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Trapping the fast-refolding state of ribonuclease A at subzero temperatures

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Unfolded ribonuclease A consists of a mixture of fast- and slow-refolding species. It is generally accepted that the slow-refolding states arise from isomerization of proline residues. We show that unfolding at subzero temperatures may be used to trap the fast-refolding species U_F, since the rate of proline isomerization slows down at a much faster rate than protein unfolding. The unfolding was carried out in 5 M guanidine hydrochloride; at $-15°C$ the protein unfolding process is complete within 30 s and under these conditions there is $< 1.5\%$ proline isomerization. By using ribonuclease in which Tyr-115 was nitrated it was possible to rule out significant isomerization of Pro-114 in the observed slow-unfolding step.

Protein folding; RNase A; Subzero temperature; Proline isomerization

1. INTRODUCTION

The existence of fast- and slow-refolding states of RNase A was first reported by Garel and Baldwin [1]. Brandts et al. [2] suggested that the slow phase might be due to proline isomerization. Two of the four Pro residues in RNase A are *cis* in the native state, residues 93 and 114 [3]. Subsequent studies [4–6] showed that there are a minimum of two or three slow-refolding species. Numerous investigations, especially by Schmid and co-workers (e.g. [7–12]) and Lin and Brandts [5,6,13–15], have supported the hypothesis that the slow-folding states involve proline isomerization. However, the exact role of the isomerization has remained controversial [15].

Most investigations of RNase folding have focused on the slow-refolding states, since they are more accessible to study due to their longer lifetimes. Clearly, from the point of view of the conformational processes involved in the transformation of unfolded to folded states, it would be desirable to observe the folding in the absence of the complications of proline isomerization. Experimentally this would be facilitated by a straightforward method of preparing unfolded protein under conditions where the isomerization did not occur and all the protein was in the form of the fast-refolding state, U_F. The inherently high energy of activation of proline isomerization (around 20 kcal·mol^{-1}) suggests an obvious approach, namely to unfold at a sufficiently low temperature such that negligible isomerization occurs over the time period for unfolding.

Here, we have investigated the unfolding of RNase A over the range 0 to $-25°C$, using 5 M Gdn-HCl to bring about unfolding. We have previously characterized the equilibrium unfolding process at subzero temperatures in aqueous methanol cryosolvents as a function of Gdn-HCl and methanol concentrations [16,17] and shown that refolding may be followed at subzero temperatures [18–21].

2. EXPERIMENTAL

2.1. *Materials*

Bovine pancreatic RNase A was purchased from Calbiochem or Sigma (chromatographically pure grade) and further purified

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by chromatography on Sephadex SPC-25 [16]. The concentration of RNase A stock solutions was determined from ϵ_{278} = 9800 $M^{-1} \cdot cm^{-1}$. The preparation of RNase in which Tyr-115 and Tyr-76,115 are nitrated has been described elsewhere [19]. Ultrapure Gdn-HCl (Research Plus Laboratories or Schwarz-Mann) and HPLC-grade methanol (J.T. Baker) were used; all other chemicals were analytical reagent grade. Cryosolvent solutions were prepared on a v/v basis as in [16,22,23]. By using buffer systems based on carboxylate the temperature effect on pH* was minimized. 5 M Gdn-HCl solutions in various concentrations of methanol (v/v) using sodium formate (pH* 2–3) or acetate buffers (pH* 4–6) were prepared. The buffer salt concentrations were 0.033 M.

2.2. *Methods*

Absorbance experiments were performed using a Cary model 118 spectrophotometer, the unfolding of the nitrated RNase derivatives being monitored at 300 nm; fluorescence measurements were made with a Perkin Elmer MPF-4 instrument, with excitation at 280 nm and emission at 305 nm. Slit widths were 2 and 7 nm, respectively. Temperatures were measured using a thermocouple and digital thermometer. The temperature of the samples was controlled by thermostatting the cells in brass blocks, using circulating ethanol or ethylene glycol/water from Neslab constant-temperature baths. The sample compartment was purged with dry N_2.

The standard protocol used to monitor the unfolding reaction was as follows: an aliquot of a stock solution of RNase A (5 mg/ml), in aqueous or 35% methanol, pH* 3.0, was injected into an appropriate volume of precooled 5 M Gdn-HCl solution in the spectrometer cell to bring the final protein concentration into the range 7–20 μM. The sample was added using a Hamilton syringe and mixed with a precooled vibrating stirrer.

Refolding, following unfolding, was performed as follows. A stock solution of 50 mg/ml of RNase was prepared in 25% methanol, pH* 3.0. Unfolding was initiated by adding 200 μl enzyme solution to 1.0 ml of 6 M Gdn-HCl, 35% methanol, 0.033 M formate, pH* 3.0, at −15°C. The unfolding reaction was carried out in an insulated brass-block cell holder attached to the circulating bath described above. After stirring for 30 s under the unfolding conditions 50 μl of the solution was added to 1.0 ml of 35% methanol, 0.033 M formate, pH* 3.0, at −15°C. The refolding reaction was followed by the change in absorbance at 286 nm [20].

Stock solutions were 0.37 or 0.77 mM for fluorescence experiments and 0.52 mM for the nitrated RNase. All solutions were filtered through 0.45 μm filters prior to use.

The data on the kinetics were accumulated with a microcomputer for subsequent analysis. Data analysis was done using the REDUCE analysis program [24].

3. RESULTS AND DISCUSSION

Experiments were carried out to map the rate of unfolding in 5 M Gdn-HCl, monitored by fluorescence or absorbance from nitrated tyrosine derivatives, as a function of pH, temperature and methanol concentration. The fluorescence emission originates from the six tyrosine residues in RNase A, although Schmid et al. [10] have shown that only Tyr-92 contributes significantly to the observed slow phase in refolding, and that this fluorescence emission of RNase A is an excellent probe for monitoring the isomerization state of Pro-93 [8,10].

The generally accepted model for the RNase A unfolding system is as follows:

$$N \xrightarrow{\text{fast}} U_F \xrightarrow{\text{slow}} U_S$$

where U_F to U_S involves the isomerization of proline.

When the unfolding process was followed by fluorescence emission at subzero temperatures biphasic kinetics were observed at the lowest temperatures, but at the higher temperature range the rate of the faster transient was too rapid to measure with the manual mixing method used. Its existence, however, was confirmed from comparison of the observed and expected amplitudes [20]. At −15°C, 35 or 50% methanol, 5 M Gdn-HCl the fast phase was complete within 40 s. The amplitude of the slow phase was 25% of the total expected fluorescence change under these conditions. This is in good agreement with the relative amplitude of the slow phase in refolding measured by fluorecence in aqueous solution, which is reported to be in the range of 20–30% [6,8].

3.1. *Effect of methanol concentration on the unfolding rate*

Solutions of 5 M Gdn-HCl freeze around −16°C (they easily supercool to below −20°C). In order to alleviate potential problems of the denaturing solution freezing we chose to add methanol to depress the freezing point further. We measured the rate of unfolding at 0 and −15°C as a function of methanol concentration. In these experiments we were interested in two main factors: (i) that the formation of U_F, i.e. the fast phase of unfolding, was rapid, and (ii) the observed rate of the slow phase corresponding to the formation of U_S, the proline isomerization.

The time-dependent change in fluorescence emission intensity during unfolding at −20°C, pH* 2.0, 5 M Gdn-HCl is shown in fig.1. Only the slow phase is shown. The rate of the slow phase was independent of methanol concentration,

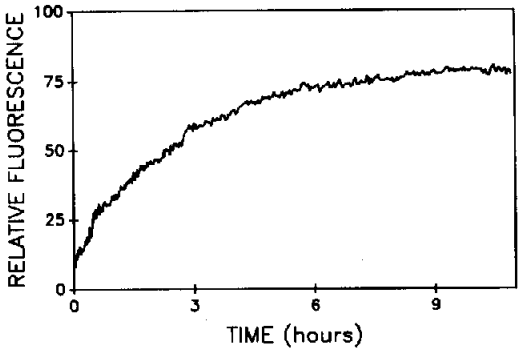

Fig.1. Unfolding of RNase A in 50% methanol, pH* 2.0, 5 M Gdn-HCl at −20°C. The reaction was monitored by the fluorescence intensity at 305 nm. Only the slow-unfolding phase is shown.

within experimental error, over the range 0–50% methanol at 0°C, and over the range 25–50% at −15°C. Some of the data are listed in table 1. The kinetics of proline isomerization are expected to be independent of methanol concentration, thus the results are consistent with the slow phase being proline isomerization.

3.2. *Effect of pH on the slow-unfolding rate*

The effect of pH* on the rate of the slow-unfolding phase was measured in 50% methanol at −15°C over the range pH* 2.0–6.2. Within experimental error, there was no effect of pH* on the rate (table 1) also consistent with the slow transient reflecting the isomerization of proline.

3.3. *Effect of temperature on the rate of the slow-unfolding phase*

A linear Arrhenius plot (not shown) was obtained for the rate of the slow-unfolding step in 50% methanol at pH* 2. Some of the data are given in table 1. The calculated energy of activation for the slow step was 17 ± 2 kcal·mol^{-1}. This value is consistent with the reaction being proline isomerization. Extrapolation of the data indicates that at −50°C the slow-unfolding step would take about 2 months to go to completion. At −20°C the slow step is complete in about 6 h. Lin and Brandts [6] used fluorescence to measure the rate of unfolding of RNase A in 8 M urea, pH 2, at 10.5 and 16.0°C. The rates for the slow phase, also assigned to proline isomerization, are very close to those extrapolated from our Arrhenius plot for unfolding at pH 2 in 5 M Gdn-HCl, 50% methanol.

Table 1

Kinetics of the slow phase of unfolding of RNase A in 5 M Gdn-HCl, as measured by fluorescence emission at 305 nm

[MeOH] (%)	pH*	Temperature (°C)	k_{obs} ($s^{-1} \times 10^4$)
0	2.0	0	19 ± 2
25	2.0	0	22 ± 3
35	2.0	0	34 ± 8
50	2.0	0	26
50	2.0	−15	2.7 ± 0.1
50	3.0	−15	2.1 ± 0.1
50	4.5	−15	2.8
50	6.2	−15	2.9 ± 0.15
25	2.0	−15	2.2
35	3.0	−15	2.7 ± 0.1
50	2.0	−20	1.6 ± 0.4

The amplitude of the slow phase was about 25% of the total expected change

Assuming that the observed slow phase represents the U_F to U_S transition, and the faster phase the N to U_F reaction, then U_F is formed rapidly, even at −20°C, whereas at temperatures of −15°C and below its conversion to U_S is very slow. Thus, conditions of 5 M Gdn-HCl, pH* 2–6, and temperatures of −15°C and below can be used to trap U_F before the $U_F \longrightarrow U_S$ transformation occurs.

3.4. *Unfolding of the nitrated derivatives*

The unfolding of the nitro-Tyr-115 and nitro-Tyr-76,115 derivatives of RNase in 5 M Gdn-HCl, 35% methanol, pH* 3.0, was followed by the change in absorbance at 300 nm. The difference in molar absorbance at this wavelength between the folded and unfolded states is 650 $M^{-1} \cdot cm^{-1}$ for the mononitro compound and 1390 $M^{-1} \cdot cm^{-1}$ for the dinitro derivative at −15°C. Various enzyme concentrations were used in the experiments, over the range 24–72 μM. At 72 μM, the expected change in unfolding would be 0.047 absorbance units for the nitro-Tyr-115 derivative. After the initial jump in absorbance on initiating the reaction there was less than a 0.0005 increase in absorbance over a 3 h period, corresponding to the time for the U_F to U_S reaction. Thus, only 1% of the expected change was noted. This suggests that the entire reaction occurred within the dead-time of the manual mixing.

For the dinitro derivative at 24 μM a change of 0.034 absorbance units was expected. Essentially no change in absorbance was observed after mixing. Again the entire reaction was complete within the dead-time of the manual mixing.

Since Tyr-115 is adjacent to Pro-114, which is *cis* in the native protein, one would expect that the absorbance signal of nitro-Tyr-115 would be a sensitive probe of the isomerization of Pro-114. The results, therefore, indicate that negligible isomerization of Pro-114 occurs in the slow phase of unfolding. This implies that Pro-114 isomerization does not occur in the U_F to U_S transition. Thus, either the isomerization of Pro-114 does not occur in unfolding or it is very fast compared to that of Pro-93. This finding is also consistent with our observations in refolding, which show that nitro-Tyr-115 attains its native signal at a much faster rate than the two slowest slow-refolding phases [19], and with Lin and Brandts' report that isomer-specific proteolysis shows only 5% of Pro-114 in the non-native *trans* state in unfolded RNase [13].

3.5. *Refolding of trapped U_F*

These experiments were undertaken to confirm that negligible U_S was formed during unfolding at -15°C for short time periods. Unmodified RNase was unfolded for 30 s at -15°C to trap the fast-refolding state. An aliquot was then refolded in 35% methanol, pH* 3.0, at -15°C. The refolding process was followed by the change in absorbance at 286 nm. The difference in molar absorbance between the native and unfolded states under these conditions is 3000 $M^{-1} \cdot cm^{-1}$. An enzyme concentration of 30 μM was employed in the reaction, thus a total absorbance change of 0.087 units (including dilution effects) is expected. On the time scale of the slow-refolding reaction (6×10^3 s) an increase in absorbance of 0.001 A was observed. This corresponds to less than 1.5% of the expected change in absorbance for the refolding reaction starting from the U_S state. Thus, we can conclude that a maximum of less than 1.5% of U_S had accumulated during the unfolding.

The present investigation demonstrates that the transformation of U_F into N can be studied in the absence of the U_S to N reaction by unfolding the protein at temperatures of -15°C or below, which prevents the conversion of U_F into U_S, and that the transformation of U_F into U_S involves the isomerization of Pro-93, but not Pro-114. By maintaining the temperature below -20°C the U_F state may be stabilized for long time periods, thus permitting the possibility of various types of studies on this state.

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