Temperature-jump-induced refolding of ribonuclease A: a time-resolved FTIR spectroscopic study

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Received 22 March 1995

Abstract FTIR difference spectroscopy has been used for the first time to investigate the kinetics of secondary structure formation during refolding. The refolding process of ribonuclease A (RNase A) as a model system was induced by applying a temperature-jump of 60 degrees. The temperature-jump was triggered by rapidly injecting a small volume of the thermally unfolded protein solution at 80°C into a special cuvette system kept at 20°C. The dead-time of the injection and the time resolution of the FTIR spectrometer permitted the observation of refolding processes in a time window ranging from 170 ms to several minutes. Specifically, the formation of β-structures and the disappearance of irregular conformations could be observed in this time interval.

Key words: FTIR spectroscopy; Protein folding; Folding kinetics; Ribonuclease A

1. Introduction

Protein folding is one of the fundamental problems not only of academic life science but also of biomedical and other applied research. A considerable number of publications has been dedicated to this subject in recent years (for review, see [1–4]). Several models of protein folding have been proposed focussing the attention on different phases and processes of protein folding [5–8].

A great variety of biophysical methods (UV absorbance, fluorescence, circular dichroism, dilatometry, and especially, NMR in combination with isotopic exchange and pulse labelling) has been used to follow different aspects of the folding process [5,7–14]. In practically all these experiments, the proteins were unfolded by chemical denaturants. Refolding, initiated by rapid dilution of the denaturant, was followed by a variety of different method-specific probes. On the other hand, refolding processes after thermal unfolding have been studied infrequently. Exceptions are dynamic light scattering [15], NMR [16,17], continous recycled flow [18], and UV absorbance [19] studies with a dead-time of the experiments in the range of seconds.

Notwithstanding its great potential for the elucidation of conformational changes in proteins [20,21], to our knowledge infrared spectroscopy has not been used to analyse refolding processes in proteins. Herein, we describe an experimental technique to induce large temperature-jumps in protein solutions and to follow the early and middle events during the transition from the unfolded to the folded state by time-resolved FTIR spectroscopy. A particularly interesting aspect of this communication is that the refolding process was induced by a temperature jump and not by dilution of a denaturant as usually done by other authors.

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2. Materials and methods

2.1. Materials

Highly purified lyophilised RNase A from bovine pancreas (Type XII-A) was purchased from Sigma Chemie GmbH (Deisenhofen, Germany) and was used without further purification.

2.2. Experiments applying a temperature-jump (T-jump)

Infrared spectra were recorded using a Bruker IFS-48 FTIR spectrometer equipped with a water cooled globar and a liquid nitrogen-cooled MCT detector. Nominal physical resolution was 8 cm^{-1}; encoding interval of data points was approximately one point per wavenumber. The protein solutions were injected into a home-made (based on a Specac micro-cuvette) flow-through cell device equipped with CaF_2 windows. The optical path-length was 50 μm. The cell device was connected by steel HPLC tubing with a Hamilton gas-tight syringe localised outside the sample chamber. This injecting syringe was equipped with a water jacket which allows adjustment of the temperature of the sample to be injected (for details of the experimental set-up, see Fig. 1). The temperatures of the IR cell and the injecting syringe were controlled by different temperature baths. The temperature of the cell was measured with a thermocouple placed on the cuvette body near the cell window. The temperature of the syringe was measured with a thermocouple placed within the syringe bore.

RNase A was dissolved in 10 mM sodium cacodylate D_2O buffer (uncorrected pH 7.0) to obtain a protein concentration of ~25 mg/ml. Prior to injection the protein solutions were incubated at 60°C for 30 min to ensure the H-D exchange of all amide protons. Then, the solution of the completely exchanged protein was filled into the injecting syringe which was then thermostatted at 80°C well above the phase transition temperature, T_m. To initiate the refolding of the thermally unfolded protein, 80 μl of the protein solution (twice the cuvette dead-volume) were injected by a fast jerk of the hand into the IR cell thermostatted at 20°C. The *Rapid Scan* IR-measurement program was started before injecting the protein solution into the IR cell. One scan takes 47 ms in the *Rapid Scan* mode on the Bruker IFS-48. Due to the specifications of the instrument, two successive scans were effectively separated by a time interval of 171 ms which defines the time-resolution of the experimental set-up. Totally, 50 single beam interferograms were collected for each independent experiment. The single beam interferograms were then converted to single channel spectra using a Blackman-Haris-3-Term apodisation function. The absorbance difference spectra were calculated from the ratio of a running single beam spectrum and the last single beam spectrum.

In a second type of experiment, possible long-term spectral changes in the time range of 0.6–160 s were studied in the normal scan mode with lower time resolution (~0.6 s).

2.3. Experiments applying a linear temperature gradient

Infrared spectra were recorded using a Bruker IFS-66 FTIR spectrometer equipped with a water-cooled globar and a DTGS detector as described recently [21]. The protein solutions were placed in a home-made, demountable cell equipped with CaF_2 windows. The path-length

SSDI 0014-5793(95)00387-8

was 50 μm. To compensate for D_2O absorption, the buffer solutions were placed in the same cell but with slightly shorter path lengths. The temperature of the gas-tight IR cell device was controlled by a programmable temperature bath purchased from Haake, Germany. FTIR spectra of the protein and the buffer solutions were recorded at a nominal resolution of 4 cm^{-1}. While the temperature of the sample was linearly increased at a rate of 0.5 degrees/min, 227 interferograms per 1°C temperature change were collected, averaged, and Fourier-transformed using a Boxcar apodisation function.

3. Results and discussion

A T-jump of 60°C (from 80°C down to 20°C) was induced in the aqueous sample well below the time resolution of the spectrometer (~170 ms). This is demonstrated by the difference spectra obtained in the time range of 0.17–7 s from a typical T-jump experiment with the pure buffer solution (see control experiment 1, Fig. 2A). Even minor inconstancies of temperature of the buffer solution in the IR-cell would lead to characteristic difference-spectroscopic features between 1700–1500 cm^{-1} (D_2O-buffer absorptions) which sensitively detect the temperature-dependent structure of the D_2O water matrix. The fact that all difference spectra only show a flat base-line proves that the final temperature was reached in less than 170 ms. The fast cooling and the temperature equilibration of the solution was only possible by injecting a *small* volume of the sample. For this purpose we used a thermostatted syringe which was connected to the thermostatted cuvette by steel HPLC tubing (Fig. 1). Under these conditions, the cell has a nearly 'infinitely' high heat capacity compared to that of the injected sample. A fast thermal equilibration of the sample was achieved by the low thickness of the cell (50 μm) which gives rise to a large (heat exchange relevant) surface area (~25 mm^2) of the sample.

Fig. 2B shows a series of difference spectra obtained from a typical T-jump experiment performed with a solution of RNase A. The difference spectra were obtained by calculating $\lg(S_n/S_l)$, where S_n is the single channel spectrum of the n^{th} scan and S_l is the single-channel spectrum of the last scan of an injection experiment. The spectrum exhibiting the most intense positive and negative difference peaks represents the first scan of a run, the spectra showing mere noise were recorded during the last milliseconds of a run. Consequently, the decrease of negative peaks (minima) which display increasing band intensities can

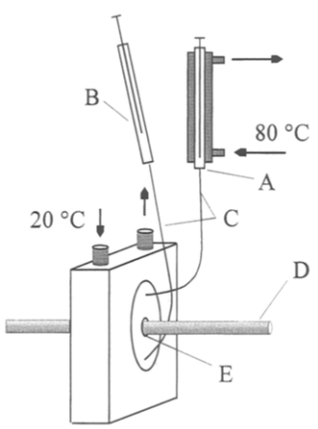

Fig. 1. Experimental set-up of the cell-device used for the temperature-jump experiments. (A) Thermostatted injecting syringe. (B) Collecting syringe. (C) Steel capillaries. (D) IR beam. (E) CaF_2 optical window.

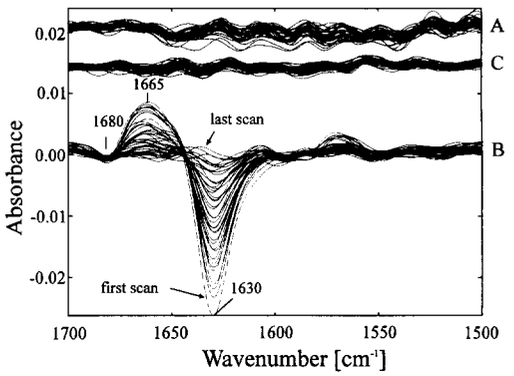

Fig. 2. Typical difference spectra obtained from measurements during an injection experiment. (A) Control experiment 1: buffer injection. Syringe temperature: 80°C, cuvette temperature: 20°C. (B) Injection of the RNase A solution: syringe temperature 80°C; cuvette temperature: 20°C. (C) Control experiment 2: injection of protein solution. Syringe temperature: 20°C, cuvette temperature: 20°C. The difference spectra werte calculated according to $\lg(S_n/S_l)$, where S_n is the single channel spectrum of the n^{th} scan and S_l is the single-channel spectrum of the last scan of an injection experiment.

be attributed to developing secondary structure elements. Decreasing positive peaks (maxima) correspond to decreasing band intensities in the absorbance spectra and, consequently, indicate disappearing structures.

Fig. 2C shows a series of spectra from a second control experiment in which an RNase A solution was injected isothermally, which proves that the spectral changes observed after the T-jump (see Fig. 2B) reflect conformational changes in RNase A during its refolding.

For a better interpretation of the spectra given in Fig. 2B the infrared spectra of RNase A were also measured while the temperature was lowered from 80°C to 20°C along a linear temperature gradient of 0.5 degrees/min. Two different sets of difference spectra were calculated from such experiments (see Fig. 3). Fig. 3A shows spectra which were obtained by directly calculating the differences between the absorbance spectra of RNase A measured at different temperatures. The strong shifts of the background reflect the temperature-induced changes of the buffer spectra. These effects could be eliminated when the spectra of the protein solution were corrected for the spectral contributions of the buffer solution at the corresponding temperatures (Fig. 3B). Interestingly, the negative and positive peaks of the difference spectra in Fig. 3B are located at similar wavenumbers such as those observed after the T-jump experiments (Fig. 2B). The absolute changes in intensity, however, are significantly lower in the latter case.

The FTIR difference spectra obtained from the gradient experiment also render intelligible why little or no information can be obtained on the development of the α-helices by the kinetic experiments: The small fraction of α-helices present in *RNase A* does not give sufficient peak intensity changes to be detected during folding by FTIR difference spectroscopic techniques. Moreover, the detection of conformational changes of the α-helices may be complicated by strongly overlapping IR bands usually assigned to α-helical and irregular structures.

In order to estimate the amount of structural species which could be detected within the given time-window of the injection experiment, we normalised the area of the negative difference

peak at 1630 cm^{-1} of both experiments to the integral peak intensity of the amide I band taken from spectra of the completely refolded protein. From this simple approximation, the percentage of refolding species during the injection experiment was estimated to be of the order of 20%. In other words, already 80% of the corresponding structures are refolded during the dead-time of the experiment.

It is well known from steady-state experiments that FTIR spectroscopy is particularly sensitive to the presence of and to changes in β-strands of proteins [20,21]. As demonstrated herein for the first time, FTIR spectroscopy also allows the kinetics of formation of β-structure in proteins to be followed. The negative difference bands observed at 1630 cm^{-1} (strong) and at 1680 cm^{-1} (weak) can be assigned to the anti-parallel β-pleated structures [22,23], while the broad positive band near 1667 cm^{-1} may result predominantly from overlapping of different irregular structures, usually observed in the range 1690–1640 cm^{-1}. The kinetics of the formation of β-structures in RNase A was examined in two different acquisition modes: (i) in the *Rapid Scan* mode which covered the time range of 0.17–7 s after the T-jump (Fig. 4A); and (ii) in the normal scan mode which covered the time range of 0.6–160 s (Fig. 4B). Fig. 4B shows that the spectral changes are apparently completed after about 60 s. The temporal intensity change of the amide I band component at 1630 cm^{-1} suggests first-order kinetics with a velocity constant of about 0.2 1/s. The velocity constant estimated herein is almost one order of magnitude lower than those obtained from T-jump experiments between 45 and 29°C (with a dead-time of 6 s) at extreme pH conditions (pH 1.2) of the RNase A solution [16]. By analysing the time behaviour of the NMR signals related to the amino acid residues His-48 and His-119, which are both localized in β-sheet regions of the folded RNase A velocity constants of approximately 0.03 1/s were estimated. Most likely, the extreme pH conditions are causative for these lower velocity constants.

Though a vast amount of data has been collected on RNase A folding [6,7,11–13,16,18,19,24], it is difficult to compare them with our results. Most of related publications focus on the late,

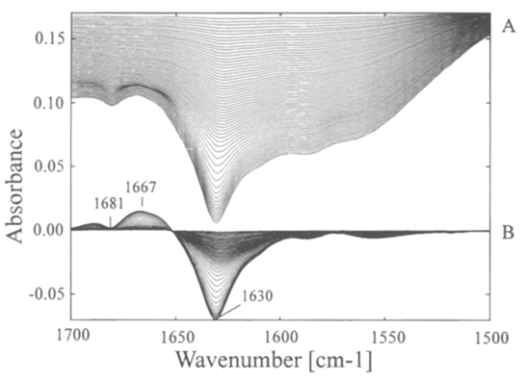

Fig. 3. Difference spectra obtained from a linear temperature gradient run from 80°C to 20°C. (A) Difference spectra obtained from the uncorrected absorbance spectra of RNase A in D_2O buffer. (B) Difference spectra obtained from the absorbance spectra of RNase A in D_2O buffer after subtraction of the buffer spectra at the corresponding temperatures. The spectra were calculated according to $A_t - A_{20°C}$, where A_t are the absorbance spectra recorded at temperatures 80°C > t > 20°C and $A_{20°C}$ is the last absorbance spectrum recorded at 20°C. One averaged spectrum was obtained per one degree temperature change.

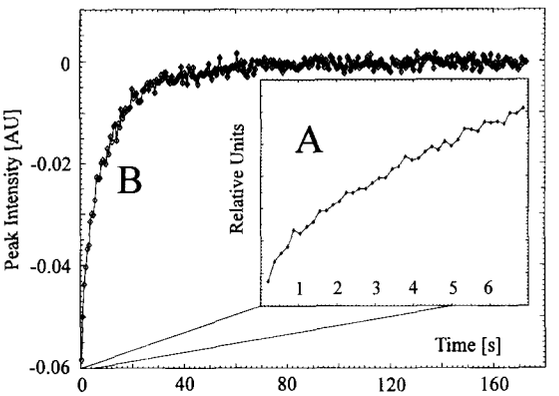

Fig. 4. Time dependence of the intensity of the difference peak at 1630 cm^{-1} (β-structure, see Fig. 2A). (A) Recorded in the rapid scan mode. (B) Recorded during a 'normal' scan mode.

rate limiting events of folding, especially on the proline-isomerisation reaction and in most cases experimental techniques were used which gave significantly longer dead-times and lower time-resolutions. Moreover, both the unfolding and the folding conditions in these experiments differ substantially from our T-jump experiments by the presence of chaotropic reagents such as guanidinium chloride, urea, and $LiClO_4$. or by extreme pH conditions.

In addition, only a few authors paid explicitly attention to the processes of secondary structure formation in RNase A. An exception are pulse hydrogen exchange (^2H–^1H) experiments [11] which indicated that all hydrogen bonds localised in β-sheet regions of RNase A are formed in the early folding intermediate I_1 and which suggested that the β-sheet conformations are formed rapidly and cooperatively. These interpretations are consistent with our IR-data, which indicate the synchronous formation of β-structures in RNase A by the nearly identical difference spectra detected for both, the gradient and the T-jump experiments performed essentially under thermodynamic and kinetic control, respectively. NMR data recently published [16] were also taken to support the notion that the folding of RNase A at low pH is a cooperative process envolving all regions of the protein molecule. The similarity between the first FTIR difference spectra of the kinetic experiment and the difference spectra obtained from the folding of RNase A under thermodynamic control refers to the fact that a substantial amount of protein molecules with conformations similar to that of the heat-denatured RNase A is still present during the first seconds of refolding. Indications for folding intermediates of RNase A similar to that of the heat-denatured protein were also obtained by other authors using continuous recycled flow NMR spectroscopy [18].

4. Conclusions

FTIR difference spectroscopy has been used for the first time to investigate protein refolding kinetics triggered by a fast temperature change of 60°C with a dead-time of ~170 ms. By its specific time-window, the new method permits a view on processes of protein folding termed 'early' and 'middle' events [25]. The early and middle events occur between the very earliest reactions, during which in the millisecond range substantial secondary structure appears and the rate-limiting steps in fold-

ing, the so-called 'late' events. The late events finally adjust the tertiary structure and lead to the fully native conformation. The proline-isomerisation is stated to be such an important rate-limiting step of RNase A refolding [5–7].

The processes which are detected by the FTIR technique certainly belong to those early and middle events. From the spectral changes it is evident that in this time range a distinct residual amount of β-sheet structures are formed in RNase A. This indication is important since RNase A contains a high portion of β-structures [26]. The time resolution of the FTIR instrument and the dead-time of the injection technique have not allowed the recording of spectral changes related to earliest events of the protein folding yet. Furthermore, from the present IR data it is not possible to comment on the course and time constants of the α-helix formation in RNase A. It is conceivable, however, that future improvement of the sensitivity of the FTIR instrumention, will also allow evaluation of some amino acid side-chain bands to follow specifically changes in the miocroenviroments of the related groups during the folding process.

The fact that FTIR spectroscopy, as demonstrated herein, allows the detection of the formation of β-structures cannot be underestimated. Although β-sheets are important structural elements, little is known about how a β-sheet in proteins folds. The information on the formation of β-structure is derived almost exclusively in an indirect way, that means by analysing the protection of backbone amide deuterons from exchange with solvent protons as a function of folding time [11,12,27,28]. Infrared spectroscopy as a direct probe for characterising the kinetics of β-structure formation during folding is now at disposal and will complement the NMR and circular dichroism-based techniques with their specific advantages.

Acknowledgements: We thank Klaus Gast, Diane Reinstädler, and Wolfram Saenger for fruitful discussions. This work has been supported by the Deutsche Forschungsgemeinschaft (Grant Na 226/3-1).

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