STUDIES OF THE INTERMEDIATES IN THE FOLDING OF RIBONUCLEASE A

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Previous work from this laboratory has shown that proline isomerization can be used as a kinetic trap for intermediates in the folding of RNase A. There are two classes of unfolded RNase A: a fast-folding class, U_F, and a major (80%) slow folding class, U_S (1). The $U_F \rightleftharpoons U_S$ interconversion reaction in unfolded RNase A has been shown to be acid-catalyzed and to have other specific properties of proline isomerization (2). Thus, it is probable that U_S contains one or more proline isomers in wrong (i.e., non-native) *cis* or *trans* conformations, as first proposed by Brandts et al. (3). However, contrary to the model proposed by Brandts et al. (3), we have evidence that, in the folding of RNase A at low temperatures, structural intermediates accumulate before proline isomerization. An assay has been devised for wrong proline isomers which can be used during folding. The results show that, at 0°–10°C, the folding reaction monitored by tyrosine absorbance occurs well in advance of proline isomerization (4). Moreover, specific structure is formed in the reaction monitored by tyrosine absorbance because a binding site for the specific inhibitor 2′CMP is formed in the reaction (4). These data point to the existence of a quasi-native intermediate, I_N, formed late in the folding reaction. I_N appears to be folded, as measured both by tyrosine absorbance and by 2′CMP binding, but has one or more prolines in the wrong cis-trans conformation, as measured by the assay for wrong proline isomers (4). Schmid (5) has recently found that the kinetic progress curve for the quenching of fluorescence emission during RNase A folding follows the same curve as the assay for wrong proline isomers, demonstrating that the proline isomerization reaction is also detectable by a spectral technique. Proline isomerization provides a suitable trap for intermediates in folding since: (*a*) proline isomerization is the final and slowest step of the folding reaction, (*b*) folding does occur before proline isomerization takes place, and (*c*) the kinetic trap is applicable to the major form of the unfolded protein (U_s).

By using this kinetic trap, we have studied the folding reaction of U_S. This has been done by radioactively labeling exchangeable amide protons and making use of the fact that folding reactions (especially H-bonding) greatly retard the rate of amide proton exchange (6). The factors which slow down exchange in native proteins (exclusion of H_2O, and H-bonding) retard exchange in bovine pancreatic trypsin inhibitor (BPTI) by as much as 10^9 (7, 8) and in RNase A by as much as 10^6 (7, 9).

Previous work from this laboratory has used hydrogen exchange to identify an intermediate early in the folding reaction of RNase A (9). After ^{3}H-labeling the amide protons of the unfolded RNase A, the labeled protein is separated from the ^{3}H-H_2O, and refolding is initiated. This sets up a competition between exchange-out from the unfolded protein ($U^* \rightarrow U$) and folding ($U^* \rightarrow I_N^* \rightarrow N^*$). It is possible to choose conditions where only a small number of protons are trapped in the competition, if there are no folding intermediates before I_N. The results show that >20 of a possible 50 protons are stably trapped after folding is complete (9). These results demonstrate the existence of an intermediate, I_1, which traps protons early in the folding reaction.

Here we report hydrogen exchange studies done over the entire time course of the folding reaction of RNase A. The method used differs from the one used previously; instead of

measuring the trapping of protons due to folding reactions, we have measured exclusion of label from partially folded structures. This approach has allowed us to study the formation of structure at any time point along the folding pathway. The experimental design is as follows. (*a*) Initiate refolding in the absence of radioactivity. (*b*) After allowing folding to occur for a variable period of time, add ^3H-H_2O. Those protons whose exchange rates are retarded by the formation of folding intermediates will be protected from labeling. (*C*) After 10 s of exposure to ^3H-H_2O, the exchange reaction is quenched by lowering the pH to 3. (*d*) Folding is allowed to go to completion and the ^3H-H_2O is separated from the partially labeled protein on a Sephadex G-25 column. (*e*) Exchange out is allowed to occur for 20 h at 10°C, pH 6, so that only the stably protected protons remain. (*f*) The number of stably trapped protons is determined using a filter assay (10).

The RNase A is unfolded for 30 min in 3.0 M GuHCl; 50 mM glycine; pH 2; 25°C. Refolding is initiated in either (*a*) strongly native conditions (0.25 M GuHCl; 0.20 M $(NH_4)_2SO_4$; 50 mM MOPS; pH 7.5; 10°C) or (*b*) in conditions in which no intermediates can be detected spectroscopically, but folding does go to completion (4, 11, 12) (2.5 M GuHCl; 50 mM MOPS; pH 7.5; 10°C). The exchange-in at pH 7.5 occurs in <500 ms (6, 7), so that additional folding during labeling can be ignored. Exposure of the protein to the ^3H-H_2O at pH 7.5 is kept to a minimum (10 s), to reduce the uptake of label by the folded protein in "breathing" reactions (6). Control experiments demonstrate that these breathing reactions can be ignored when a 10 s pulse of labeling is used.

The data from these experiments have been compared to spectroscopic measurements of the folding reaction of RNase A: tyrosine absorbance (monitoring formation of I_N) and fluorescence quenching (monitoring formation of the native protein, N). Our results show that when folding is initiated under strongly native conditions, the ^3H-labeling results fall below the spectroscopically measured curves for folding indicating that, before I_N is formed, at least one additional intermediate is formed which provide protection against exchange. However, when folding is initiated under conditions where there are no spectroscopically detectable intermediates, then the ^3H-labeling results fall on the same line as the spectroscopic measurements of folding indicating that there are also no intermediates detected by ^3H-labeling.

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