

The effect of methanol and temperature on the kinetics of refolding of ribonuclease A

Anthony L. Fink, William D. Anderson, James E. Hattersley and Brooke S. Lustig

Department of Chemistry, University of California, Santa Cruz, CA 95064, USA

Received 20 June 1988

Unfolded ribonuclease A consists of 20% fast refolding (U_F) and 80% slow refolding material (U_S). The latter consists of at least two different forms which refold at different rates. We have used absorbance and fluorescence spectrophotometry to compare the kinetics of refolding in aqueous and aqueous-methanol solutions. At 1°C and pH 3.0, the addition of increasing concentrations of methanol (to 50%, v/v) had negligible effect on the rates and amplitudes of the slow refolding U_S states. The effect of temperature on the two slow phases of refolding was determined in 35 and 50% methanol. From Arrhenius plots the energies of activation were found to be in the vicinity of 20 kcal/mol for both processes. The results suggest that both slow phases correspond to proline isomerization, and that the presence of methanol does not significantly perturb the overall refolding process. It is possible that the faster of the slow refolding phases corresponds to the isomerization of a proline residue which is *trans* in the folded native state but which undergoes extensive isomerization to the *cis* conformation in the unfolded state.

Protein folding; RNase; Cosolvent effect; Subzero temperature; Proline isomerization; Kinetics; Solvent effect

1. INTRODUCTION

Studies of ribonuclease folding using aqueous-methanol cryosolvent systems may be valuable in elucidating the pathway of folding [1–6]. However, since alcohols are known to have the potential to induce helical structure in proteins [7], it is important to determine the relationship between the folding process in aqueous solution and in aqueous-methanol solvents. One simple way to gain insight into this question is to conduct experiments at 0°C where direct comparisons of the kinetics of folding in the two solvents can be made. Here, we have examined the kinetics of refolding of ribonuclease A as a function of methanol concentration, and for 50% methanol, determined the

energies of activation for the observed phases in refolding. The results from these experiments also provide information on the role of proline isomerization in the refolding process.

There has been considerable debate regarding the origin of the slow-refolding species in ribonuclease and other small globular proteins (e.g. [8–16]). Brands et al. [17] proposed that the isomerization of proline was responsible; if one or more proline residues in the native conformation was *cis* then on unfolding it could isomerize to the more stable *trans* configuration. On refolding the necessary isomerization back to the *cis* configuration (a slow process) could become rate-limiting. Subsequent studies have shown that there are at least three different unfolded states of ribonuclease. One, U_F , corresponding to 20% of the total, refolds very rapidly and is assumed to have the Pro residues in their native configurations. U_S^I and U_S^{II} correspond to minor and major slow refolding species, respectively [18]. In native ribonuclease two of the four Pro residues are in the less stable *cis* form, namely Pro-93 and Pro-114.

Correspondence address: A.L. Fink, Department of Chemistry, University of California, Santa Cruz, CA 95064, USA

Abbreviations: RNase, ribonuclease; Gdn·HCl, guanidine hydrochloride; C_m , the concentration of denaturant at which half the protein is unfolded

The exact relationship between the isomerization of Pro-93, Pro-114 and the two major transients observed in the refolding of U_S has remained controversial [10–16].

2. EXPERIMENTAL

2.1. Purification of RNase A

Chromatographically 'pure' ribonuclease A was purchased from Calbiochem-Behring and further purified with Sephadex SPC-25 [1]. The purified material was homogeneous by analytical IEF, and was lyophilized and stored at -20°C . The enzyme stock solution was 0.5 mM in protein, pH* 3.0, 0.033 M sodium formate in 35% methanol.

2.2. Materials

Aqueous methanol solvents were mixed on a v/v basis using HPLC grade methanol. Either sodium acetate (pH* 6.0) or sodium formate (pH* 3.0) buffers were used at 0.033 M. Carboxylate buffers were used to minimize the effect of temperature on the pH.

2.3. Methods

Absorbance measurements of folding were performed at 286 nm on a Cary 118 spectrophotometer. Fluorescence measurements were made with a Perkin-Elmer MPF-4 instrument. Circular dichroism data were collected with a modified Jasco J-20 instrument; a 0.2 mm path length cell was used. An insulated, thermostatted brass-block cell holder was used to maintain constant temperature in the sample cuvette. The cell compartment was constantly purged with dry N_2 .

In a typical refolding experiment, monitored by absorbance, a 20–50 μl aliquot of concentrated protein solution (0.75 mM) was taken up into a gas-tight microsyringe (Hamilton) and incubated for 10 min at 70°C in a water bath. Previous experiments using NMR had demonstrated that these conditions result in the apparent complete unfolding of RNase [1]. In some experiments the protein was unfolded with 5 M Gdn·HCl, pH 2.0, 25 or 35% methanol. No difference in results was noted as a function of unfolding method. The syringe contents were then injected into a thermostatted cuvette containing 1.0 ml cryosolvent. Immediately after injection, the solution was mixed with a pre-cooled, vibrating stirrer. The time-dependent changes in absorbance were directly accumulated on a microcomputer interfaced to the spectrophotometer. By appropriate signal averaging the sensitivity of the instrument was significantly increased.

Refolding was also monitored by fluorescence emission. Ribonuclease A was unfolded in 5 M Gdn·HCl, 35% methanol, 0.033 M formate (pH 2.0), at room temperature. The enzyme concentration was 10.5 mg/ml. A 50 μl aliquot of this solution was pipetted into 1.0 ml of either 35 or 50% methanol, 0.033 M formate (pH 3.12) to initiate refolding at pH 3.0 at various temperatures. The solution in the cell was stirred for 30 s with a motor-driven stirrer. Refolding was followed by the change in fluorescence emission at 305 nm, with excitation at 280 nm.

Data were analyzed by the curve-fitting program, REDUCE [19]. Other details of low-temperature experiments in cryosolvent have been reported [20,21].

3. RESULTS AND DISCUSSION

3.1. Effect of methanol on the rate of refolding

In aqueous solution the refolding of U_S shows two distinct kinetic phases [18] attributed to two different unfolded forms, U_S^I and U_S^{II} , with amplitudes corresponding to about 30 and 50% of the total absorbance change observed under native-like refolding conditions [11]. The kinetics of refolding of ribonuclease A at pH* 3.0 were followed by absorbance at 1°C at methanol concentrations between 0 and 50% (v/v). Fig.1 shows the effect of increasing methanol concentration on the rates of the two phases observed in the refolding of U_S , as monitored by ΔA_{286} . The rates of both slow refolding phases were insensitive to the presence of the methanol. We do not consider the small decrease in observed rate for the faster phase at higher methanol concentrations to be significant, since the error associated with k_{obs} is $\pm 10\%$. This was corroborated in a further set of experiments in which the rates of refolding in 0, 25 and 50% methanol were compared in quadruplicate runs. Again, the rates were identical within experimental error. The rates of the slow refolding phases under our conditions of 0% methanol are in good agreement with such rates previously reported under reasonably similar conditions [8,15,18].

The amplitudes of the two slow-refolding phases detected by ΔA_{286} remained essentially independent of the concentration of methanol present during refolding, at values of about 50% of the

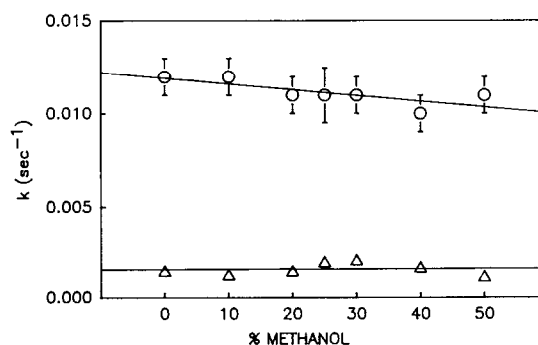


Fig.1. Effect of methanol on the kinetics of slow refolding ribonuclease A. The kinetics were measured by the change in absorbance at 286 nm. Conditions: pH* 3.0, 0.5°C . Data for (○) faster slow refolding, (Δ) slower phase.

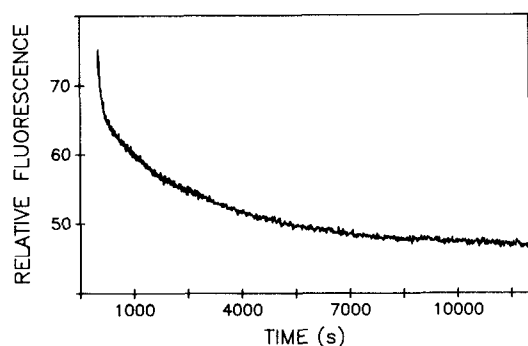


Fig.2. Refolding of Gdn·HCl-unfolded RNase in 35% methanol, pH* 3.0, -20°C , as monitored by fluorescence emission at 305 nm. Only the slow refolding phases are shown.

expected total for the major, fast phase, and about 30% for the slower phase. When fluorescence was used to monitor the refolding at 0, 35 and 50% methanol biphasic kinetics were observed. The presence of methanol had no significant effect on the rates and amplitudes.

3.2. Effect of temperature on the rate of refolding in the presence of methanol

The rates for the two slowest phases observed during refolding of U_S in 50% methanol, pH* 6.0, were measured by ΔA_{286} at temperatures between 0 and -25°C . Linear Arrhenius plots were obtained, with both processes showing energies of activation of 21 ± 2 kcal/mol. It is probably no coincidence that the energy of activation of proline isomerization is 20 kcal/mol. At temperatures of -15°C and below, additional faster transients were detected in these experiments in which the refolding was monitored by ΔA_{286} . The significance and details of these additional processes have been discussed elsewhere [3–5].

The temperature dependence of refolding was also measured using tyrosine fluorescence emission at pH* 3.0, over the range 0 to -20°C , in both 35 and 50% methanol systems. The reaction was biphasic throughout this temperature range. A typical trace of the fluorescence change during refolding is illustrated in fig.2, which shows the reaction at -20°C in 35% methanol, pH* 3.0. The Arrhenius plots (fig.3) for both phases of refolding, as measured by fluorescence, give energies of activation of 16 ± 2 kcal/mol.

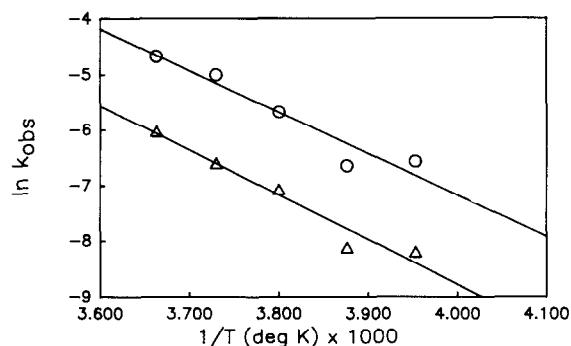


Fig.3. Effect of temperature on the kinetics of slow refolding ribonuclease A. Conditions: 35% methanol, pH* 3.0; refolding was monitored by fluorescence. Data for (Δ) the slowest phase, (○) faster observed phase.

Several investigations in aqueous solution have led to reports of the energies of activation for the two slow refolding processes. These have been mostly in the vicinity of 15–20 kcal/mol for both processes, measured by both absorbance and fluorescence [11,14,15,22,23].

3.3. Role of proline isomerization

In aqueous solution Rehage and Schmid [22] have shown that the major source of the fluorescence emission of RNase is Tyr-92, which is adjacent to Pro-93 which is *cis* in the native state, and that fluorescence emission can thus be used to monitor the isomerization of Pro-93 [13]. Many experiments in aqueous solution are consistent with the slowest step detected in ribonuclease A refolding being rate-limited by the isomerization of Pro-93 [10,13,15,22].

Our observations for refolding in aqueous methanol are entirely consistent with this hypothesis:

- (i) The rate of the slowest phase is independent of [methanol].
- (ii) Its energy of activation is in the vicinity of 20 kcal/mol.
- (iii) Its amplitude is unaffected by the presence of methanol in refolding.
- (iv) 30% of the total absorbance change is associated with the slow step. Lin and Brandts [10,24] have shown with isomer-specific proteolysis that 30% of Pro-93 is in the non-native *trans* configuration in U_S .

- (v) When Tyr-115 is nitrated, the signal from the nitrotyrosine during refolding indicates that the environment around Tyr-115 has become native-like at a much faster rate than the slowest phase observed by Tyr absorbance or fluorescence [3] indicating that the isomerization of Pro-114 is not involved in the slowest phase of refolding.

It is significant that with both absorbance and fluorescence measurements, at pH* 3 and 6, two transients are detected with essentially identical energies of activation, but differing in rate by an order of magnitude. It is possible that the underlying process for both transients is the same. Schmid [16,25] has proposed that the rate of proline isomerization can be affected by the local protein environment, however, this interpretation has been questioned [26]. It is thus possible that both of the observed slow transients correspond to the isomerization of Pro-93, as has been suggested for the situation in aqueous solution [13,15], although it is difficult to find a reasonable physical explanation if this is the case.

The kinetics of folding for proteins normally vary with the concentration of denaturant, reaching a minimum at the C_m . The known exceptions to this rule are cases where proline isomerization is rate-limiting [27]. In the present investigation the methanol concentrations used were all below the C_m at 0°C and pH* 3. Consequently, for protein conformational changes during folding the rate should have been dependent on the methanol concentration, and decreased with increasing methanol concentration. We have observed such a dependence in the case of staphylococcal nuclease (Nakano and Fink, in preparation). Since such a dependence of rate on methanol was not observed for either process it strongly suggests that both observed slow refolding transients correspond to Pro isomerization. Support for this comes from the recent report that peptidyl-prolyl isomerase catalyzes both slow refolding steps in the refolding of ribonuclease S protein [16].

Our investigations of ribonuclease in which Tyr-115 was nitrated [3,28] indicate that the isomerization of Pro-114 (which is *cis* in the native state) is not the rate-limiting step in the faster of the two slow refolding phases. One explanation for

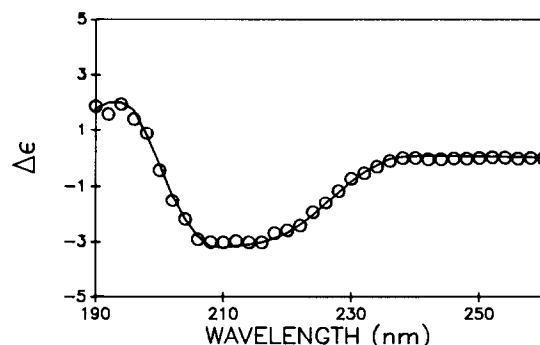


Fig.4. Circular dichroism spectra for native ribonuclease A in aqueous solution (—) and in 50% methanol (○), pH* 3.0. Conditions: 0°C for the aqueous sample, and -15°C for the 50% methanol sample.

two slow refolding paths, each with rate-limiting Pro isomerization, which has not previously been suggested, is that the minor pathway involves molecules in which one of the Pro residues which is in the *trans* conformation in the native state, isomerizes to the *cis* conformation to a significant extent in the unfolded state. Studies on model peptides indicate that substantial equilibrium concentrations of the *cis* conformer may exist, and that the exact amount is highly dependent on both the local sequence as well as the solvent conditions [29]. In this model the isomerization of the non-native *cis* proline is more rapid than that of Pro-93 and leads to the minor, faster slow refolding pathway.

3.4. Folding in aqueous methanol

Comparison of the refolding properties of ribonuclease in aqueous solution with those in the presence of methanol suggest that there are no significant effects of methanol in the later stages, at least, of refolding. This is corroborated by the fact that the final product of refolding in methanol is conformationally very similar or identical to the native conformation in aqueous solution, as judged by NMR [1,2] and circular dichroism (fig.4). Thus, whatever effects methanol may have on the early stages of refolding, they do not result in a different final product, nor do they affect the overall rate-limiting processes significantly.

Acknowledgements: This research was supported by a grant from the National Science Foundation. We thank Roger Biringer and Linda Calciano for their contributions.

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