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Temperature-jump NMR study of protein folding: Ribonuclease A at low pH

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SUMMARY

The kinetic process of folding of bovine pancreatic ribonuclease A in a ^2H_2O environment at pH 1.2 was examined by a recently developed temperature-jump NMR method (Akasaka et al., (1990) Rèv. Sci. Instrum. **61**, 66-68). Upon temperature-jump down from 45°C to 29°C, which was attained within 6 s, the proton NMR spectral changes were followed consecutively in time intervals of seconds. There was a rapid spectral change, which was finished within the jump period, followed by a much slower process which lasted for a minute or longer. Rates of the slower process were measured at different positions of the polypeptide chain as intensity changes of individual His and Tyr proton signals of the folded conformer and as intensity changes of aliphatic and His protons of the unfolded conformer. Most of these rates coincided with each other within experimental error with an average value of $2.8 \times 10^{-2} s^{-1}$. The result gave clear experimental evidence that the slow folding of RNase A at low pH is a cooperative process involving most regions of the molecule, not only thermodynamically, but kinetically as well.

INTRODUCTION

The kinetic process of protein folding has been studied rather extensively with optical spectroscopy (Kim and Baldwin 1982). However, the use of NMR spectroscopy has been severely limited for technical reasons, although NMR spectroscopy has enormous potential utility because of its possibility of giving structural information from all parts of the molecule simultaneously. In applying NMR spectroscopy to protein folding, which might occur in a time range of milliseconds to minutes, some technique must be introduced to produce chemical nonequilibrium rapidly within an NMR sample tube. Temperature-jump may be a desired technique for this purpose, since, under favorable conditions, protein folding is brought about reversibly with temperature without additional reagents so that signal accumulation is possible when synchronized with heat cycles.

A first temperature-jump NMR study of protein folding was reported by Blum et al. (1978) who attained a temperature-jump down of 35°C within one minute by manually transferring the NMR sample tube from a heat bath to an NMR probe. More recently, Adler and Scheraga (1988) introduced recycled flow spectrometry to attain an effective temperature-jump by passing the protein solution through two temperature baths successively before bringing it into the NMR probe and then continuously recycling it for signal accumulation or for 2D NMR study. Recently, we developed a temperature-jump NMR apparatus by which the temperature of the protein solution may be jumped up or down directly in an NMR sample tube (Akasaka et al., 1990). Cooling of the sample by 15°C may be attained within several seconds by using gated gas flows with three different temperatures. In the present work, by using this temperature-jump NMR apparatus, the process of folding of ribonuclease A (RNase A) upon temperature-jump down was followed in time sequence of events at intervals of seconds on essentially all observable proton signals of the protein.

MATERIALS AND METHODS

RNase A (from bovine pancreas) obtained from Sigma (Type XII-A) was passed through a CM-cellulose column. The purified, lyophilized RNase A sample was dissolved in diluted ^{2}HCl containing 0.2 M NaCl to give a final solution of approximately 10% RNase A and a measured pH of 1.2 for temperature-jump NMR measurements.

The basic NMR spectrometer system was a JEOL GX-400 high-resolution NMR spectrometer equipped with a high-sensitive proton probe (NM-CH5), to which the gas flow temperature-jump equipment was connected to attain the desired temperature-jump in the NMR sample tube. The sample tube was made up of a pyrex capillary with ca. 2.3 mm inner diameter (outer diameter 3 mm), and contained about 40 μL of the protein solution. No sample spinning was executed. Both the radiofrequency pulse and gas flows were controlled by a pulse programmer of the spectrometer, and the entire pulse sequence was repeated for signal accumulation. Details of the apparatus and the pulse sequence are described elsewhere (Akasaka et al., 1990).

RESULTS AND DISCUSSION

Under the present conditions (pH 1.2, 0.2 M NaCl in ^2H_2O), RNase A was found to undergo a reversible conformational transition with a transition temperature of about 35°C; the proton NMR spectrum showed a typical pattern of fully folded protein below 30°C, whereas above 40°C it showed a pattern of the fully unfolded protein. The C_ε proton signals of the four His residues (His-12, -48, -105 and -119) of RNase A in its native conformation have been assigned (Lenstra et al., 1979, Tanokura 1983). These four signals turned into almost a single peak at 8.7 ppm, when the protein was unfolded by heat denaturation.

Figure 1 shows time-dependent spectral changes in the aromatic proton region upon temperature-jump down from 45°C to 29°C. The temperature-jump was attained within approximately 6 s, after which the time elapsed is depicted as positive values in Fig. 1. It is recognized that at $t = -2$ s before the temperature reached the final temperature at $t = 0$, about 30% of the His C_ε proton resonances of the native protein appeared, while the rest of the spectrum (about 70%) remained close to that of the heat-denatured protein (the spectrum before the jump). This observation is consistent with the simplest folding scheme (1) proposed for the unfolding of RNase A in

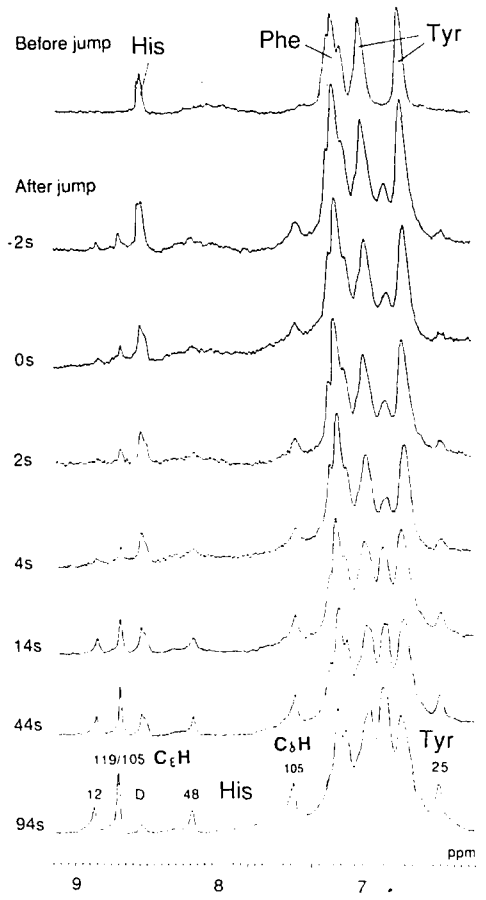

Fig. 1. Proton NMR spectral changes in the aromatic region of RNase A (10% in ^2H_2O, pH 1.2) upon temperature-jump down, measured at 400 MHz. Chemical shifts are referenced to the methyl signal of trimethylsilyl propionate. The temperature-jump down from 45 C to 29 C of the RNase A solution was brought about within 6 s by gated pressurized gas flows (3 kg/cm^2) (see Akasaka et al., (1990) for details). As the temperature of the sample went down, the radiofrequency sampling pulses were applied consecutively at intervals of 2 s or longer, to acquire time-dependent free induction decay (FID) signals at 29 C, after which the sample was brought back to 45 C in about 80 s. The entire cycle was repeated 16 times for coherent signal accumulation. Finally, the accumulated FIDs were Fourier-transformed to give time-dependent NMR spectra (NMR 'snap shots') as shown.

the acidic pH range from UV absorption temperature-jump studies (Hagerman and Baldwin, 1976),

$$U_s \overset{\text{slow}}{\rightleftharpoons} U_f \overset{\text{fast}}{\rightleftharpoons} N \qquad (1)$$

in which U_f and U_s are both denatured species with fast- and slow-folding properties, respectively, and may differ in proline isomerization. U_f folds rapidly (within 50 ms at 25°C and pH 3), well within the 'dead time' needed for the temperature-jump NMR (6 s). Thus those species that remain after the 'dead time', showing a spectral pattern close to that of heat-denatured RNase A, may be identified with the slow-folding species (U_s) and with the refolding ensemble observed by

Adler and Scheraga (3). The spectral pattern at 0 s is indistinguishable from that of heat-denatured RNase A at equilibrium, which is a mixture of U_f and U_s species (Fig. 1, top), showing that the NMR spectral patterns of the U_f and U_s species are similar.

After the sample temperature reached 29 ± 1°C at t = 0, the signal of His C_ε protons of the unfolded RNase A(designated as 'D') decreased gradually with concomitant increase of the signals of the individual His C_ε protons of folded RNase A. Figure 2 shows the plot of signal intensities of individual His and Tyr protons against time at 29°C, from which first-order rate constants were obtained as follows; 2.5 ± 1.2 × $10^{-2}s^{-1}$ (C_ε proton of His-12), 3.0 ± 0.5 × $10^{-2}s^{-1}$ (C_ε proton of His-48), 3.0 ± 0.3 × $10^{-2}s^{-1}$ (C_ε protons of His-109 and -115 combined), 3.0 ± 0.3 × $10^{-2}s^{-1}$ (C_δ proton of His-105), 2.7 ± 0.8 × $10^{-2}s^{-1}$ (C_ε proton of Tyr-25). The results indicate that rates of folding of RNase A monitored at different polypeptide segments coincide within experimental error, with an average of 2.8 × $10^{-2}s^{-1}$.

Figure 3 shows time-dependent spectral changes in the aliphatic proton region, which also depicts the slow folding process after the temperature reached 29 ± 1°C. The top spectrum depicts dissolved signals of methyl and methylene protons of several amino acid side chains (labeled by numbers and assigned as in the figure legend (Wüthrich 1986)) of the slow-folding species (U_s) of unfolded RNase A. Since these signals become increasingly overlapped with those of the folded species with time, only the initial parts of their decay cuves may be worthy of quantitative analysis (Fig. 4). The first-order rate constants were obtained from a least-squares fit as given in the legend of Fig. 4. These rate constants fall within a narrow range between 2.6 – 3.7 × $10^{-2}s^{-1}$ (except for Thr and Lys), with an average value of 3.1 × $10^{-2}s^{-1}$, so they are in reasonable agreement with those obtained from Fig. 2. The rate constants obtained here should represent time constants of reaction of U_s into U_f and N in scheme (1). The formation of N from U_f could not be observed separately in the present experiment, because this reaction was too rapid and because U_f and U_s have similar NMR spectra as pointed out before.

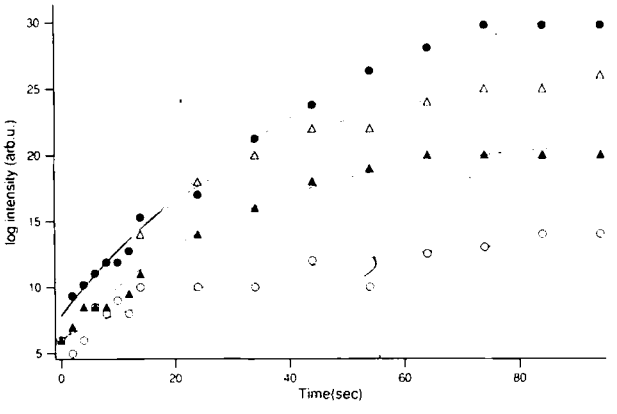

Fig. 2. Plot of individual signal intensities of the C_ε and C_δ protons of His and the C_ε protons of Tyr-25 of the folded RNase A (Fig. 1) against time after the temperature of the sample was brought down to 29 ± 1 C. ○ His-12 $C_\varepsilon H$, △ His-48 $C_\varepsilon H$, ● His-119/105 $C_\varepsilon H$, ▲ His-105 $C_\delta H$, □ Tyr-25 $C_\varepsilon H$. Rate constants estimated from individual signals are (in units of $10^{-2}s^{-1}$): 2.5 ± 1.2, 3.0 ± 0.5, 3.0 ± 0.3, 3.0 ± 0.3 and 2.7 ± 0.8, respectively. Curves represent least-squares fit to the experimental data.

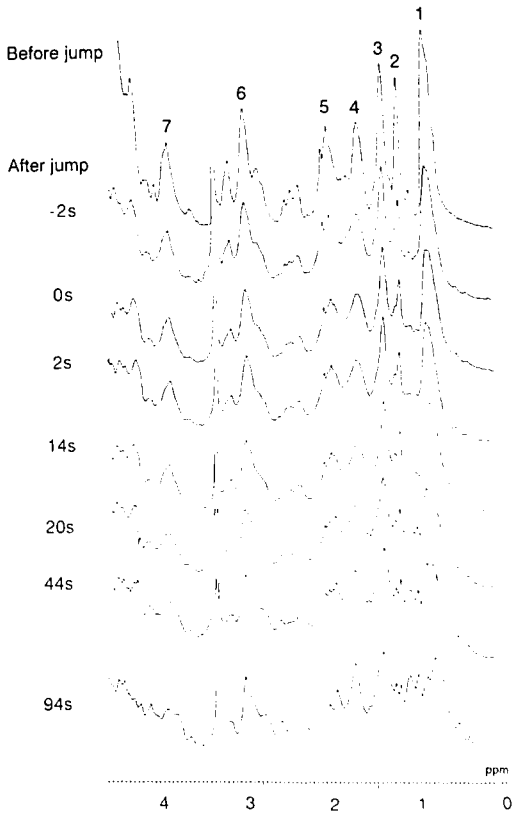

Fig. 3. Proton NMR spectral changes in the aliphatic region of RNase A upon temperature-jump down to 29 ± 1 C. The experimental conditions were the same as in Fig. 1. Major peaks of the unfolded RNase A are numbered and represent approximately the following protons: Peak 1, the methyl protons of Val, Leu and Ile; 2, the methyl protons of Thr; 3, the methyl protons of Ala; 4 (1.75 ppm), the methylene protons of Leu, Lys and Arg; 5 (2.0 ppm), the methyl and methylene protons of Met, Pro, Glu and Gln; 6 (3.0 ppm), the C_ϵ protons of Lys; 7 (3.9 ppm), the C_β protons of Ser.

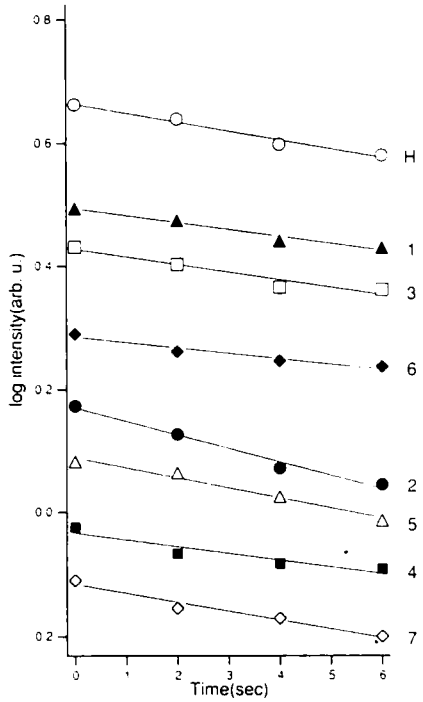

Fig. 4. Similogarithmic plot of signal intensities of the His C_ϵ and aliphatic protons of the unfolded RNase A (Fig. 3) as a function of time upon temperature-jump down to 29 ± 1 C. The numbers correspond to those in Fig. 3 and H represents the C_ϵ protons of four His residues of the unfolded RNase A (Fig. 1, Peak D). Rate constants estimated from individual peaks are (in units of 10^{-2} s^{-1}): Peak 1, 2.6 ± 0.3; 2, 4.7 ± 0.7; 3, 2.9 ± 0.5; 4, 2.6 ± 0.6; 5, 3.7 ± 0.4; 6, 2.0 ± 0.4, 7, 3.3 ± 0.5; H, 3.3 ± 0.4.

CONCLUSION

In the present work, use of the temperature-jump NMR technique enabled us for the first time to examine the process of folding of a globular protein at different regions of the polypeptide chain simultaneously at intervals of seconds. The finding that the reaction proceeds at nearly a common rate at various positions of the polypeptide chain gave a clear evidence for the notion that the slow folding of RNase A at low pH is a cooperative process involving all regions of the protein molecule simultaneously, not only in the thermodynamic sense but in the kinetic sense as well.

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