Development of temperature-jump NMR spectroscopy and its application to the protein folding study

川上, 勝

(Degree)
博士（理学）

(Date of Degree)
1999-03-31

(Date of Publication)
2008-06-23

(Resource Type)
doctoral thesis

(Report Number)
甲1916

(JaLCDOI)
https://doi.org/10.11501/3156317

(URL)
https://hdl.handle.net/20.500.14094/D1001916

博士論文

Development of temperature-jump NMR spectroscopy
and its application to the protein folding study

(温度ジャンプNMR法の開発と
そのタンパク質フォールディングへの応用)

平成１１年１月

神戸大学大学院自然科学研究科

川上　勝

Contents

Chapter 3. Application to the protein folding study

Chapter 1. General Introduction

1-1. Kinetic nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is based on the phenomenon that nuclear spins in a magnetic field absorb electromagnetic radiation, usually in the radiofrequency region. The frequency of absorption by a particular spin is dependent not only on the externally applied magnetic field, but also on the local field determined by the chemical and structural environment of the nucleus in question. Thus, in principle, each nucleus in a molecule has a frequency of absorption characteristic of its environment. This fact, combined with two-dimensional techniques, makes it possible to attain extreme high resolution in NMR spectroscopy that enables to differentiate all individual environments of atoms even in a complex molecule such as a protein.

From NMR spectra, information on chemical structure, conformation and dynamics is obtained. Furthermore, the pulsed Fourier transform technique and the multidimensional NMR concept have provided an explosive development of NMR spectroscopy. As a result, NMR spectroscopy is now a powerful technique for studying structures and dynamics of molecules. Currently, NMR spectroscopy and X-ray crystallography are the only techniques that can examine structures of macromolecules at atomic resolution.

Application of NMR spectroscopy, however, has been limited usually to a system in thermodynamic equilibrium. Kinetic application of NMR spectroscopy for a system in a nonequilibrium state has been rather limited. Although NMR is capable of studying rate processes even in a system in thermal equilibrium,[1,2] observation of transient species appearing in the process of fast reactions requires a combination of NMR spectroscopy with a suitable jump technique.

Concentration-jump or stopped-flow NMR was first developed by Grimaldi and Sykes,[3] and has been used for enzymatic reactions and metal ligand interactions as well as macromolecular reactions such as protein folding. Recently, new evidence for early folding and unfolding intermediates was reported with this technique,[4-9] and showed the potential of this kinetic NMR method. However, because of the irreversibility of mixing procedure, stopped-flow NMR is not suited for signal accumulation with repeated reactions, except for slow reactions for which signal accumulations may be made during a single reaction.

Temperature and pressure are the external parameters that may change thermodynamic equilibrium in a reversible manner. Temperature- or pressure-jump cycle can be repeated for the same sample, as long as the reaction is reversible. If such a cycle is incorporated into an NMR pulse sequence and the signal is acquired synchronized with the jump, accumulation of kinetic (or transient) NMR signals is possible by repeating the cycle. It is also possible to conceive a two-dimensional correlation spectrum between two phases

of different temperature or pressure. Although a construction of a pressure-jump NMR apparatus was initiated by Yamada et al.,[10] it has not been completed for regular use. In the temperature-jump (T-jump) spectroscopy developed by Eigen et al., the jump was achieved by an electric discharge through an aqueous solution made conductive by the addition of ions. Laser is also used for T-jump in recent studies.[11-14] These methods are suited for monitoring extremely fast reactions (in picosec~nanosec range), which, unfortunately, is outside the range of NMR spectroscopy. A first T-jump NMR experiment was reported by Blum et al.[15] in which a conventional NMR system was used and the dead time of T-jump was nearly a minute. In 1988, Adler and Scheraga[16] introduced continuous recycled flow (CRF) NMR in which a protein solution is heated by heat conduction from a thermal bath. Using a gas flow, Akasaka and co-workers[17] developed a T-jump NMR apparatus. Both of these techniques require at least several seconds for a T-jump by 10°-20° and are applicable only for relatively slow reactions. A laser T-jump NMR was also introduced,[18,19] but it has been used for solid materials, and has not become a widely applicable technique to liquids.

1-2. Temperature-jump NMR by microwave heating

Microwave heating using a high-power magnetron was introduced for T-jump ^{1}H NMR in our laboratory. The microwave TJ NMR device has gained success for liquid crystals,[20] but for aqueous solutions, it has gained only limited success because of its limited sensitivity, significant temperature gradient after the jump and interference between the microwave and radiowave coils.

A primary objective of my Ph.D. work was to develop a microwave T-jump NMR apparatus with tolerable sensitivity, less temperature gradient after the jump and no interference between the radiowave and microwave coils. These problems were resolved by introducing a nonconducting dielectric resonator as a microwave mediator to the sample solution and by introducing a mechanical sample mixing apparatus. Details of the design and the performance of the system are described in chapter 2.

1-3. Application of microwave temperature-jump NMR to the protein folding study

A protein molecule is composed of a polypeptide chain in which amino acids are connected by peptide bonds. Under physiological conditions, the polypeptide chain folds into a unique three-dimensional structure (or a tertiary structure); hence it gains its biological activity. At high temperature or at extreme acid or alkaline condition, the tertiary structure is destroyed and the activity is lost. Until the first half of the twentieth century, it was commonly believed that if once a protein is denatured, it can not regain its biological activity. This perception was, however, overturned by the Anfinsen's discovery of the spontaneous renaturation of reduced and unfolded ribonuclease A.[21] After Anfinsen's finding of reversibility of protein denaturation, various efforts have been made to understand how a protein folds into its characteristic three-dimensional structure and to know the relationship between the amino acid sequence and the three-dimensional structure of the protein. Protein folding is now one of the major problems in life science as well as in biomedical and other applied research.[22-25]

To understand protein folding, structural information about intermediates and the transition state in the folding pathway is required. NMR is an only spectroscopic method in solution that is capable of giving such information at atomic resolution. When

combined with a jump technique, NMR is potentially capable of providing structural information at atomic resolution on intermediates in the folding and unfolding pathway. The number of reports using NMR to follow folding and unfolding reactions is increasing. However, in these studies, unfolding is initiated by T-jump[15-17] or concentration-jump of a denaturant,[4-8] in which the dead time is several seconds or more. By introducing the microwave T-jump technique, direct observation of faster folding and unfolding reactions (within 1s) is expected.

Recently, the study of denatured and partially folded states is receiving considerable attention. It is considered that a residual structure in the denatured state has a resemblance to that of a folding intermediate or to the structure of a chain folding initiation site.[26,27] Therefore it is expected that the structural information on the denatured and partially folded states provide critical clues for solving the mechanism of protein folding.[28-31] A large number of studies have been performed on the denatured and partially folded states, but the information on their structure has been limited to the macroscopic level. Microscopic details of the structures of the denatured and partially folded states are expected to be obtained by NMR spectroscopy. For the last decade, progress in NMR techniques has enabled the characterization of these states by hydrogen exchange,[32] magnetization transfer experiment[33] and heteronuclear multidimensional NMR.[34,35] In our laboratory, the State-Correlated two-dimensional (SC-2D) NMR

spectroscopy in which a T-jump event is included into the two-dimensional NMR pulse sequence has been developed.[36] This technique is capable of assigning NMR signals of unfolded species or transient unfolding species. In chapter 3, direct NMR observation of protein unfolding processes is reported. In addition, transient unfolding species are detected, and their structures are characterized based on SC-2D experiments.

1-4. References

1. H. S. Gutowsky, H. S., D. M. McCall, and C. P. Slichter, J. Chem. Phys. 21, 279 (1953).

2. G. S. Huang and T. G. Oas, *Proc. Natl. Acad. Sci. USA* 92, 6878 (1995).

3. J. J. Grimaldi and B. D. Sykes, *Rev. Sci. Instrum.* 46, 1201 (1975).

4. S. D. Hoeltzli and C. Frieden, *Proc. Natl. Acad. Sci. USA* 92, 9318 (1995).

5. J. Balbach et al., *Nature Struct. Biol.* 2, 865 (1995).

6. T. Kiefhaber, A. M. Labhardt, and R. L. Baldwin, *Nature (London)* 375, 513 (1995).

7. J. Balbach et al., *Science* 274, 1161 (1996).

8. X. Y. Liu, D. L. Siegel, P. Fan, B. Brodsky, and J. Baum, *Biochemistry* 35, 4306 (1996).

9. J. Baum and B. Brodsky, *Folding Design* 2, R53 (1997).

10. Yamada et al., 35th NMR meeting (Kyoto) P74 (1996).

11. C. M. Phillips, Y. Mizutani, and R. M. Hochstrasser, *Proc. Natl. Acad. Sci. USA* 92, 7292 (1995).

12. R. M. Ballew, J. Sabelko, and M. Gruebeke, *Proc. Natl. Acad. Sci. USA* 93, 5759 (1996).

13. S. Williams, T. P. Causgrove, R. Gilmashin, K. S. Fang, R. H. Callender, W. H. Woodruff, and R. B. Dyer, *Biochemistry* 35, 691 (1996).

14. V. Munoz, P. A. Thompson, J. Hofrichter, and W. A. Eaton, *Nature (London)* 390, 196 (1997).

15. A. D. Blum, S. H. Smallcombe, and R. L. Baldwin, *J. Mol. Biol.* 118, 305 (1978).

16. M. Adler and H. A. Scheraga, *Biochemistry* 27, 2471 (1988).

17. K. Akasaka, and A. Naito, *Rev. Sci. Instrum.* 61, 66 (1991).

18. D. B. Ferguson, T. R. Krawietz, and J. F. Haw, *J. Magn. Reson.* Ser. A 109, 273 (1994).

19. T. Mildner, H. Ernst, D. Freude, and W. F. Holderich, *J. Am. Chem. Soc.* 119, 4258 (1996).

20. K. Akasaka et al., *J. Phys. Chem.* 99, 9523 (1995).

21. C. B. Anfinsen, *Science* 181, 223 (1973).

22. T. E. Creighton, (ed.) Protein Folding, Freeman, New York (1992).

23. C. K. Woodward, *Curr. Opin. Struct. Biol.* 4, 112 (1994).

24. J. T. J. Lecomte, and C. R. Matthews, *Protein Eng.* 6, 1 (1993).

25. H. Frauenfelder, and P. G. Wolynes, *Physics Today* 2, 58 (1994).

26. P. E. Wright, H. J. Dyson, and R. A. Lerner, *Biochemistry*, 27, 7167 (1998).

27. J. Moult, and R. Under, *Biochemistry*, 11, 150 (1991).

28. P. Kim, and R. L. Baldwin, *Annu. Rev. Biochem.* 59 631 (1990).

29. T. E. Creighton, *Curr. Biol.* 1, 8 (1991).

30. C. M. Dobson, *Curr. Opin. Struct. Biol.* 1, 22 (1991).

31. C. M. Dobson, *Curr. Opin. Struct. Biol.* 2, 6 (1992).

32. C. M. Dobson, P. A. Evans, and K. L. Williamson, *FEBS Letters*, 168, 331 (1984).

33. P. A. Evans, K. D. Topping, D. N. Woolfson, and C. M. Dobson, *Proteins: Struct. Funct. Genet.* 9, 248 (1991).

34. D. Neri, G. Wider, and K. Wuthrich, *Proc. Natl. Acad. Sci. USA* 89, 4397 (1992).

35. D. Neri, M. Billeter, G. Wider, and K. Wuthrich, *Science*, 257, 1559 (1992).

36. A. Naito, H. Nakatani, M. Imanari, and K. Akasaka, *J. Magn. Reson.* 87, 429 (1990).

Chapter 2. Development of the Temperature-jump NMR apparatus

2-1. Introduction

In 1990, microwave heating using a high-power magnetron was introduced for T-jump NMR,[1] and the method was used to develop two-dimensional NMR spectroscopy using phase transition, i.e., state-correlated 2D NMR spectroscopy for liquid crystals[2-4] and proteins in solution.[5] Initially, the TJ NMR technique employed a simple one-turn coil for microwave irradiation.[1] This device has gained success for liquid crystals, but for aqueous solutions, it has gained only limited success because of its limited sensitivity, significant temperature gradient after the jump, and interference between the microwave and radiowave coils. These drawbacks are expected to be greatly improved by introducing a cylindrically shaped, nonconducting dielectric resonator as a microwave mediator to the sample solution in place of the copper wired antenna. A dielectric resonator has recently been introduced successfully to pulsed electron paramagnetic resonance (EPR) spectroscopy.[6,7] In this article, the basic design of the microwave T-jump NMR apparatus utilizing a dielectric resonator is described, together with typical pulse sequences (both microwave and radiowave) for one dimensional (1D) TJ NMR experiments. The actual performance of the system is demonstrated in an example of the T-jump unfolding reaction of ribonuclease A.

2-2. The microwave temperature-jump NMR system

The entire set-up of the microwave TJ NMR spectrometer system is shown in Fig. 2.1. As a basic NMR spectrometer, a Lambda 400 high resolution NMR spectrometer (JEOL, Tokyo) was used with a 9.4 T super conducting magnet with a bore size of 54 mm (Oxford Instrument, Oxford), although other spectrometers may also be used. To cause a T-jump in the NMR probe, a pulsed microwave generator specially designed for this experiment (manufactured by IDX Corporation, Tokyo) was employed. The generator utilizes a 1.3 kW magnetron (Toshiba) as the microwave source at a frequency of 2.46 GHz (S band) corresponding to a wavelength of 12.2 cm. This type of magnetron has been used commercially for a microwave oven and is easily available. To avoid interference with the magnetic field of the superconducting magnet, the microwave generator is placed at a distance (~2.5 m) from the magnet. A dc pulse of 5 V output from the input/output board of the spectrometer opens the gate of the microwave generator, thus allowing microwave pulses to be incorporated into TJ NMR pulse sequences. Then, the microwave travels in the waveguide, and is sent to the bottom part of the probe *via* a semirigid coaxial cable (8 mm outer diameter). The coupling between the waveguide and the coaxial cable is assured by adjusting the position of the microwave terminator at the end of the waveguide. Within the NMR probe body, another semi rigid coaxial cable (3 mm

outer diameter) brings the microwave to a coupling loop, which works as an antenna to couple the microwave very efficiently to the dielectric resonator.

2-3. The temperature-jump NMR Probehead

Fig. 2.2 shows the design of the T-jump NMR probehead. The probe was constructed by modification of a commercial NMR probe supplied from JEOL (Tokyo). The radio-frequency circuit for the deuterium field-frequency lock and the temperature control unit were not modified from the original ones.

A. Dielectric resonator.

As a microwave power mediator to the sample solution, we introduced a slotted-tube dielectric resonator [16 mm height, 22 mm outer diameter, 11 mm inner diameter; Fig. 2.2, (b)], designed for this purpose and manufactured by Murata Seisakusho Co. (Kyoto). A major ingredient of this resonator is a ceramic with a high dielectric constant of 37.4 ± 0.5. The dielectric resonator is supported by a glass tube (10 mm outer diameter) with a soft Teflon tape as filling [Fig. 2.2, (a)].

B. Microwave coupling loop.

Within the NMR probe body, the end part of the semirigid coaxial cable (3 mm outer diameter) is made into a loop [13 mm in diameter, Fig. 2.2(c)], which works as an antenna to couple the microwave to the dielectric resonator. The distance between the coupling loop and the bottom of the dielectric resonator is approximately 2 mm. The resonance frequency of the dielectric resonator is sensitive to this distance, so that the distance can be changed for minute adjustment of the microwave tuning with the aid of a microwave tuning unit (network analyzer 8753D, Hewlett Packard).

C. Radiofrequency coil.

The radiofrequency coil is hand-made from a copper foil of 0.1 mm thickness and is gold plated. The coil consists of a two-turn saddle, and is fixed on a 6 mm outer diameter glass tube with a fluorocarbon polymer sheet as insulator [Fig. 2.2(a)]. The glass tube is centered by Teflon rings at both ends. To avoid an unfavorable interference between the radio-frequency coil and the resonator, the top and bottom parts of the radio-frequency coil (20 mm in height) are placed outside the cavity space of the dielectric resonator (16 mm in height).

2-4. The mechanical mixing device

The mixing device shown in Fig. 2.3 was introduced to reduce the temperature gradient across the sample volume immediately after the T-jump. The sample tube (Shigemi, Tokyo) consists of two parts; a thin-walled outer glass tube (5 mm in outer diameter) and a long and slender piston-like inner glass stick. The sample solution of about 100 μl is contained in the confined area of the outer glass tube, 6 mm in height, between the bottom part of the outer glass tube and the end part of the inner glass tube, and is placed in the center of the radio-frequency coil. The mixer is designed to hold both tubes separately and to rotate only the inner stick. For an effective stirring, a screw is attached to the inner stick. The screw is made of glass, which has a similar magnetic susceptibility to that of deuterium oxide. The glass screw, 1.5 mm outer diameter and 6 mm height, is carved with a groove, 0.3 mm in depth (see the blow-up in Fig. 2.3, right-hand side). The inner stick is linked indirectly to the motor through a loose cog to avoid conduction of unfavorable vibrations from the dc motor. The shaft is centered by nonmagnetic ball bearings which consist of a plastic box and glass balls. For measuring the rotation frequency, an optical tachometer is used that employs a laser light reflection from a piece of reflecting seal on the shaft. A rotation frequency of 100 Hz is usually employed.

2-5. TJ Pulse Sequences

Fig. 2.4(a) shows pulse sequences (radiowave and microwave pulses combined) suitable for one-dimensional T-jump NMR experiments. "noboru" (meaning "rise" in Japanese) is the simplest sequence for observation of fast reactions. However, since cooling air is flown within the probe all through the experiment, and since the temperature of the flow is set to the temperature before the jump, a few seconds after the jump the temperature of the sample begins to cool down.

For observation of a slower reaction, a pulse sequence "tamotsu" (meaning "keep" in Japanese) was developed [Fig. 2.4(b)]. In this pulse sequence, after the initial jump pulse, short microwave pulses are repeatedly applied at constant intervals to keep the temperature of the sample solution constant for a longer period of time. Since the experimental conditions are different for each experiment, optimal widths of the repetitive microwave pulse and intervals should be determined for each experiment. Besides these 1D pulse sequences, we also introduced a two-dimensional pulse sequence [state-correlated 2D (SC-2D)],[1] which was designed to obtain the correlation of NMR spectra between two physicochemically distinct states before and after a sudden T-jump. This sequence has gained success in separating local dipole couplings of protons in liquid crystals[17-19] and in demonstrating the possibility of assigning signals of an unfolding intermediate species

of a protein.[20]

2-6. Performance of the System

The actual performance of the system is now demonstrated in an experiment on a protein aqueous solution as shown below. A TJ NMR experiment was performed on ribonuclease A (10% in 99.96% ^2H_2O, pH 3.5) with the simple TJ pulse sequence (noboru) with a 20 ms microwave irradiation under mechanical mixing of the sample solution at 100 Hz. Changes of the NMR signals of the water proton and of the aromatic ring protons of ribonuclease A upon a T-jump from 55 °C are shown in Fig. 2.5(a). The temperature of the sample solution as well as the temperature distribution within the sample volume can be estimated directly from the peak position and the width of the water ($^1H^2HO$) resonance, respectively, as the chemical shift of water is quite sensitive to temperature, the up-field shift corresponding to the temperature increase with a slope of ~10 °C / 0.1 ppm. In Fig. 2.5(a), immediately after the jump, the mean position of the water signal is high field shifted by about 0.25 ppm, showing the immediate rise of the temperature of the sample solution by an average of 25 °C, but the broadness of the signal indicates the presence of a significant temperature distribution within the sample volume.

The mean temperature of the sample solution, together with the width of the temperature distribution, is plotted against time after the

jump in Fig. 2.5(b). The mean temperature was estimated from the mean chemical shift of the water proton ($^1H^2HO$) signal, by using the precalibrated temperature-chemical shift correlation. The temperature gradient immediately after the jump disappeared almost completely within 100 ms, thanks to the mechanical mixing of the sample solution. Without mixing, the temperature gradient persisted for about 500 ms.

In Fig. 2.5(a), the aromatic region of the ^1H NMR spectra of ribonuclease A is shown. The signal-to-noise ratio as well as the resolution is fairly satisfactory. Before the jump, the signals are well dispersed, characteristic of the folded structure of the protein. However, the spectrum immediately after the jump (second row) appears almost identical with that of the denatured conformer well thermalized after 500 ms above 75 °C. This observation clearly indicates that the tertiary structure of ribonuclease A is largely destroyed within 20 ms of the jump event. Detailed analysis of the spectrum immediately after the jump will be performed with the aid of the SC-2D pulse sequence in the following chapter.

2-7. Future Prospects

A rapid T-jump is important in NMR spectroscopy for at least two reasons. One is to carry out two-dimensional NMR spectroscopy, observing the spectral correlation between different temperatures, i.e.,

18

the state-correlated 2D NMR spectroscopy.[1,5] For a successful SC-2D experiment, we need to change the physico-chemical state of the sample within the spin-lattice relaxation time (which is on the order of a second for most proteins[10]), which is well realized in the present setup with 20 ms of the T-jump time. The other reason is to observe early events in thermally induced reactions directly by NMR. Especially in protein folding and unfolding studies, observation of intermediate species often requires detection in the time range of milliseconds to tens of milliseconds.[11,12]

For kinetic observation of such species, the current jump time, 20 ms, may not be sufficient and a faster jump in a millisecond range is desired. We believe that this is possible within the scope of the microwave T-jump technique utilizing a dielectric resonator. Our present system is capable of causing a T-jump of a 100 µl protein solution by as much as 20 °C within 20 ms. A simple calculation shows that in this case the nominal efficiency of energy absorption by the sample solution is only 30% of the 1.3 kW power of the magnetron. We must admit that our present set-up is not fully optimized yet with respect to the efficiency of energy conversion in various sections of the microwave circuit, including the wave guide-to-coaxial tube coupling and the loop-to-resonator coupling. This indicates that there is still a room for improvement of microwave irradiation efficiently within the present setup, possibly by about twofold. Furthermore, a simple introduction of a higher microwave power source commercially

available (e.g., 5 kW, IDX, Tokyo) in place of the current 1.3 kW magnetron would reduce the jump time by a factor of about 4, cutting the jump time well within 5 ms. Theoretically, if all conditions are optimized, a jump time of a couple of milliseconds is attainable.

The sensitivity of signal detection is another point that requires improvement for a wider application of this technique to proteins and other biopolymers. First, the Q value of the radiofrequency circuit will be increased by a more careful choice of the material used for the radiofrequency coil. Furthermore, a substantial increase in the sensitivity will be realized simply by the use of an NMR spectrometer working at a higher frequency, e.g., at 600-800 MHz. This will increase the signal-to-noise ratio substantially (by two- to three- fold) from the present level. If the same quality of signals that we obtain at present at 400 MHz are sufficient, we need one third of the present sample volume, and the T-jump time may be shortened by a factor of 2-3. Based on all the aforementioned estimates, construction of a microwave T-jump NMR spectrometer with 20 °C T-jump with a rise time within a millisecond appears to be feasible.

Finally, we may point out that the basic design of the currently proposed system utilizing a dielectric resonator as a microwave mediator is a useful general technique that can be applied to most other T-jump spectroscopies as well.
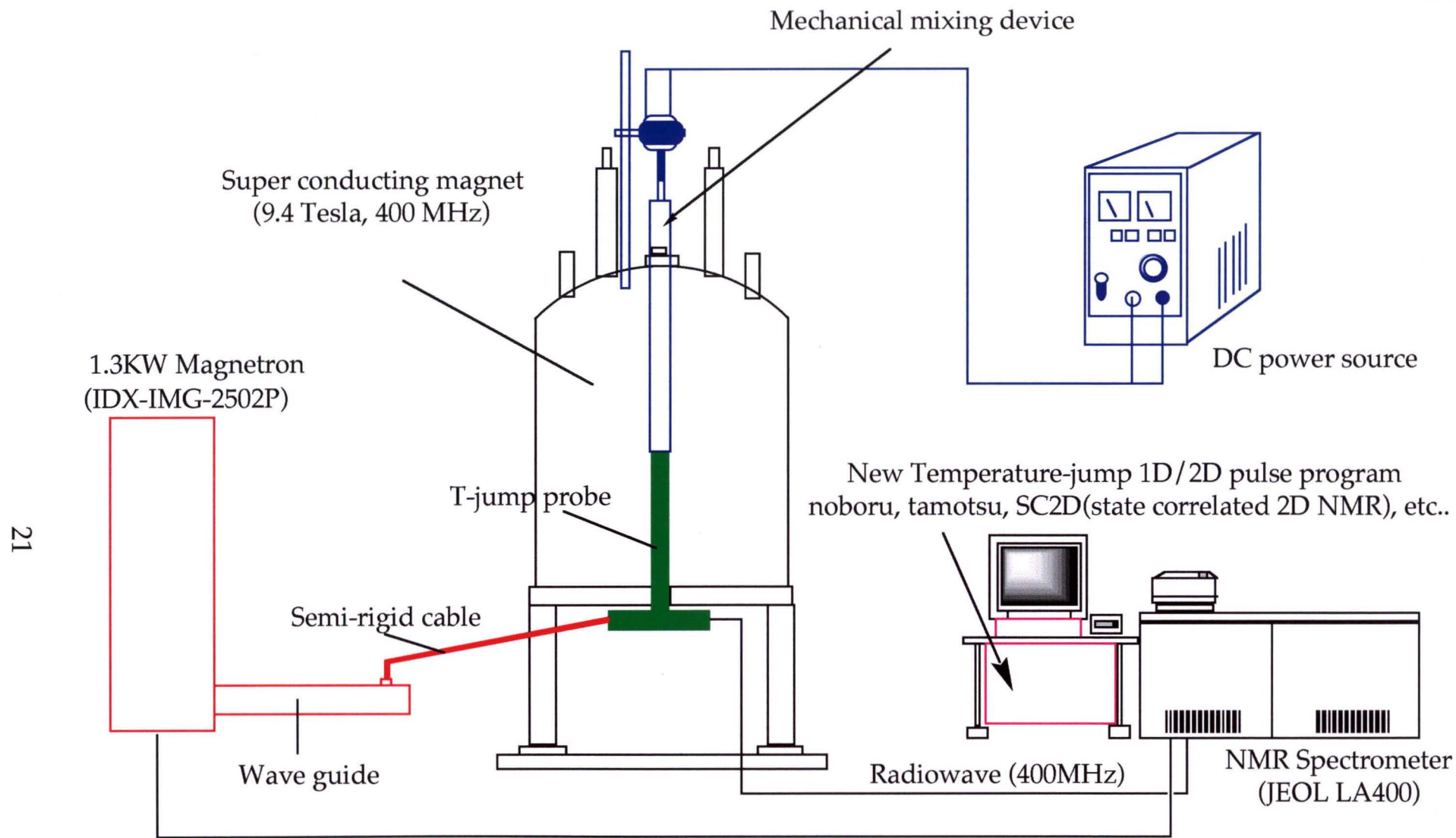
Fig.2.1. The microwave temperature jump NMR system.
An NMR spectrometer: LA-400 (JEOL, Tokyo) equipped with a super conducting magnet (9.4 T) with a bore size of 54 mm. A pulsed microwave generator: IMG-2520P (IDX Corporation, Tokyo) with a 1.3 kW Toshiba magnetron, the output of which is controlled by a pulse programmer of the NMR spectrometer. A mixing apparatus is inserted tightly into the bore of the magnet and connected with a dc motor (DN35-T151N1B, Canon, Tokyo).
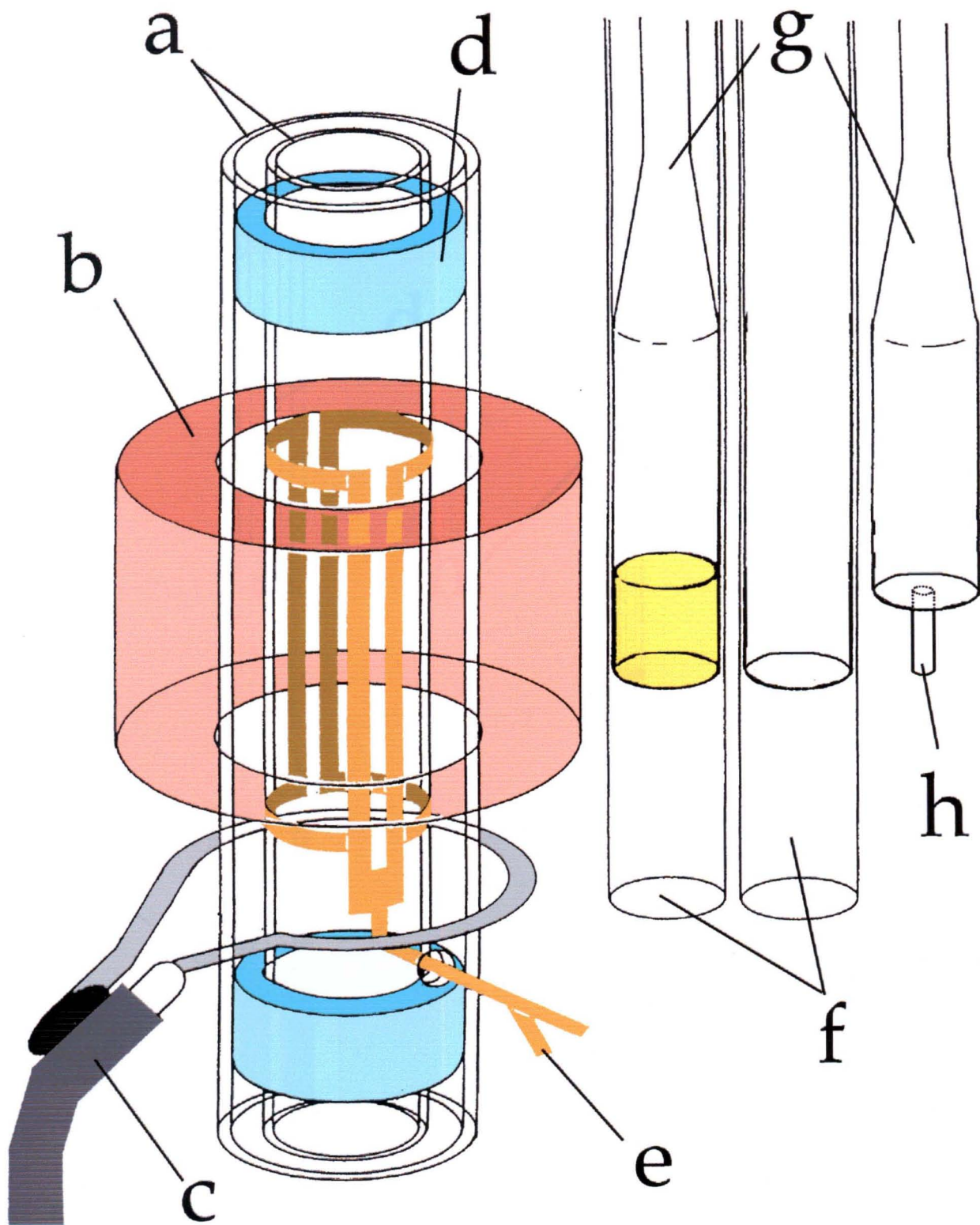
Fig. 2.2. The design of the probehead and the sample tube . For easy understanding, the resonator is drawn transparent and the sample tube is drawn to pieces (right). (a) glass tubes with 6 mm and 10 mm outer diameters, (b) dielectric resonator (MURATA products, Kyoto), (c) coupling loop, (d) spacer (fluorocarbon polymer), (e) radiofrequency coil, (f) outer tube, (g) inner tube, (h) glass screw (1.5 mm outer diameter).
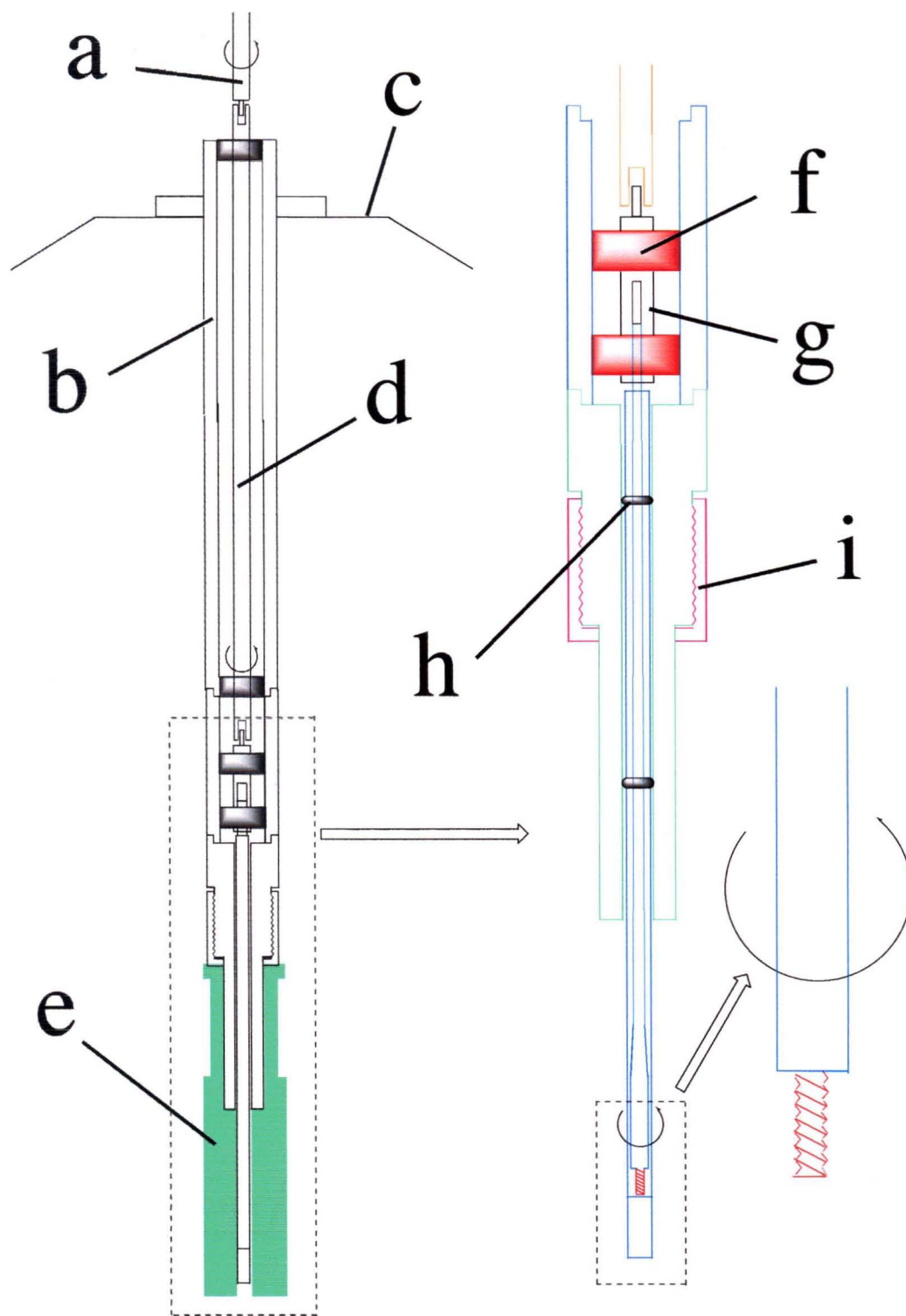
Fig. 2.3. Details of the mixing apparatus and the sample tube (modified from BMS-005J, Shigemi, Tokyo, Japan). The arrows indicate blow-ups of the parts enclosed by broken lines. (a) Rod, connected with a dc motor; (b) supporting body (vinyl plastic, 29 mm outer diameter); (c) the top of the magnet; (d) aluminum shaft; (e) the upper part of the NMR probe; (f) ball bearings (PB626, Shimizu-seiko, Osaka, Japan); (g) short shaft (fixed with ball bearings and holds the inner sample stick); (h) O ring (fixes the outer sample tube to the body); and (i) sample positional adjuster.
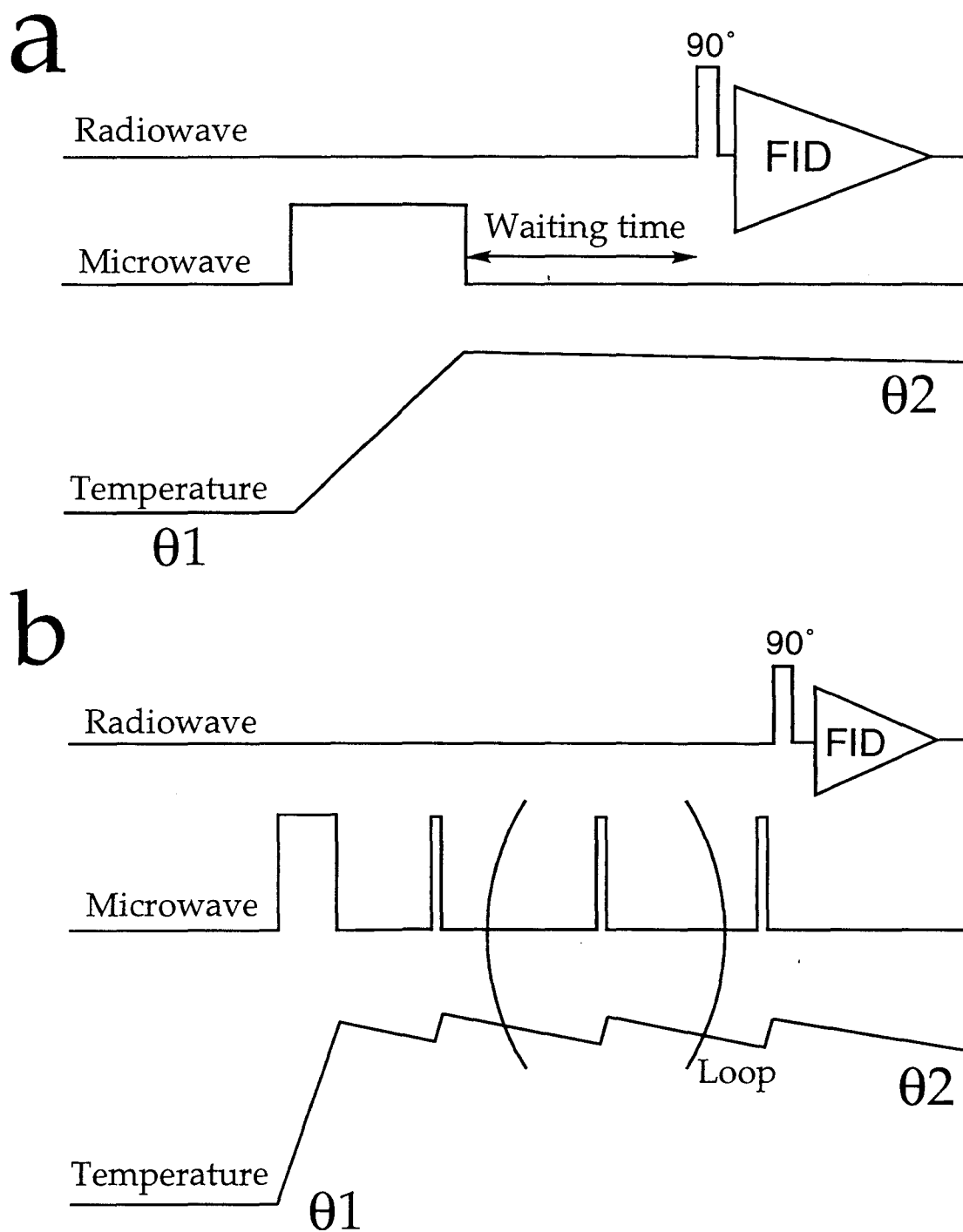
23

Fig. 2.4. Typical pulse sequences for temperature-jump NMR experiments. (a) The sequence Noboru. Before the jump, the sample solution is equilibrated at θ1. Upon irradiation of the microwave pulse, the temperature is jumped to θ2. After waiting, acquisition of free induction decay signals is executed. A repetitive delay of 180 s is allowed for sample cooling and equilibration at θ1. (b) The sequence Tamotsu. Microwave pulses are applied intermittently after the temperature-jump to keep the temperature after the jump as constant as possible for a longer period of time. For each new experiment, optimization of the length and frequency of intermittent microwave pulses must be achieved by monitoring the temperature of the sample.
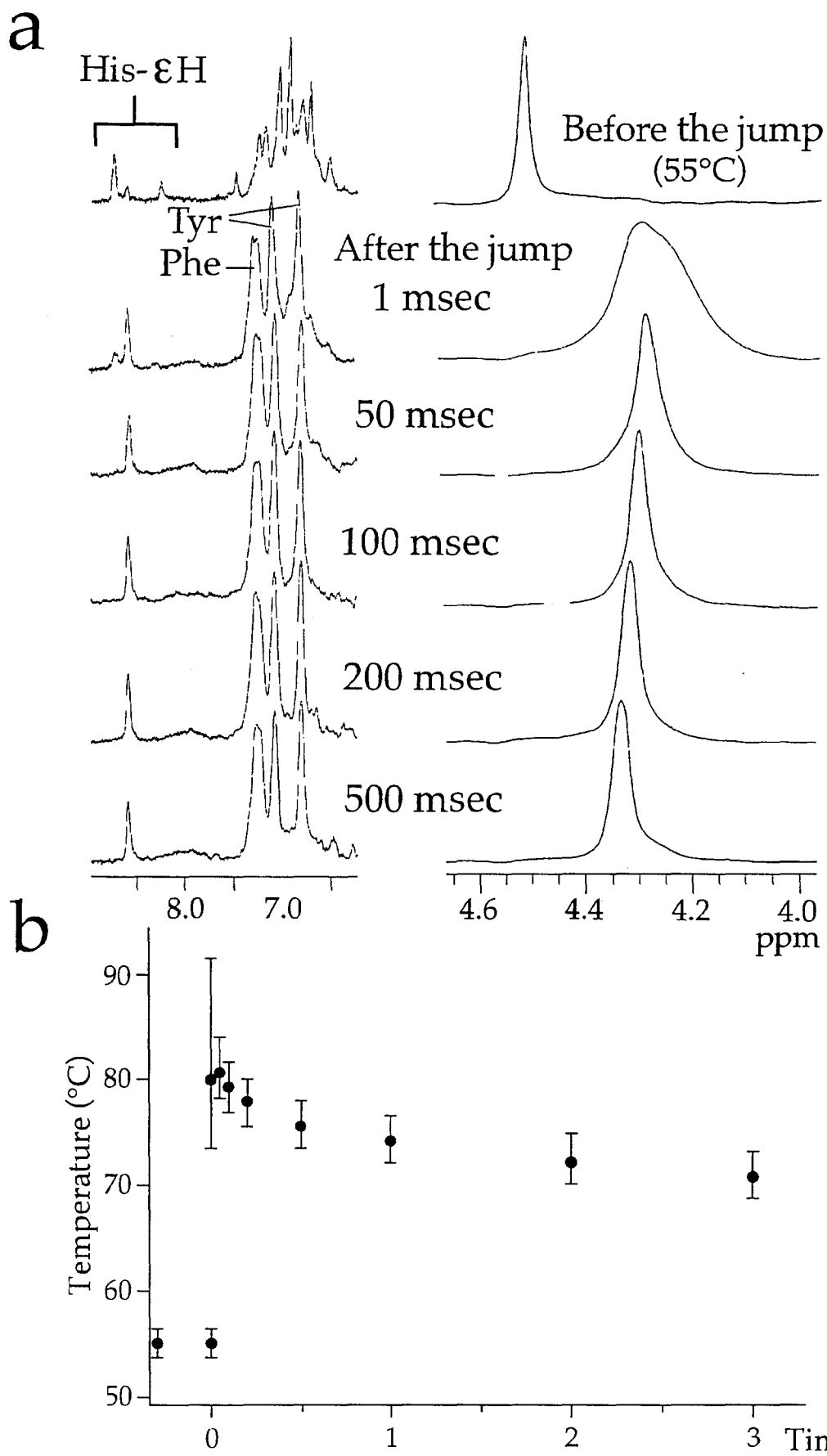
Fig. 2.5. (a) Proton NMR spectral changes of the ribonuclease A solution,
(b) Temperature variation of the sample solution against time sfter the jump
For details, see the next page.

Fig. 2.5. (a) Proton NMR spectral changes of the ribonuclease A solution (10% in 99.96% ^2H_2O, pH 3.5) upon temperature jump from 55 °C, measured at 400 MHz.

Right: the $^1H^2HO$ region. Left: the aromatic region of ribonuclease A. The experiment was performed under the rotation of the mixing screw at 100 Hz with the pulse sequence of Fig. 3(a). The temperature-jump was attained with a microwave pulse of 20 ms duration and the cooling was attained during the repetition time of 180 s. No field-frequency lock was used. Chemical shifts are referenced to the methyl signal of 3-trimethylsilyl propionate added as an internal reference.

(b) Temperature variation of the sample solution against time after the jump. The mean temperature was estimated from the mean chemical shift of the water proton ($^1H^2HO$) signal, based on the precalibrated temperature-chemical shift correlation. Likewise, the temperature distribution across the sample volume, as estimated from the half-intensity widths of the water signal, are shown with vertical bars.

2-8. References

1. A. Naito, H. Nakatani, M. Imanari, and K. Akasaka, *J. Magn. Reson.* 87, 429 (1990).

2. A. Naito, M. Imanari, and K. Akasaka, *J. Magn. Reson.* 92, 85 (1991).

3. K. Akasaka et al., *J. Phys. Chem.* 99, 9523 (1995).

4. A. Naito, M. Imanari, and K. Akasaka, *J. Chem. Phys.* 105, 4504 (1996).

5. K. Akasaka, A. Naito, and M. Imanari, *J. Am. Chem. Soc.* 113, 4688 (1991).

6. R. W. Dykstra and G. D. Markham, *J. Magn. Reson.* 69, 350 (1986).

7. W. M. Walsh and L. W. Rupp, *Rev. Sci. Instrum.* 57, 2278 (1986).

8. A. Naito, M. Imanari, and K. Akasaka, *J. Magn. Reson.* 92, 85 (1991).

9. K. Akasaka et al., *J. Phys. Chem.* 99, 9523 (1995).

10. R. Ishima, S. Shibata, and K. Akasaka, *J. Magn. Reson.* 91, 455 (1991).

11. H. Roder, G. A. Elove, and S. W. Englander, *Nature (London)* 335, 700 (1988).

12. J. B. Udgaonkar, and R. L. Baldwin, *Nature (London)* 335, 694 (1988).

Chapter 3. Application to the protein folding study

3-1. Bovine pancreatic ribonuclease A

3-1-1. Introduction

Structural information on folding intermediates is considered to be crucial for solving the protein-folding problem. However, since refolding starts from the unfolded protein whose conformation is poorly understood and since it is generally thought that the rate-limiting step in folding lies close to the native state, the transition state can be characterized by studying the unfolding reaction rather than by the folding reaction. So far, however, information available for the unfolding pathway is quite limited.

Ribonuclease A (RNase A) from bovine pancreas is one of the best-studied proteins for unfolding reactions, because of its small size, well-defined structure and extremely high reversibility of denaturation against pH, denaturant, heat and pressure. A number of kinetic studies, including the use of kinetic NMR, were made on the unfolding process of RNase A. However, the image of the unfolding process is rather diverse among scientists, as shown below.

Scheraga and his associates applied stopped-flow UV-absorption and fluorescence methods to the unfolding study of RNase A upon pH jump or the concentration jump of guanidinium hydrochloride.[1] A

very rapid unfolding phase (completed within 100 ms at room temperature) was detected, followed by two slower processes occurring in time ranges of seconds to hundreds of seconds. The two slower processes were attributed to the isomerization reaction of proline residues, while the very rapid unfolding phase was attributed to the transition from the native folded state to a fully denatured state before the proline isomerization takes place.

Kiefhaber et al. applied the ^{1}H-^{2}H exchange-pulse labeling method to monitor the hydrogen bonding state of individual amide protons with NMR in the process of unfolding of RNase A initiated by a concentration jump of guanidinium chloride.[2] It was found that most amide protons exchange at a common rate (~0.004 s^{-1}). The result was interpreted to indicate that the entire network of peptide hydrogen bonds is broken in a single rate-limiting step. Furthermore, by comparing the time change of the ^{1}H NMR spectrum with that of circular dichroism, the existence of an intermediate species with a lifetime as long as a few hundreds of seconds was suggested in the unfolding process of RNase A at 10 °C.[3] They attributed this intermediate species to a "dry molten-globule" state[4] in which the side chains are free to rotate, but are not yet hydrated.

Meanwhile, an ultrafast infrared absorption change (nanoseconds scale) was reported by Hochstrasser and his associates in the amide I region in a laser-pulsed T-jump unfolding study of RNase A [5]. The authors interpreted that water penetration into the buried β-sheet

regions has completed in this period.

Thus, opinions are diverse on the unfolding process of RNase A. Scheraga et al. found no intermediate state in the unfolding process except the proline isomers. Kiefhaber et al. consider a "dry molten-globule" as possible intermediate in the unfolding process. On the other hand, Hochstrasser et al. considers hydration is very rapid, maybe finished within nanoseconds. Although Kiefhaber et al. used the real-time NMR for their study, the mixing dead time of the apparatus was several tens of seconds. Therefore, any fast conformational change was not discussed. Moreover, probably because of the huge denaturant signal, the low-field side of the NMR spectrum was not used for analysis. Meanwhile, the pulse labeling experiment can extract information only about hydrogen-bonds of backbone amide groups, but no information is available on the packing state of the side-chains characteristic of tertiary structure.

In order to circumvent this situation, the microwave T-jump NMR method was applied for the unfolding process of RNase A. The side chain signals were followed as 1D NMR spectra in a time-sequential manner synchoronized with a T-jump. The unfolding process was also followed by 2D NMR measurement synchronized with a T-jump, i.e., SC-2D NMR, to characterize transient unfolding species.

3-1-2. Materials and Methods

Materials

Deuterium oxide was purchased from ISOTEC (Ohio, USA). Deuterium chloride and TSP (3-trimethyl-silyl-propionate-2,2′,3,3′-d4) were acquired from Merck (Darmstadt, Germany). Bovine pancreatic RNase A, types XII-A was obtained from Sigma (Tokyo, Japan) and used with no further purification.

Sample preparation.

Prior to the experiments, the labile amide protons of RNase A had been exchanged with deuterium atoms twice by incubation in 99.9 % ^2H_2O at pH 3.0, 70 °C for 15 minutes and lyophilization. The pH values cited are direct pH meter readings at room temperature without correlation for isotope effects. The lyophilized samples of RNase A were dissolved at a concentration of 100mg/ml in 99.96% ^2H_2O, an aliquot of a TSP solution being added as internal chemical shift reference. The final pH of the solution was adjusted to 3.5. After centrifugation, 120 µl of the solution was transferred into a sample-tube (BMS-005J, Shigemi, Tokyo, Japan).

One-dimensional Temperature-jump NMR experiment.

Before the T-jump, the sample solution was maintained at 50 °C where the protein molecules exist in the native state. Then the

31

temperature of the sample solution is jumped to the average temperature of 70 °C by the application of a microwave pulse. The temperature of the sample after the jump was estimated from the chemical shift of the $^1H^2HO$ signal of the solvent water, based on the precalibrated temperature-chemical shift correlation. For a rapid quenching of the temperature gradient across the sample volume after the jump, a glass-made screw is attached at the bottom of the inner tube of Shigemi (BMS-005J) and the inner tube is rotated at a rate of 100Hz. By this rotation, the temperature gradient within the sample solution is largely destroyed well within 100 msec. For each spectrum, eight scans were accumulated with 180 sec repetition. A spectral width of 6000 Hz was used with 8192 complex data points. After each T-jump, the sample solution was cooled by airflow for 3 minutes. Since the volume of sample is small (~120 μl), the cooling is complete in 3 min, during which the protein returns to its native state at 50 °C before the next jump starts. No field-frequency lock was used during the T-jump NMR measurement, but the magnetic field was sufficiently stable to allow a long time signal accumulation.

State-correlated two-dimensional NMR.

The SC-2D sequence[6] used in the present study is shown in Fig. 3.1.1. This pulse sequence resembles that of NOESY. The mixing period in NOESY is replaced by the T-jump period and the waiting period. In the evolution period, proton spin is labeled by its chemical shift in the

native state. A microwave pulse of typically 15-20 ms is applied and the temperature of sample solution is suddenly brought about from 50 °C to 70 °C. Unfolding reaction proceeds in the waiting period. By varying the waiting period, we can obtain auto-correlation spectrum of various protons in the protein between the native state before the jump and some unfolded state upon unfolding but after the waiting period. In the present study, correlation spectra were obtained between the native state and the state immediately after the jump (1 msec) and between the native state and the state at 200 msec after the jump. In each spectrum, spectral width and data points in the t1 axis were 6000 Hz and 1024, while they were 600 Hz and 32 complex points in the t2 axis. The t1 domain signals were zero-filled to 128 points prior to Fourier transformation. Eight scans were accumulated for each t1 value with 180 sec repetition for phase cycling, giving a total accumulation time of 33 h for one SC-2D spectrum. To minimize the temperature gradient within the sample solution, the sample tube was spun at 30 Hz by the commercial air-flow spinning device supplied by JEOL.

3-1-3. Results

Time sequence of events upon T-jump occurring on RNase A is shown in a series of 1D proton NMR spectra in Fig. 3.1.2. Before applying the microwave pulse, the spectrum (top) at pH 3.5, 50 °C is that of the native conformation showing well-dispersed chemical shifts characteristic of its tertiary structure. Within one millisecond after the 20 ms microwave pulse, the spectrum changed drastically into the one (second from the top) that appears almost identical with that of the thermally denatured state at equilibrium at 70 °C (bottom). The four C2 proton signals of four His residues merged into a singlet at 8.65 ppm, which is a standard chemical shift in a random-coiled polypeptide chain.[7] Likewise, proton signals of phenylalanine, tyrosine and histidine protons grouped into their specific chemical shift positions. The signals of high-field shifted methyl protons disappeared almost completely. Although some peaks remaining at the chemical shift in the native state were observed, we consider that most of these peaks are due to temperature gradient within the sample tube. At 50 msec after the jump when the temperature gradient almost disappeared, those residual "native" peaks apparently disappeared. No further prominent change was apparent in the spectrum at 100 and 200 ms after the jump.

The spectra observed after the jump were much simpler than that before the jump, since they are grouped into peaks of the same types of

protons of the same kind of amino acid residues. Assignment of signals to specific protons in the polypeptide chain cannot be made with conventional two-dimensional spectroscopic techniques. However, with a novel technique called state-correlated two-dimensional NMR Spectroscopy (SC-2D), a correlation spectrum is obtained between signals of individual protons that exist in two different states or environments related by rapid T-jump (much less than the spin-lattice relaxation time T1), i.e., between the N (native) state and the U (unfolded) state upon a T-jump.

Fig. 3.1.3a shows an SC-2D spectrum for the aromatic proton region of RNase A between the native state N (50 °C) and the state U immediately (1 msec) after the jump by more than 20 °C. Cross peaks are observed for each proton of four histidine, three phenylalanines, and six tyrosines of RNase A. We note that the signals which are well dispersed along the F1 axis in the native conformation are grouped into signals at positions nearly characteristic for a random coil along the F2 axis; 7.3-7.5 ppm for the C-2 protons of histidine, around 7.3 ppm for phenylalanine, 7.1 and 6.85 ppm for δ and ε protons of Tyrosine residues, respectively. Since the NMR signals have been assigned in the native state of RNase A,[8,9] we could assign individual proton signals in the unfolded state as well, *via* cross peaks connecting the two. Previously these assignments could be done only for histidine C2 protons using specific deuteration.[10]

One of the characteristic features of the SC-2D spectrum is that the

cross peaks appear only at one side of the diagonal line, because the magnetization is transferred unidirectionally from one state to the other, but not exchanged mutually as in conventional 2D exchange spectroscopy. If the transfer of magnetization is complete, there should be essentially no diagonal peaks, except for some accidental ones. In Fig. 3.1.3, however, we observe some diagonal peaks, e.g., ε proton of Tyr25 at 6.52 ppm and δ proton of His109 at 7.50 ppm. These peaks originate from residual native conformers that remained after the jump due to a significant temperature gradient within the sample volume. The same signals are also apparent in the spectrum right after the jump in 1D TJ experiment (Fig. 3.1.2). From the cross sectional spectra of ε protons of Tyr25 and δ protons of His109 along the F2 axis (Fig. 3.1.3a), the volumes of the remaining peaks were calculated, from which we estimate the percentage of the native species remaining 1msec after the jump to be about 20%.

Fig. 3.1.3b shows the correlation spectrum measured between the native state and the state at 200 msec after the jump; the volumes of the diagonal peaks are reduced, the percentage of which deduced from the volume of the ε proton signal of Tyr25 and the volume of the δ proton signals of His109 was decreased to 4%, while those of off-diagonal peaks increased. The above observation indicates that the temperature gradient within the sample volume disappeared in 200 ms time. Narrowing of the line width of the His-δ peaks in the F2 axis also shows the destruction of the temperature gradient in Fig. 3.1.3b.

Interestingly, a new signal appeared at F1: 6.93ppm and F2: 7.15ppm. The chemical shift value in the F1 axis shows that this signal belongs to the δ proton of Tyr92. That this new cross peak is not due to the temperature gradient is clear from the fact that, even after 200 msec, the percentage of the new peak is less than 50% of the total δ proton peak of Tyr92. The percentage should be 96% if the temperature gradient were the reason. Therefore further conformational change after the loss of chemical shift dispersion immediately after the jump is indicated. Accordingly, the possibility that the state immediately after the jump is a transient unfolding state is suggested. The origin of this new peak will be discussed in the following section.

3-1-4. Discussion

The tertiary structure is lost within 20 ms at 70 °C.

Immediately (1 ms) after the microwave pulse of duration 20 ms, the spectrum lost its characteristic feature of the native, folded state; the chemical shift dispersion in the native tertiary structure is lost almost completely. Besides, the SC-2D spectrum showed clearly that most aromatic proton peaks are grouped together in their random coil positions. The result indicates that the short-range order of the native tertiary structure (within several Å), responsible for the characteristic chemical shift dispersion of the folded state, is lost within 20 ms of the

37

T-jump event. Further change was not so apparent with time in the 1D TJ NMR spectrum measured at 70 °C in the equilibrium denatured state. This observation suggests that the magnetic environments around most side chains become similar to those of the thermally denatured protein.

From Fig. 3.1.3b, we recognize that the chemical shifts of His105 and His119 in the unfolded state are smaller than that of His12 and His48. Chemical shift of His residue is sensitive to a slight circumstantial difference.[18] However, for Phe and Tyr residues, no significant chemical shift deviation is observed. It is unlikely that a significant hydrophobic interaction exists in the state immediately after the jump. Therefore, within the jump event, the penetration of water molecules into the hydrophobic cluster or the hydration of the hydrophobic core, must be complete.

Pro-trapped transient species is detected.

Examination of the SC-2D spectra reveals some time dependence in part of the spectrum. The signal for the δ proton of Tyr-92 shifted close to its random-coiled position immediately after the jump. However, the peak showed further downfield shift with time after the jump. In the primary sequence of RNase A, Tyr92 precedes Pro93, which is conserved in all of the sequenced homologous RNase As.[11] The importance of this residue for the structural stability[12] and for the kinetics of folding/unfolding[13,14] has been pointed out. RNase A has four proline residues at positions 42, 93, 114, and 117. The Lys41-Pro42

and Val116-Pro117 peptide bonds are in *trans* conformation in the native state, while the Tyr92-Pro93 and Asn113-Pro114 peptide bonds are in 100% *cis* conformer.[15] When RNase A is unfolded, the four X-Pro peptide bonds are predominantly in the *trans* conformation in thermal equilibrium. Consequently, unfolded RNase A exists as a mixture of a number of unfolded conformers under equilibrium condition with respect to *cis-trans* isomerization of the four X-Pro bonds,[16,17] which complicates the folding kinetics.[18] The kinetics of folding was investigated with double-jump stopped-flow techniques [1,19] on samples with substitution for a proline residue.[14] In the study of unfolding of RNase A, the fast (millisecond range) and the slow (seconds to a hundred second range) phases were reported.[18,20] Recently, by the unfolding kinetics of RNase A with substitution for proline or Tyrosine residues, Juminaga et al. confirmed that the slow unfolding phase arises from the two cis-proline residues (e.g., Pro92 and Pro114) and evaluated the time constants and activation energies for the isomerization of Pro92 and Pro114 individually.[21] Using their kinetic data for P114A mutant (τ=105 s at 15 °C and E_a=21.2 kcal/mol), we estimated the time constants for the isomerization of the Tyr92-Pro93 peptide bond at 70 °C to be 290 msec. In addition, the percentages of the peptide bond in cis conformer in the unfolded state were estimated to be at least 60%.[22,23] Therefore, at least 33 % of the peptide bond should be in *trans* conformer in 200 msec after the jump. Judging from the cross sectional spectrum of SC-2D (Fig. 3.1.3b), the volume ratio

between the new peak and the peak of δ proton of Tyr92 seems to be close to this value (~33 %). Accordingly, we considered that the new peak appeared in SC-2D spectrum at 200 msec after the jump corresponds to the δ proton of Tyr92 of the species in which Tyr92-Pro93 peptide bond is in *trans* conformer. In view of the small chemical shift difference of the δ proton of Tyr92 between the transient species and the unfolded species, it is unlikely that any tertiary structure involving Tyr92 is present in the transient species. Although the same behavior is expected on the Tyr115 residue which follows the cis-proline residue (e.g., Pro114, whose time constant for isomerization at 70 °C is estimated to be 162 msec with the Juminaga's[21] kinetic data), the coincidence of chemical shifts between the native and unfolded states for Tyr115 prohibits a definite answer on this. On the other hand, the transient species detected here appears to correspond to Uvf or Uf, which was reported by Houry et al.[4] to be a denatured species lacking any native-like structure.

In conclusion, within 20 ms at 70 °C, the native structure of RNase A transforms into a transient species whose characteristic tertiary structure is almost fully lost and the side chains nearly fully hydrated, except for the cis conformation of the Tyr92-Pro93 peptide bond which gradually transforms into trans conformation with a time constant of approximately 300 ms. This result apparently contradict the dry-molten globule hypothesis of the early unfolding species by Kiefhaber et al.[3] However, the discrepancy between the two experiments may result

from different experimental conditions between the two experiments, and more experiments are needed to reach a unified conclusion.
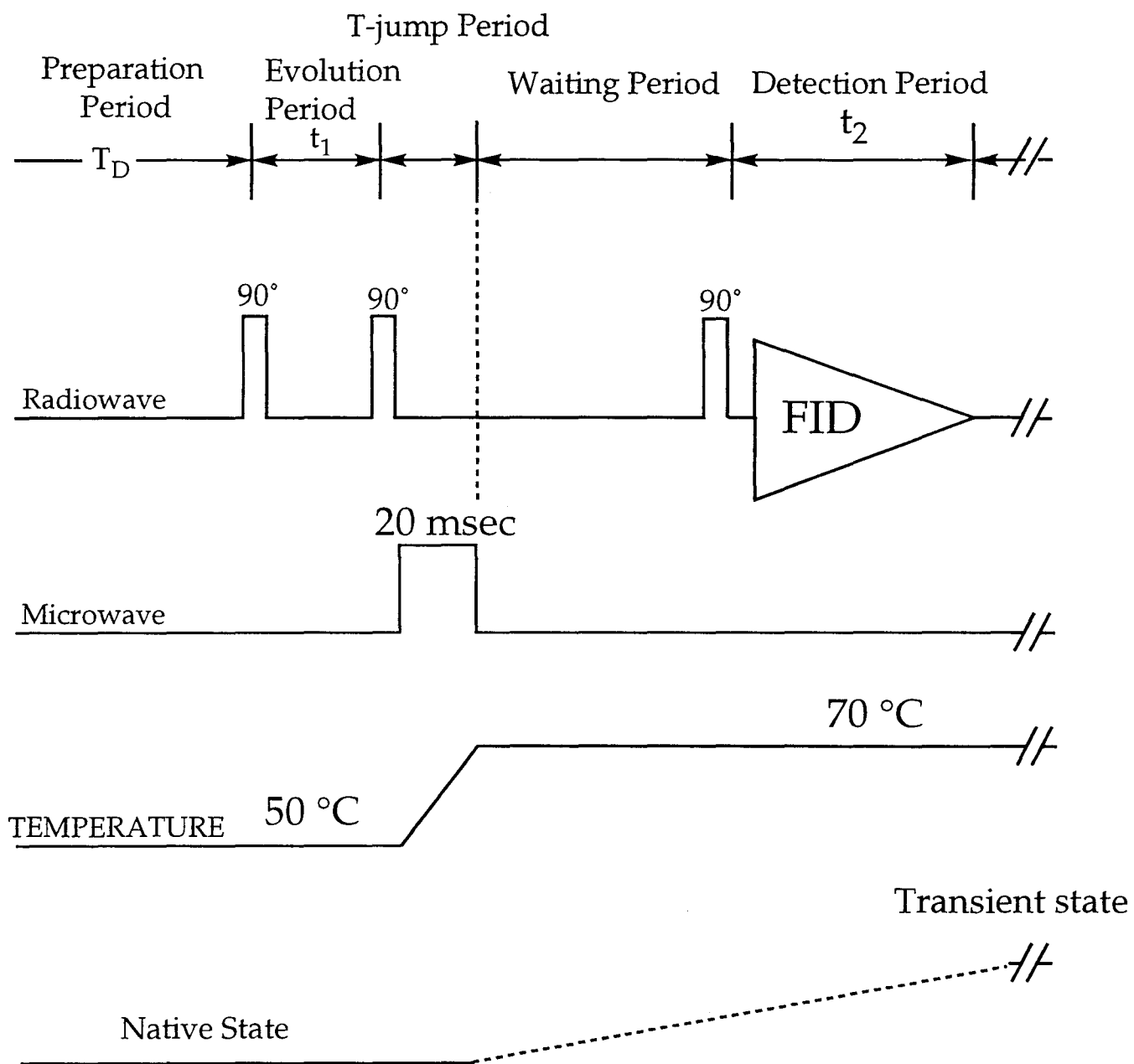
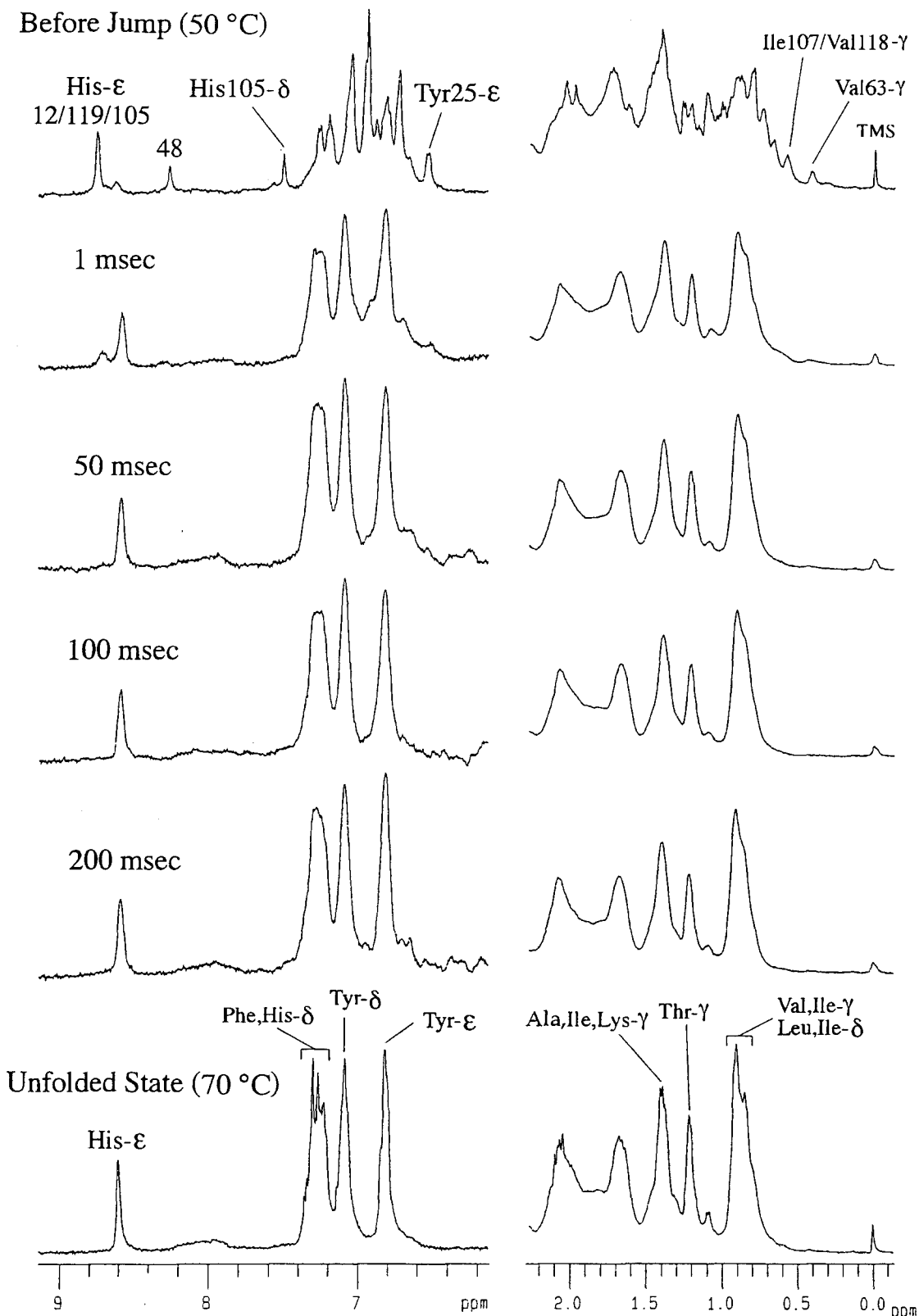Fig. 3.1.1. Pulse sequence for State-Correlated two-dimensional (SC-2D) NMR experiment.

Before Jump (50 °C)

His-ε
12/119/105

His105-δ

48

Tyr25-ε

Ile107/Val118-γ

Val63-γ

TMS

1 msec

50 msec

100 msec

200 msec

Phe,His-δ

Tyr-δ

Tyr-ε

Ala,Ile,Lys-γ   Thr-γ

Val,Ile-γ
Leu,Ile-δ

Unfolded State (70 °C)

His-ε

9   8   7   ppm   2.0   1.5   1.0   0.5   0.0 ppm

Fig. 3.1.2. Time courses of spectral change in the aromatic and aliphatic proton regions of 1H NMR spectrum of RNase A at pH 3.5.
For comparison, 1H NMR spectra obtained in equilibrium at 50 °C (top) and 70 °C (bottom) are shown.

43
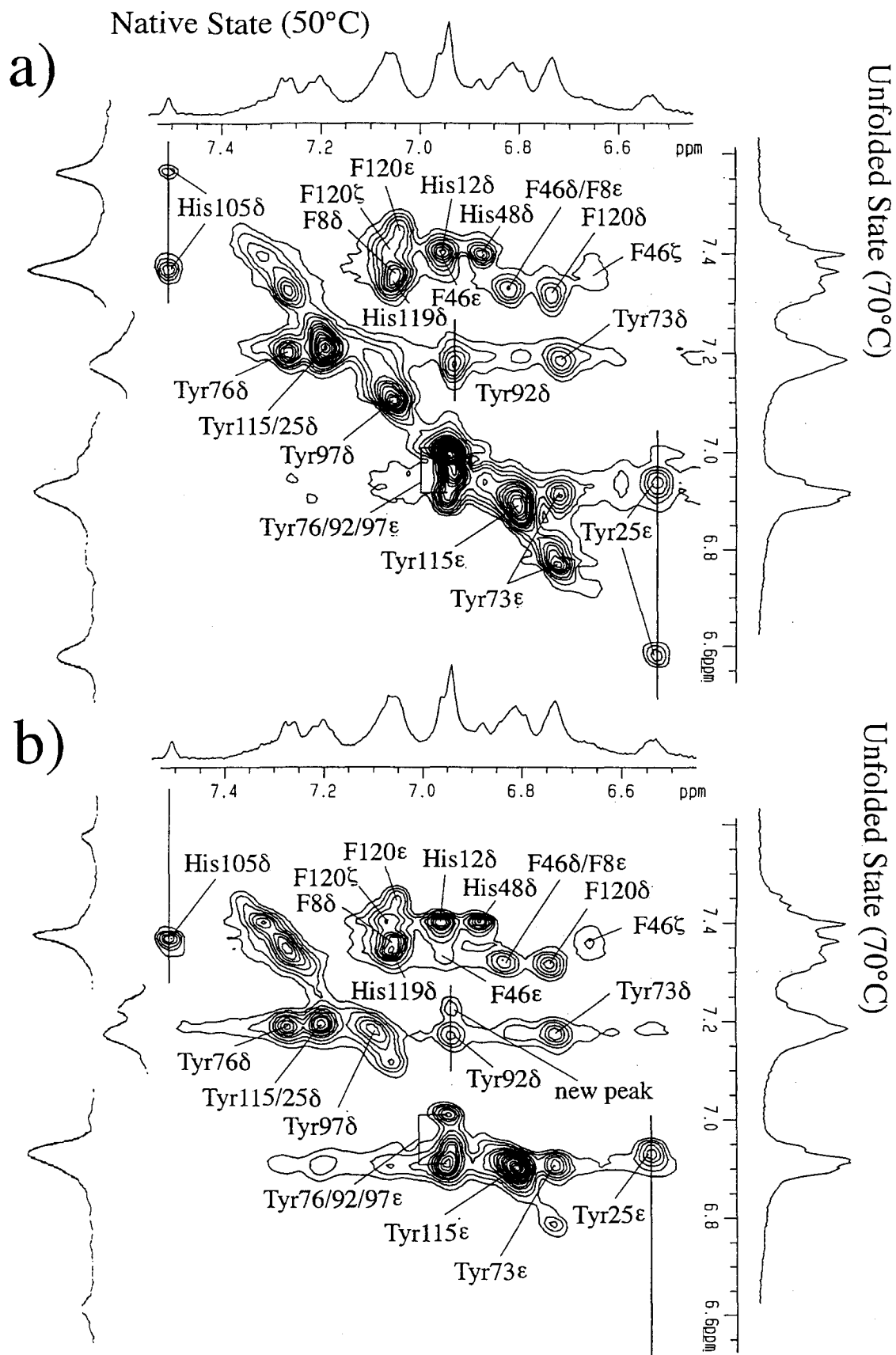
Fig. 3.1.3. Aromatic proton region of SC-2D NMR spectra of RNase A in D2O at pH 3.5. a), SC-2D spectrum between the native state at 50 °C and the state 1 msec after the jump, b), SC-2D spectrum between the native state at 50 °C and the state 200 msec after the jump.
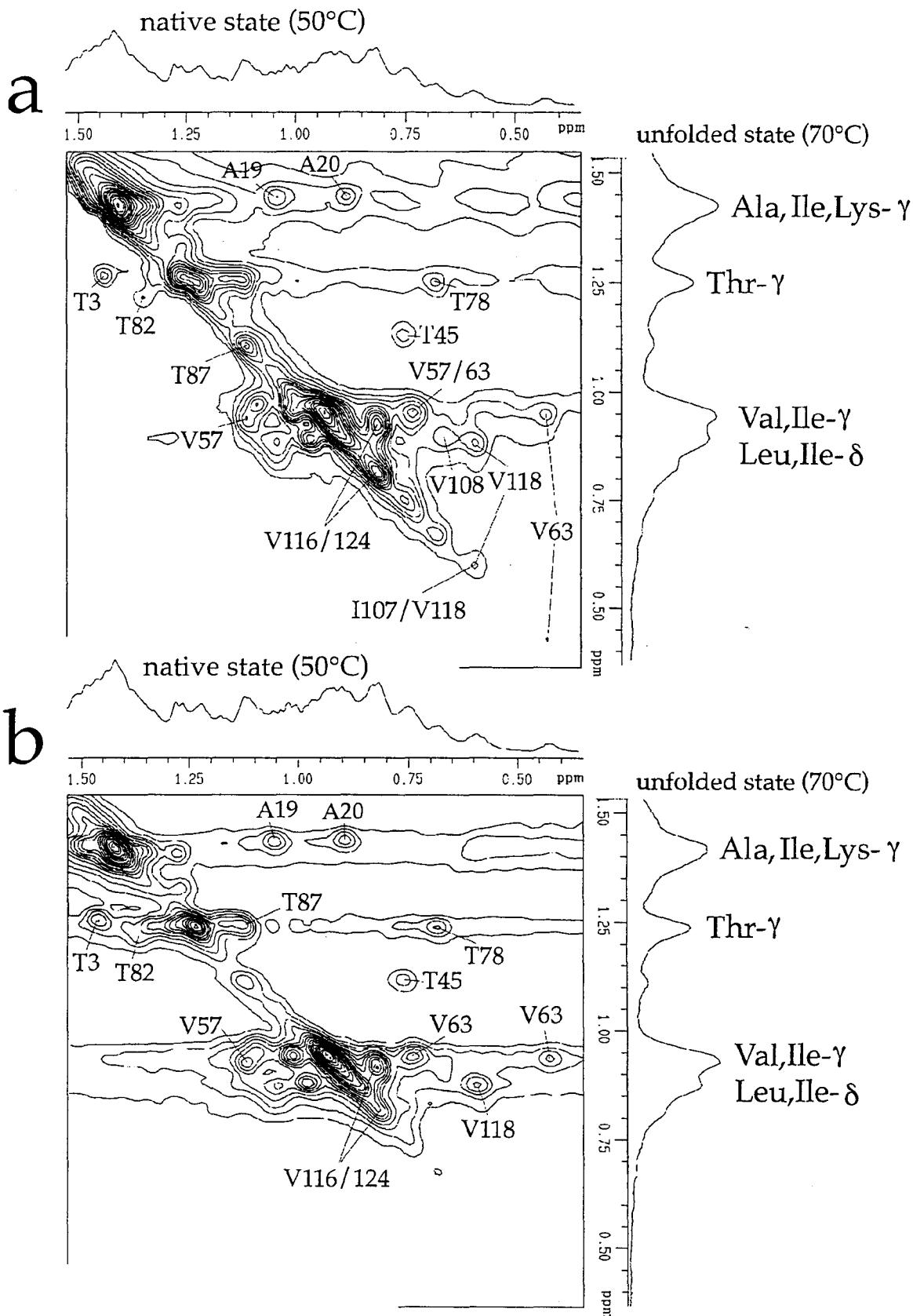
Fig. 3.1.3. Aliphatic proton region of SC-2D NMR spectra of RNase A in D2O at pH 3.5. a), SC-2D spectrum between the native state at 50 °C and the state 1 msec after the jump, b), SC-2D spectrum between the native state at 50 °C and the state 200 msec after the jump.

3-1-5. References

1. Houry, W. A., Rothwarf, D. M., and Scheraga, H. A., *Biochemistry* 33, 2516-2530 (1994).

2. Kiefhaber, T., and Baldwin, R. L., *Proc. Natl. Acad. Sci. U.S.A.* 92, 2657-2661 (1995).

3. Kiefhaber, T., Labhardt, A. M., and Baldwin, R. L., *Nature (London)* 375, 513-515 (1995).

4. Shakhnovich, E. I., and Finkelstein, A. V., *Biopolymers* 28, 1667-1680 (1989).

5. Phillips, C. M., Mizutani, Y., and Hochstrasser, R. M., *Proc. Natl. Acad. Sci. U.S.A.* 92, 7292-7296 (1995).

6. Akasaka, K., Naito, A., and Imanari, M., *J. Am. Chem. Soc.* 113, 4688-4689 (1991).

7. Wuthrich, K., NMR of Proteins and Nucleic Acids, John Wiley and Sons, New York (1986).

8. Robertson, A. D., Purisima, E. O., Eastman, M. A., and Scheraga, H. A., *Biochemistry* 28, 5930-5938 (1989).

9. Rico, M., Bruix, M., Santoro, J., Gonzalez, C., Neira, J. L., Nieto, J. L., and Herranz, J., *Eur. J. Biochem.* 183, 623-638 (1989).

10. Matthews, C. R., and Westmorel, D. G., *Biochemistry* 14, 4532-4538 (1975).

11. Beintema, J. J., Fitch, W. M., and Carsana, A., *Mol. Biol. Evol.* 3, 262-275 (1986).

12. Schultz, D. A., and Baldwin, R. L., *Protein Sci.* 1, 910-916 (1992).

13. Schultz, D. A., Schmid, F. X., and Baldwin, R. L., *Protein Sci.* 1, 917-924 (1992).

14. Dodge, R. W., and Scheraga, H. A., *Biochemistry* 35, 1548-1559 (1996).

15. Wlodawer, A., *Acta. Crystallogr.* B36, 1826-1831 (1980).

16. Garel, J. R., and Baldwin, R. L., *J. Mol. Biol.* 94, 611-620 (1975).

17. Garel, J. R., Nall, B. T., and Baldwin, R. L., *Proc. Natl. Acad. Sci. U.S.A.* 73, 1853-1857 (1976).

18. Tsong, T. Y., Baldwin, R. L., and Elson, E. L., *Proc. Natl. Acad. Sci. U.S.A.* 68, 2712-2715 (1971).

19. Brandts, J. F., Halvorson, H. R., and Brennan, M., *Biochemistry* 14, 4953-4963 (1975).

20. Tsong, T. Y., and Baldwin, R. L., *J. Mol. Biol.* 63, 453-469 (1972).

21. Juminaga, D., Wedemeyer, J., and Scheraga, H. A., *Biochemistry*, 37, 11614-11620 (1998).

22. Lin, L. N., and Brandts, J. F., *Biochemistry* 22, 553-559 (1983).

23. Adler, M., and Scheraga, H. A., *Biochemistry* 29, 8211-8216 (1990).

3-2. Bovine α-lactalbumin

3-2-1. Introduction

Various origins of α-lactalbumin have been used as models for the protein folding study.[1-4] Although the primary structure and the tertiary structure of α-lactalbumin is homologous to those of lysozymes, the function and folding behavior are quite different for the two proteins. When decalcificated under extremes of low pH, α-lactalbumins form a state with poor tertiary structure but with native-like secondary structure. A similar conformational state, called "molten-globule state", is realized under mildly denaturing conditions such as the moderate concentration of GdnHCl[5] or the reduction of disulfide bonds. Interestingly, a "molten-globule state" is also observed as a transient species in the kinetic folding experiment[6]. Hence, α-lactalbumin has received considerable attention in the study of protein folding.

Structures of α-lactalbumins from different living organisms were studied by NMR in their native [7-10] and molten-globule states.[2,11] The refolding of bovine α-lactalbumin was monitored by stopped-flow NMR and it was found that in the dead time of the experiments (~5 s), the spectrum of BLA changed to that of molten-globule state which then slowly turned into that of the native state.

For the unfolding kinetics of α-lactalbumins by NMR spectroscopy, however, no study has been reported. In the present work, the

unfolding process of bovine α-lactalbumin (BLA) was followed by one-dimensional T-jump NMR spectroscopy, which suggested the existence of transient unfolding species. Furthermore, the SC-2D spectroscopy was used to assign signals of unfolded and transient unfolding species.

3-2-2. Materials & Methods

Materials.

Calcium chloride, Urea and Tris-HCl were purchased from nacalai tesque (Kyoto, Japan). All chemical reagents used were guaranteed reagent grade. BLA was obtained from Sigma (Tokyo, Japan) and used without further purification.

Circular Dichroism measurements.

All Circular Dichroism (CD) measurements were performed in 10 mM Tris-HCl Buffer (pH 7.1) containing 150 μM $CaCl_2$ and urea whose concentration ranged between 0M and 6M. The protein concentration was 0.7 mg/ml (~50μM). Thermal unfolding transitions of the protein were measured with CD absorption at 222 nm, 270 and 290 nm in a JASCO J-720 spectropolarimeter.

Preparation of NMR samples.

Prior to the NMR sample preparation, labile amide protons were

replaced with deuterons by heating the sample at 327 K for 15 min in 99.9 % ^2H_2O at pH 7.0. Then lyophilized samples of BLA were dissolved at a concentration of 70 mg/ml in 99.96% ^2H_2O containing 100 mM Tris-HCl and 4M Urea. An excess of $CaCl_2$ (25mM) was dissolved in the protein solution to ensure complete complexation of the protein by Ca^{2+}. An aliquot of TSP solution was added as internal chemical shift reference. The final pH of the solution was adjusted by adding a small amount of ^2HCl or NaO^2H solution in 99.96 % ^2H_2O. All pH values cited here are direct pH meter readings without isotope corrections. After centrifugation, 120 µl of the sample solution was transferred into the Shigemi NMR tube (BMS 005J).

One-dimensional temperature-jump NMR.

For one-dimensional TJ NMR experiment, the combined radiofrequency and microwave pulse sequence "noboru" was used. The number of data points was 4096 and the spectral width was 6000 Hz. A T-jump from 40 °C to approximately 65 °C was realized by a microwave pulse of 15 msec duration. Acquisitions of free induction decay signals were executed after a certain waiting time. Sixteen scans were accumulated for each spectrum with a repetition time of 180 s for the sample cooling and the refolding of the protein.

State-correlated two-dimensional NMR spectroscopy.

The radiofrequency and microwave pulse sequence used for the state-correlated NMR spectroscopy is given in chapter 3-2-1. The T-jump was carried out similarly as in the case of one-dimensional TJ NMR. The waiting period was 100 msec. The number of data points was 64 and the spectral width was 1600 Hz in the t1 domain, while the corresponding numbers were 2048 complex points and 6000 Hz in the t2 axis, respectively. The t1 domain signals were zero-filled to 128 points prior to Fourier transformation. Sixteen scans were accumulated with 180-sec pulse delay time.

3-2-3. Results

Equilibrium thermal unfolding followed by Circular Dichloism

All the T-jump experiments were carried out in 4M urea to minimize the effect of protein aggregation. First, the effects of urea on the stability and the cooperativity of unfolding were examined with CD spectroscopy. The native form of BLA gives negative ellipticity in the near and far UV regions, with minima at 270 nm and 222 nm, respectively. The minima at 270 nm results from aromatic rings buried in the hydrophobic core, indicating the existence of tertiary structure. The minimum at 220 nm results mainly from the peptide chain and shows the existence of the secondary structure. In Fig. 3.2.1a, the

thermal transition curves of BLA monitored by the mean residue ellipticity at 270 nm at various concentrations of urea are presented. Below 4M, the sigmoid type transition with common baselines for the native and unfolded species is observed with a decrease of Tm.

Fig. 3.2.1b shows transition curves monitored at three wavelengths (222, 270 and 290 nm) in the presence of 4M urea, pH 7.1. The three curves coincided well with each other, indicating that the two-state transition is maintained even in the presence of 4M urea.

One dimensional Temperature-jump NMR measurements.

Before applying the microwave pulse, both aromatic and aliphatic proton regions of the NMR spectrum of BLA (at pH 7.2, 40 °C, Fig. 3.2.2, top) show wide dispersions of chemical shift, which is characteristic of a globular protein, reflecting the highly specific inter-residue interactions within a compact folded structure. Since the chemical shifts in this spectrum agreed with the spectrum at 0M urea (data not shown), it is considered that urea itself does not influence the native structure of BLA. The bottom spectrum in Fig. 3.2.2 was obtained for BLA in thermal equilibrium at 65 °C where BLA exists fully in the fully unfolded state. The wide dispersion of chemical shift is lost, which is typical for the random-coil state of a protein. Nevertheless, in the aromatic region one notice that chemical shifts of some peaks are deviated from the average shift positions, for example, the peaks at 6.6, 6.7 and 7.72 ppm. The origin of these peaks will be discussed in the next

section.

The spectrum taken immediately after the jump from 40 °C to 65 °C (Fig. 3.2.2, second row) appears almost identical with that of the unfolded state after the thermal equilibrium is reached at 65 °C (Fig. 3.2.2, bottom). However, a close inspection of Fig. 3.2.2 indicates that the peaks at 6.6, 6.7 and 7.72 ppm are considerably broadened immediately after the jump and become shaper with time. In addition, shoulders grow with time at 6.98 and 7.05 ppm.

The above results indicate that the tertiary structure of BLA is largely destroyed within the jump event (15 ms), but that some further conformational changes may take place after the jump.

State-correlated two-dimensional NMR spectroscopy.

An SC-2D spectrum of BLA between the native conformer (40 °C) and the conformer at 100 msec after the jump (65 °C) is shown in Fig. 3.2.3 for the aromatic region. BLA has three histidine, four phenylalanine, four tyrosine and four tryptophan residues. In the SC-2D spectrum of Fig. 3.2.3, the signals which are dispersed along the F1 axis by the characteristic chemical shifts in the native conformation are grouped into chemical shifts characteristic for a random coil along the F2 axis. Since assignments of the aromatic ring proton signals of BLA in the native state have been completed,[12] cross peaks in the SC-2D spectrum can easily be made as shown in the Fig. 3.2.3. Unfortunately, assignments of most other protons of phenylalanine residues except for

the δ protons of Phe31 could not be made because of the poor chemical shift dispersion in the native state (F1 axis) and the serious signal overlap in the unfolded state (F2 axis). For clarity, correlated peaks are colored in their respective amino acid group.

In Fig. 3.2.3, while most signals are grouped by the types of the protons of amino acids, some peaks show deviations from the average values of the group. The peaks with significant chemical shift deviations from random coil positions at 65 °C were assigned to the δ and ε protons of Tyr103 (6.6 and 6.72 ppm in F2 axis) and to the ε protons of His107 (7.73 ppm in F2 axis). In addition, the chemical shift of the δ protons of His107 was also found to be considerably upfield-shifted in the unfolded state (6.83 ppm in F2 axis). Small deviations of chemical shift as observed here cannot be detected without the use of the SC-2D method due to severe overlap of signals in the F2 axis.

Generally speaking, the line-width of the unfolded protein is narrower than that of the native protein, hence the shape of the cross peak should be an ellipsoid with a long axis along the F1 axis (for example, see the ε proton signal of His32 in Fig. 3.2.3 (F1; 8,12 ppm, F2; 7.96 ppm). However, the shapes of some peaks are evidently more elongated along the F2 axis than along the F1 axis. The extreme can be seen for signals of Tyr103 and for the signal of the ε proton of His107 in comparison to those of His32 and His68. These observations indicate that there are differences in the line widths even among the same protons of the same type of amino acid in the transient spectrum.

54

Moreover, it was found that some signals, namely the signals of Trp104 C4, Trp26 C5 and Trp26 C6, exhibit considerable line-broadening. These peaks become sharper with time, as judged from the gradual growth of the shoulders at 6.98 and 7.05ppm in the 1D T-jump spectra.

3-2-4. Discussion

Immediately after the T-jump, the 1D NMR spectrum lost its characteristic feature of the native state. Accordingly, it is concluded that the aromatic residue clusters are largely destroyed in the unfolded state. However, proton signals of Tyr103 and His107 are significantly upfield-shifted. Moreover these signals are abnormally broadened immediately after the jump, but become normally sharp with time after the jump. The broadening of the signals suggests the existence of some kind of inter-residue interactions involving Tyr103 and His107.

The tertiary structure of α-lactalbumin is presented in Fig. 3.2.4. There are two clusters of aromatic residues (cluster I, cluster II) which make up part of the hydrophobic core of the helical domain of the native BLA.[14] Tyr103 forms part of cluster II and His107 is located close to cluster II (Fig. 3.2.5). In the early studies, the chemical shift deviation of some residues in the acid-denatured state (A-state) has been reported for bovine,[14] guinea pig[2] and human α-lactalbumin. According to these

reports, in the A-state, no ordered structure for either of the two major clusters of aromatic residues was detected, but a new aromatic cluster was found by some well-defined NOE effects.[2] They concluded that the residual structure in the A-state results from the rearrangement of the side chains of Tyr103, Trp104 and His 107. Meanwhile, from the studies on the model peptides which correspond to the 101-110 peptide region of bovine[15] and human[16,17] α-lactalbumin, it was found that the side chains of these peptides tend to form non-native hydrophobic clusters of α-lactalbumin. In view of the coincidence of residues (Tyr103 and His107) which show upfield-shift chemical shifts in the A-state and in the spectrum immediately after the jump, the cluster formation of side chains of Tyr103 and His 107 is quite likely.

Furthermore, the line broadening is also recognized for the signals of Trp104 C4, Trp60 C4, Trp26 C5 and Trp26 C6 (Fig. 3.2.3). Trp104 is considered to be included in the non-native hydrophobic cluster as discussed in the A-state. Although Trp26 is distant from the 101-110 peptide region in the primary structure, in the native structure of BLA, it is located close to Trp104 (see Fig. 3.2.5). On the other hand, Trp60 is a member of the cluster II, whose NOE with Tyr103 is detected in the native conformer.[13] Therefore, the amino acid residues which showed line broadenings in the T-jump experiment are located close to each other in the native structure. Therefore, although the original native cluster is not maintained in the unfolded species, the same groups of amino acid residues form a different form of a cluster in the

transient unfolding species found immediately after the jump or in the stable thermal denatured state.

One possibility is that the residual structure is fluctuating like molten-globule state. In the A-state, considerable line-broadening is observed. In particular, the line-broadening is remarkable for Tyr103 and His107. Immediately after the jump, this region may form a group of structures similar to the A-state. The sharpening with time may reflect the disruption of that structure.

The residual structure is assumed to be fluctuating. The point to note is that the line-broadening was observed not for overall residues, but only for the localized residues. Thus, it means that a partially folded state is detected. Therefore, the part consisting of residues 101-110, Trp26 and Trp60 is suggested as the chain folding initiation site.
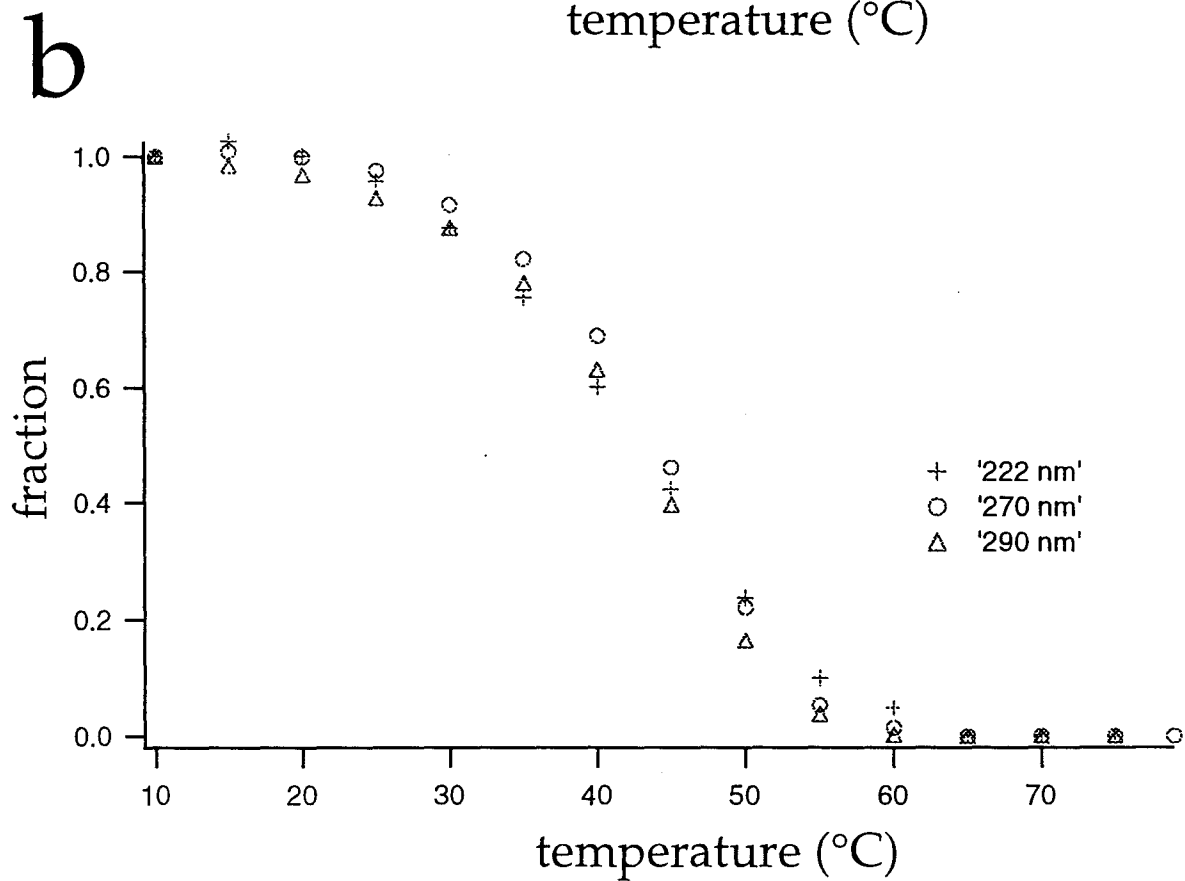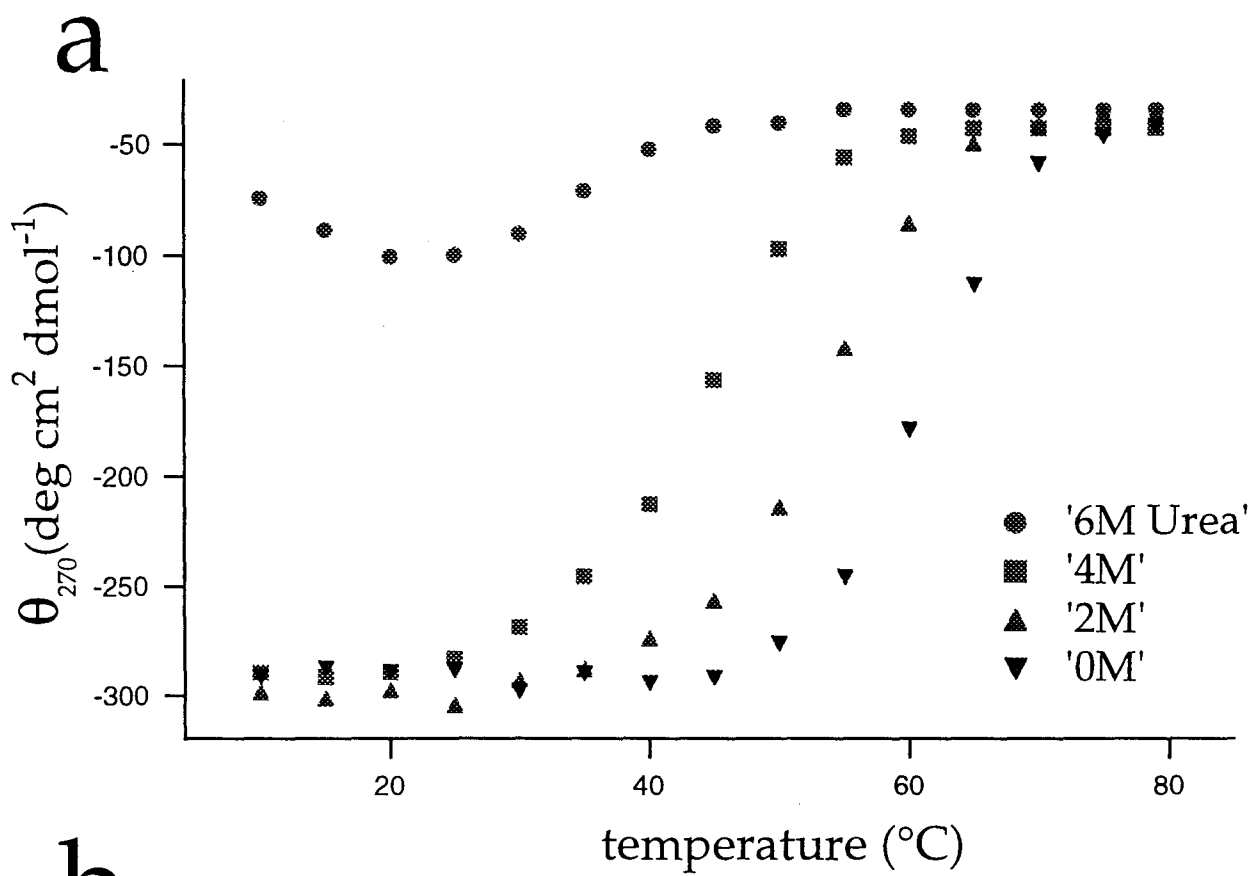
Fig. 3.2.1. Thermal transition curves of BLA measured by the ellipticity change.

before the jump (40 °C)

1 msec

50 msec

100 msec

1000 msec

unfolded state (65 °C)

8.5   8.0   7.5   7.0   6.5   ppm

2.0   1.5   1.0   0.5   0.0   -0.5   ppm
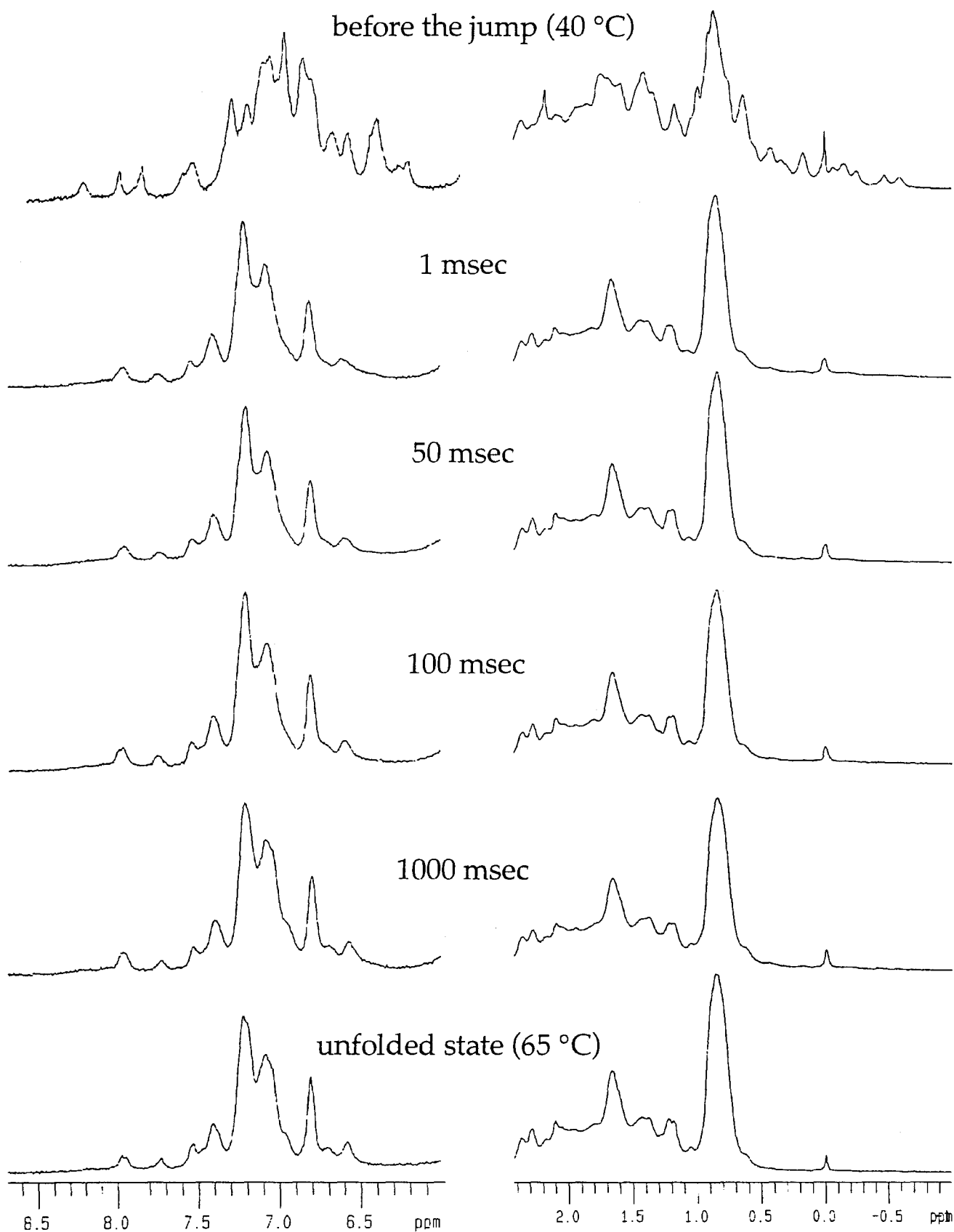
Fig. 3.2.2. Time courses of spectral change in the aromatic and aliphatic proton regions of ^{1}H NMR spectrum of BLA at pH 7.2.
For comparison, ^{1}H NMR spectra obtained in equilibrium at 40 °C (top) and 65 °C (bottom) are shown.

59
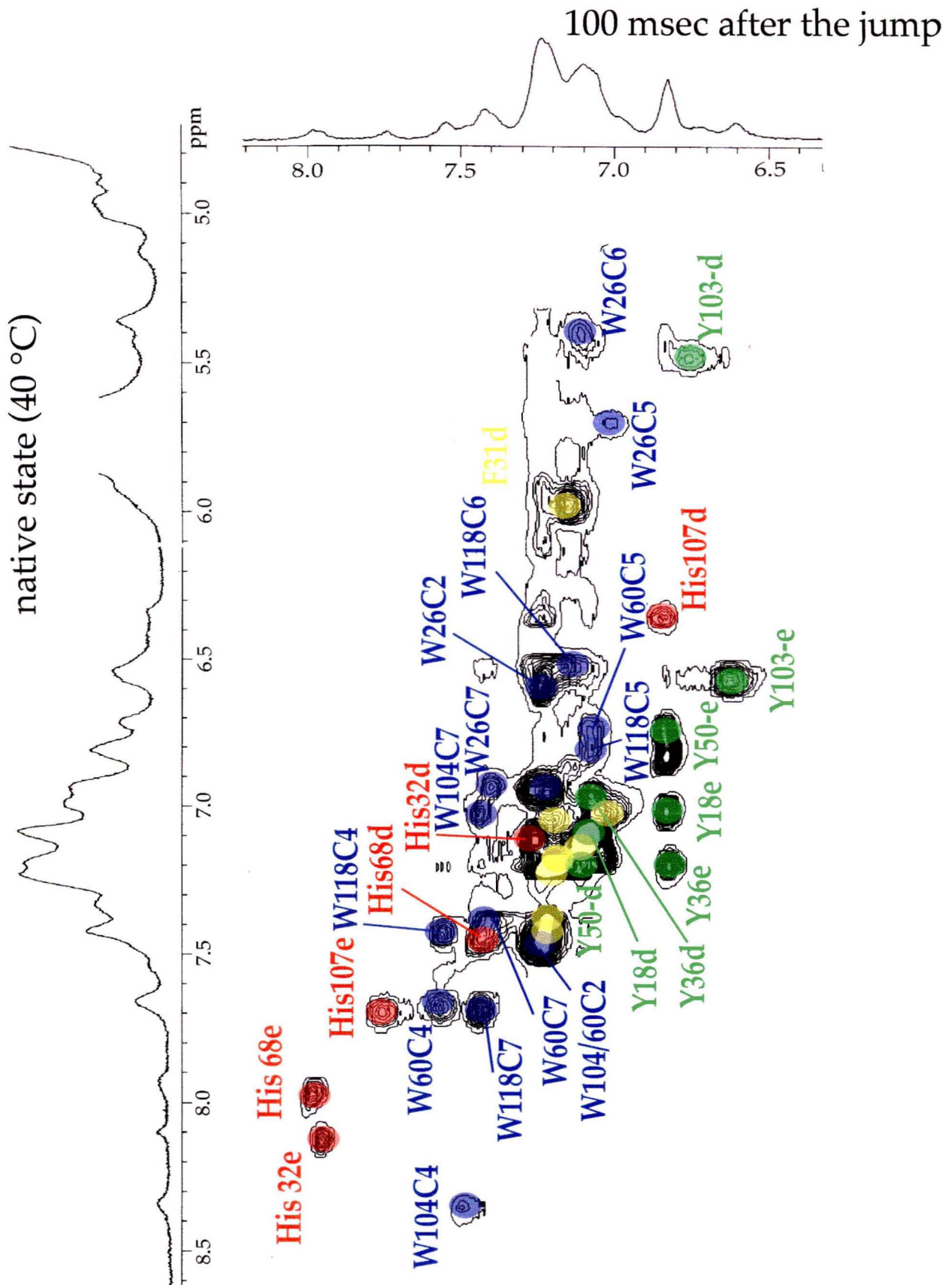
Fig. 3.2.3. Aromatic proton region of SC-2D spectrum of BLA at pH 7.2.
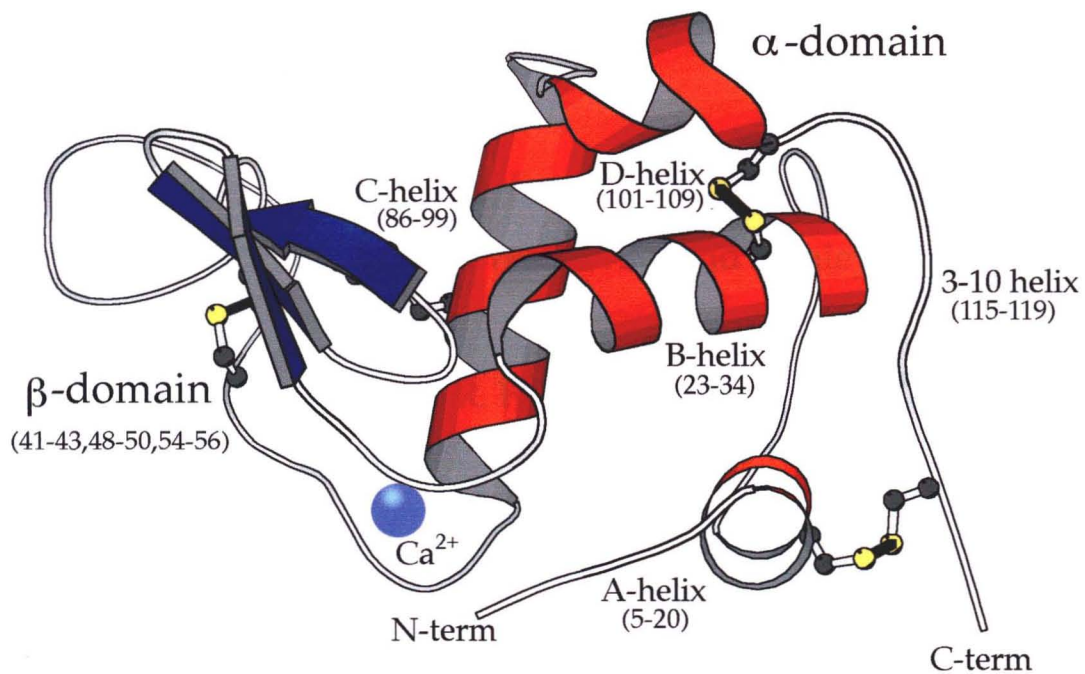
Fig. 3.2.4. Ribbon diagram of α-lactalbumin. The structure was taken from PDB. The diagram was prepared with the program MOLSCRIPT.
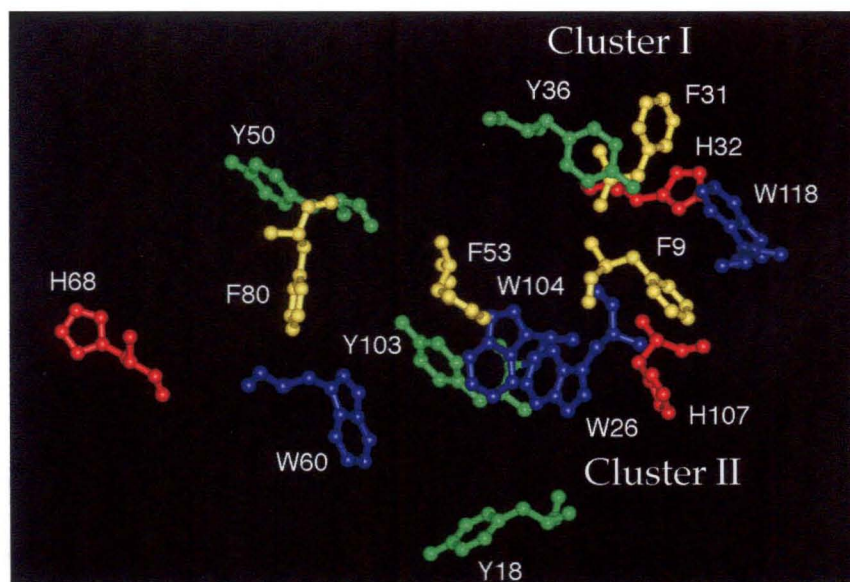
Fig. 3.2.5. Aromatic residue clusters in bovine α-lactalbumin.

3-2-5. References

1. Kuwajima, K., *Proteins Struct. Funct. Genet.* 6, 87 (1989).

2. Baum, J. B., Dobson, C. M., Evans, P. A., and Hanley, C., *Biochemistry* 28, 7 (1989).

3. Chrintensen, H., and Pain, R. H., *Eur. Biophys. J.* 19, 221 (1991).

4. Ptitsyn, O. B., *FEBS Lett.* 285, 176 (1991).

5. Kuwajima, K., Nitta, K., Yoneyama, M., and Sugai, S., *J. Mol. Biol.* 43, 411 (1976).

6. Kuwajima, K., Hiraoka, Y., Ikeguchi, M., and Sugai, S., *Biochemistry* 24, 874 (1985).

7. Bradbury, J. H., and Norton, R. S., *Eur. J. Biochem.* 53, 387 (1975).

8. Berliner, L. J., and Kaptein, R., *J. Biol. Chem.* 255, 3261 (1980).

9. Koga, K., and Berliner, L. J., *Biochemistry* 24, 7257 (1985).

10. Harushima, Y., and Sugai, S., *Biochemistry* 28, 8568 (1989).

11. Alexandrescu, A. T., Evans, P. A., Pitkeathly, M., Baum, J., and Dobson, C. M., *Biochemistry* 32, 1707 (1993).

12. Alexandrescu, A. T., Broadhurst, R. W., Wormald, C., Chyan, C. L., Baum, J., and Dobson, C. M., *E. J. Biochem.* 210, 699 (1992).

13. Pike, A. C. W., Brew, K., and Achara, K. R., *Structure (London)* 4, 691 (1996).

14. Shimizu, A., Ikeguchi, M., Kobayashi, T. and Sugai, S., *J. Biochem.* 119 (5), 947 (1996).

15. Alexandrescu, A. T., Ng, Y.-l., Evans, P. A., Baum, J., and Dobson, C. M., *J. Cell Biochem.* 15G 187 (1991).

16. Demarest, S. J., Fairman, R. and Raleigh, D. P., *J. Mol. Biol.* 283 (1), 279 (1998).

17. Smith, L. J., Alexandrescu, A. T., Pitkeathly, M., and Dobson, C. M., *Structure* 2, 703 (1994).

Chapter 4. Conclusion & Future Prospects

The performance of the microwave T-jump NMR system and its application to protein unfolding study have been demonstrated. On this system, fast reaction (millisecond range) can be followed at atomic resolution. Besides, the new SC-2D technique introduced in chapter 3-1 is a novel spectroscopy in which the multidimensional NMR technique is combined with time resolution.

Although in this thesis the applications of the T-jump NMR technique are shown only for the unfolding process, it is also possible to follow the folding process with this technique, since it is widely known that protein unfolds at low temperature. By performing a T-jump experiment on a cold denatured protein, a folding process may be directly observed by NMR.

Recently, a pulsed laser TJ technique with IR or fluorescence detection, observing early events in protein folding and unfolding in the picosecond to nanosecond range, was introduced by other workers. The microwave T-jump NMR technique introduced here works for the detection of much slower events in protein folding and unfolding, but with a spatial resolution far beyond that attainable by other spectroscopic techniques. Moreover, the signals can be observed from the entirety of the protein molecule. With these unique features, the microwave TJ NMR spectroscopy introduced in the present work will continue to be a crucial technique in the study of protein folding.

Finally I point out that this technique can be applied to a wide variety of systems with temperature-dependent properties. This study provides the possibility that chemical, physical and biological reactions are analyzed at atomic resolution by NMR spectroscopy.

Publication list

Kawakami, M., & Akasaka, K. (1998) Microwave T-jump NMR for aqueous solutions, Rev. Sci. Instrum. 69, 3365-3369.

Kawakami, M., & Akasaka, K. (1999) Proline-trapped unfolding transient species of ribonuclease A detected by state-correlated two-dimensional T-jump NMR spectroscopy, *to be submitted*.

Acknowledgments