 M GdmCl, 0.16 M $(NH_4)_2SO_4$, pH 6, 10°C, and coincide with absorbance- and fluorescence-detected refolding (fig.1A). No intermediates are found under these marginally stable conditions. In the second experiment, where I_N was populated by prefolding, Pro 93 *trans* ⟶ *cis* isomerization is much faster under the same final conditions (fig.1B). The kinetics can be decomposed tentatively into a major process (about 70%) with a time constant of 100 s and a minor process (about 20%) with τ = 500 s. This small slow phase originates from Pro 93 *trans* ⟶ *cis* isomerization during refolding of the residual

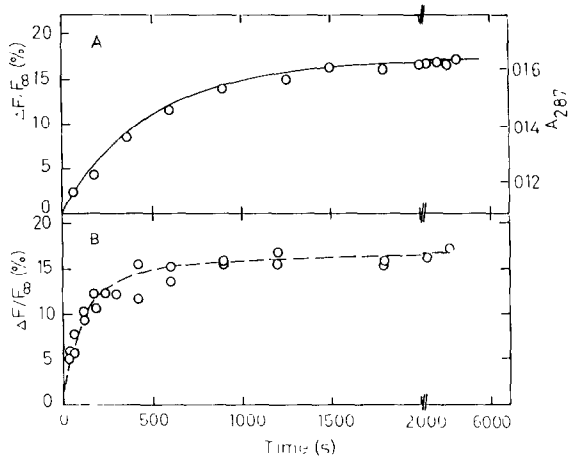

Fig.1. Kinetics of *trans* ⟶ *cis* isomerization of Pro 93 during refolding of RNase A (○) at 10°C in the presence of 2.0 M GdmCl, 0.16 M $(NH_4)_2SO_4$, 0.05 M sodium cacodylate, pH 6. Pro 93 *trans* ⟶ *cis* isomerization was measured by fluorescence unfolding assays at 305 nm (excitation at 268 nm). The assays were carried out by 10-fold dilution of aliquots withdrawn after the indicated times of refolding to final 5.0 M GdmCl, 0.1 M glycine, pH 2.0, at 10°C. The dependence of the amplitudes of the slow fluorescence increase after unfolding on the duration of the refolding step is shown. Amplitudes are given as percentage of the final fluorescence emission. (A) One-step refolding. Refolding was initiated by 5-fold dilution of unfolded RNase A (7 mg/ml in 4.0 M GdmCl, 0.1 M glycine, pH 2) with 1.5 M GdmCl, 0.2 M $(NH_4)_2SO_4$, 0.05 M sodium cacodylate, pH 6.2, to give final conditions of 2.0 M GdmCl, 0.16 M $(NH_4)_2SO_4$, pH 6, at 10°C. The kinetics of Pro 93 *trans* ⟶ *cis* isomerization (left ordinate) are compared with absorbance-detected (right ordinate) slow refolding (——) under the same conditions (but 30 μM RNase). Both kinetics show a time constant of 500 s. Fluorescence-detected refolding (not shown) also coincides with these kinetics. (B) Kinetics of *trans* ⟶ *cis* isomerization of Pro 93 following a 15 s refolding pulse to populate I_N. Unfolded RNase A (40 mg/ml in 4.0 M GdmCl, 0.1 M glycine, pH 2) was diluted 10-fold to give 0.8 M ammonium sulfate, 0.4 M GdmCl, 0.05 M sodium cacodylate, pH 6, at 0°C. After 15 s the resulting mixture of N, I_N, and U_S species was transferred to 2.0 M GdmCl, 0.16 M $(NH_4)_2SO_4$, at 10°C in the same buffer by 5-fold dilution. (– – –) Decomposition of the observed kinetics into two phases (τ_1 = 500 s, ΔF_1 = 4%; τ_2 = 100 s, ΔF_2 = 11%). The small amplitude at t = 0 originates from about 10% native RNase formed on U_S^{II} ⟶ N during the 15 s prefolding step.

amount of U_S species still present after the prefolding step. It shows the same rate as isomerization during direct refolding (cf. fig.1A). The major phase originates from molecules which had been converted to I_N by the folding pulse. This represents Pro 93 *trans* ⟶ *cis* isomerization during the I_N ⟶ N reaction.

The following conclusions can be drawn from these results. (i) Unfolded molecules with an incorrect *trans* Pro 93 can refold to a native-like intermediate, I_N. (ii) In the presence of I_N Pro 93 *trans* ⟶ *cis* isomerization is about 5-fold faster than without I_N. The use of identical conditions for this comparison rules out solvent effects on Pro 93 *trans* ⟶ *cis* isomerization as a potential cause of the observed differences in rate. (iii) Acceleration of Pro 93 *trans* ⟶ *cis* isomerization occurs only in the presence of I_N. This indicates that formation of I_N and Pro 93 *trans* ⟶ *cis* isomerization cannot occur on different refolding pathways as proposed [10].

These findings rule out the simple model of Lin and Brandts [9] for refolding of the major U_S^{II} species, in which Pro 93 *trans* ⟶ *cis* isomerization must take place before any folding occurs. Moreover, these results suggest that the Pro 93 *trans* ⟶ *cis* isomerization is the Y ⟶ X reaction of Lin and Brandts [9] and not the T ⟶ C reaction as they propose. Their simple model may be adequate for refolding under unfavourable conditions (e.g. in the transition region) where intermediates are unstable and folding is very slow.

Although folding of RNase molecules with an incorrect *trans* Pro 93 isomer is not blocked, it is strongly decelerated from the millisecond to the second time range and the product of refolding (the intermediate I_N) is less stable than native RNase [20]. This reflects the destabilizing effect of an incorrect proline isomer on the structure of folding intermediates.

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