Stopped-Flow Photo-CIDNP Observation of Protein Folding

P. J. Hore,*,† Stephen L. Winder,† Claire H. Roberts,† and Christopher M. Dobson‡

Oxford Centre for Molecular Sciences
Physical and Theoretical Chemistry Laboratory
and New Chemistry Laboratory, University of Oxford
South Parks Road, Oxford OX1 3QZ, U.K.

Received December 27, 1996

Recent experiments have shown that stopped-flow NMR spectroscopy can provide detailed structural and kinetic information on the processes involved in protein folding at the level of individual amino acid residues.[1−4] Rapid dilution of a denatured protein into a refolding medium can be followed by acquisition of either a series of one-dimensional spectra[2] or a complete two-dimensional spectrum[4] while refolding takes place. Though extremely valuable, the approach has so far been restricted to rather slow folding events ($\gtrsim$10 s) which allow several free induction decays to be acquired before the native state is fully formed. Here we demonstrate that coupling the stopped-flow experiment with photo-CIDNP (chemically induced dynamic nuclear polarization) detection provides an opportunity to study folding processes up to two orders of magnitude faster than previously shown to be possible.

Photo-CIDNP[5−8] probes the accessibility of aromatic sidechains in proteins by means of a cyclic photochemical reaction that results in enhanced ^{1}H NMR signals from exposed residues. Conventionally, a solution of the protein together with a small quantity of flavin photosensitizer is irradiated inside the NMR probe with visible light from an argon-ion laser. Electron or hydrogen atom transfer from the amino acid to the excited triplet flavin produces a radical pair, in which nuclear polarization is generated through the influence of the electron-nuclear hyperfine interactions on the radical pair recombination probability. In the stopped-flow experiment, CIDNP spectra are recorded for different delays between the initiation of refolding and the light pulse so as to monitor changes in histidine, tyrosine, and tryptophan accessibility as the protein resumes its native state.

The success of the technique is crucially dependent on the speed with which the protein solution can be transferred into the NMR tube, the efficiency of mixing, and the recovery of the NMR line shape from the shock of the stopped-flow. Exploratory experiments were performed by injecting small quantities of aqueous tyrosine or tryptophan into solutions of flavin mononucleotide (FMN), from a pneumatically driven gas-tight syringe via ∼2 m of 0.5 mm internal diameter PTFE tubing. The 5 mm NMR tube was fitted with a Wilmad coaxial Pyrex insert with its stem, intended for an external reference compound, cut down to allow the end of the PTFE tube to dip into the flavin solution, just above the sensitive region of the radiofrequency coil. Both the NMR tube and the insert were spun at ∼15 Hz around the stationary transfer line. The spins in the NMR tube were presaturated prior to the light flash so that only light-induced signals were observed. A fresh sample was used for each spectrum.

A 50 ms light pulse at various times after the beginning of the injection produced the spectra of *N*-acetyl tyrosine shown in Figure 1. Strikingly well resolved, strongly enhanced NMR signals are seen even in the earliest spectra, indicating that mixing had occurred on the microscopic scale necessary to bring photoexcited FMN and amino acid molecules into intimate contact. The integrated intensity of the emissive resonance of the H3,5 protons, ortho to the phenolic hydroxyl group, hardly changes during the first 500 ms but falls by ∼20% over the next 2 s, reflecting the spreading of a portion of the injected solution away from the ∼50 μL volume illuminated by the laser. The resolution, as judged by the splitting of the H3,5 doublet, is already acceptable in the earliest spectra, 25−35 ms after the beginning of the injection, and is noticeably better after ∼40 ms. That efficient mixing occurs within a few tens of milliseconds was confirmed by filming, outside the magnet, the injection of small quantities of an intensely colored dye solution into 400 μL of water in an NMR tube. Frame-by-frame analysis showed the injection of 50 μL of dye to be complete within 25 ms (40 ms for 100 μL), with dye well spread over all but the extremes of the NMR sample within these times.

The refolding of hen lysozyme was chosen to illustrate the potential of stopped-flow CIDNP. At 20 °C in H_2O at pH 5.2, recovery of the fully native state occurs with a time constant of ∼350 ms.[10] Pulsed H-exchange experiments, in which nearly half of the 126 amide protons were used as probes of refolding,[11] have shown, however, that the amide hydrogens are protected from exchange with a variety of exponential time constants, τ, between 5 and 500 ms as a consequence of the formation of intermediates. Although the amides of most of the potentially CIDNP-active residues have τ < 100 ms, one, Tyr-53, is protected more slowly ($\tau \simeq$ 350 ms in ∼70% of molecules).

Figure 2B shows stopped-flow CIDNP spectra recorded during the refolding of hen lysozyme in D_2O. In contrast to the spectra of tyrosine, the enhancements for lysozyme showed a marked time dependence, the emissive tyrosine resonance(s) decaying and the absorptive tryptophan signals growing as the delay between the injection pulse and the light flash was increased. The spectrum after a 2 s refolding period is essentially the same as that of the native protein in the absence of urea (Figure 2C). The latter is dominated by signals from Trp-62 and Trp-123 in the range 6.9−7.7 ppm and also contains a small emission at 6.71 ppm from Tyr-23.[12−14] The other four tryptophans, two tyrosines, and the single histidine are fully protected from attack by triplet FMN by secondary and tertiary structure in the native state.[12−14] The earliest spectrum (at 30 ms), however, differs considerably from that of lysozyme in 10 M urea (Figure 2A), showing a much larger tyrosine polarization relative to tryptophan. During the course of the refolding, the chemical shifts and relative enhancements of the tryptophan peaks barely change. The tyrosine resonance at 6.76 ppm decays, again without change in chemical shift, at the same time as the much weaker native Tyr-23 peak grows in. The two sets of polarized signals, from tryptophans and tyrosines,

* To whom correspondence should be addressed: E-mail: hore@physchem.ox.ac.uk.
† Physical and Theoretical Chemistry Laboratory.
‡ New Chemistry Laboratory.

(1) Frieden, C.; Hoeltzli, S. D.; Ropson, I. J. *Prot. Sci.* **1993**, *2*, 2007−2014.
(2) Balbach, J.; Forge, V.; van Nuland, N. A. J.; Winder, S. L.; Hore, P. J.; Dobson, C. M. *Nature Struct. Biol.* **1995**, *2*, 865−870.
(3) Hoeltzli, S. D.; Frieden, C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9318−9322.
(4) Balbach, J.; Forge, V.; Lau, W. S.; van Nuland, N. A. J.; Brew, K.; Dobson, C. M. *Science* **1996**, *274*, 1161−1163.
(5) Kaptein, R.; Dijkstra, K.; Nicolay, K. *Nature* **1978**, *274*, 293−294.
(6) Kaptein, R. *NMR spectroscopy in molecular biology*; Pullman, B., Ed.; D. Reidel: Dordrecht, 1978; pp 211−229.
(7) Kaptein, R. *Biol. Magn. Reson.* **1982**, *4*, 145−191.
(8) Hore, P. J.; Broadhurst, R. W. *Prog. NMR Spectrosc.* **1993**, *25*, 345−402.
(9) Lu, H.; Dobson, C. M. Unpublished results.
(10) Itzhaki, L. S.; Evans, P. A.; Dobson, C. M.; Radford, S. E. *Biochemistry* **1994**, *33*, 5212−5220.
(11) Radford, S. E.; Dobson, C. M.; Evans, P. A. *Nature* **1992**, *358*, 302−307.
(12) Hore, P. J.; Kaptein, R. *Biochemistry* **1983**, *22*, 1906−1911.
(13) Stob, S.; Scheek, R. M.; Boelens, R.; Dijkstra, K.; Kaptein, R. *Isr. J. Chem.* **1988**, *28*, 319−327.
(14) Broadhurst, R. W.; Dobson, C. M.; Hore, P. J.; Radford, S. E.; Rees, M. L. *Biochemistry* **1991**, *30*, 405−412.

S0002-7863(96)04413-7 CCC: $14.00 © 1997 American Chemical Society

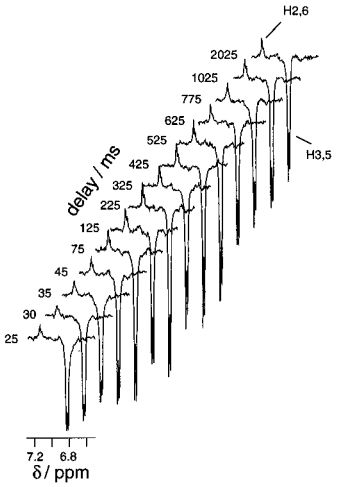

Figure 1. 400 MHz ^{1}H CIDNP spectra (aromatic region) of *N*-acetyl tyrosine at various times after the stopped-flow injection of 50 μL quantities of 3 mM amino acid solution into 400 μL of 0.5 mM flavin mononucleotide in D_2O. The delay times shown are from the beginning of the (~25 ms) injection pulse to the beginning of the 50 ms laser flash, which was immediately followed by a radiofrequency pulse and acquisition of the free induction decay. The integrated intensities of the emissive H3,5 resonance show much smaller fluctuations than do the peak heights, which are affected by slight differences in the line widths. A further demonstration of the efficiency of mixing (not shown) was provided by 10-fold dilution of denatured hen lysozyme in dimethyl sulfoxide into D_2O, to initiate a refolding process known to be complete within ~200 ms.[9] The CIDNP spectra were characteristic of the native protein in aqueous solution, even at the earliest times.

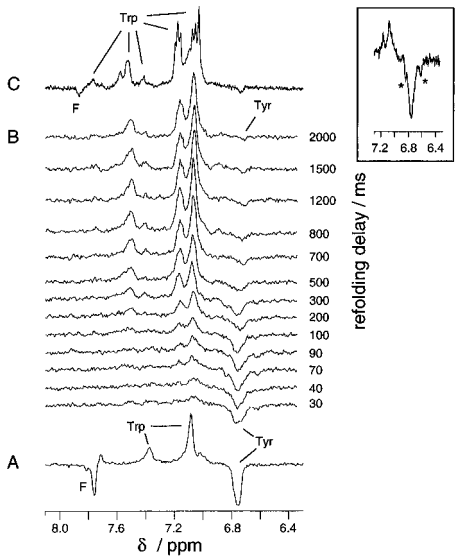

Figure 2. 400 MHz ^{1}H CIDNP spectra (aromatic region) of hen lysozyme: (A) in the presence of 10 M urea; (B) at various times after stopped-flow initiation of refolding; and (C) in the absence of urea. For the stopped-flow measurements, 70 μL volumes of 3 mM denatured protein in 10 M urea at pH 1.1 were injected into 430 μL of deuterated sodium acetate buffer at 20 °C. Both solutions contained 0.5 mM FMN. The final pH was 5.2, and the urea concentration was 1.4 M. The refolding times represent the delays between the end of the 30 ms injection pulse and the beginning of the 50 ms light flash. The resonances marked F are due to the FMN. The inset shows part of the 70 ms spectrum, with subsidiary tyrosine peaks, at 6.66 and 6.84 ppm, indicated by asterisks.

display similar kinetics with time constants of 450 ± 30 ms and 510 ± 30 ms, respectively.

These observations are consistent with the following tentative interpretation. Immediately after dilution from 10 M urea, and within the dead time of the experiment, the protein undergoes a rapid collapse,[10,11] from a denatured state in which both tyrosine and tryptophan residues are to a large degree accessible, to an intermediate whose tryptophans have a much greater protection against attack by flavin. Such a situation would result from the preferential burial and exclusion from solvent of the more hydrophobic tryptophan residues. This intermediate then converts into the native state, at a rate of ~2 s^{-1}, by a rearrangement which partially buries the tyrosines and exposes Trp-62 and Trp-123, required for functional reasons to be on the surface of the protein, causing the CIDNP spectra (Figure 2B) to be linear combinations of the spectra characterizing the intermediate and the native state. Allowing for the solvent isotope effect on the refolding rate constant,[15] $k(H_2O)/k(D_2O) \simeq 1.5$, the ~500 ms CIDNP time constant in D_2O agrees well with the ~350 ms time constants measured for the recovery of the active site[10] and the protection of the amide proton of Tyr-53[11] in H_2O. Indeed, there is some evidence in the spectra recorded between 50 and 100 ms (see inset in Figure 2) for a second or even a third emissive tyrosine peak, possibly due to Tyr-20 and/or Tyr-23 whose amide protons are protected with $\tau < 100$ ms.[11]

Although further work is clearly needed to clarify the origin of the time dependence observed for lysozyme, our results indicate that stopped-flow CIDNP can be used to follow refolding processes that are essentially complete within a second. The reduction in experimental dead time compared to conventional NMR detection may be seen to have two origins. First, the nuclear polarization is produced during a ~50 ms laser light flash, a somewhat faster process than waiting for spin−lattice relaxation to polarize spins that have been transferred into the NMR probe from a lower field region of the magnet. Second, the CIDNP signal comes from only the ~50 μL portion of the 500 μL NMR sample illuminated by the laser. Homogeneous mixing in this volume, which is considerably smaller than the sensitive region of the NMR receiver coil, can be achieved in ~50 ms, even for viscous solutions containing high concentrations of denaturant. Additionally, the technique benefits from the sensitivity advantage associated with the CIDNP enhancement, from the alleviation of spectral crowding—typically only a handful of residues are polarizable—and from the direct information photo-CIDNP gives on side-chain accessibility.[7,8]

The time resolution of the experiments described here is limited by the time required for mixing and the duration of the CIDNP-generating light flash (~100 ms). This time scale can, however, undoubtedly be improved by the use of faster and more turbulent mixing techniques, smaller illumination volumes, and nanosecond pulsed lasers. It may also be possible to boost the sensitivity of the experiment by polarizing the protein in a weak magnetic field[16] before injecting it into the NMR probe. Other types of CIDNP measurements should also be possible. For example, a partially folded protein, e.g., formed at low pH, could be polarized with a light flash and subjected to a pH jump to initiate rapid refolding. Observation of the pattern of polarization in the well dispersed and previously assigned spectrum of the native state would give insight into the structure of the partially folded precursor. Such possibilities are under investigation.

Acknowledgment. We acknowledge support by the EPSRC, BBSRC, and MRC through the Oxford Centre for Molecular Sciences. The research of CMD is supported by an International Research Scholars award from the Howard Hughes Medical Institute. We thank Nico van Nuland, Jochen Balbach, Vincent Forge, and Hui Lu for valuable advice.

JA9644135

(15) Itzhaki, L. S.; Evans, P. A. *Prot. Sci.* **1996**, *5*, 140−146.
(16) Salikhov, K. M.; Molin, Yu. N.; Sagdeev, R. Z.; Buchachenko, A. L. *Spin polarization and magnetic effects in radical reactions*; Elsevier: Amsterdam, 1984; pp 199−217.