LA-UR-

96-0237

Title:

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APR 0 1 1996

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Submitted to:

DOE Office of Scientific and Technical
Information (OSTI)

Los Alamos
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Fast Events in Protein Folding

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Abstract

This is the final report of a three-year, Laboratory-Directed Research and Development (LDRD) project at the Los Alamos National Laboratory (LANL). The primary objective of this work was to develop a molecular understanding of how proteins achieve their native three-dimensional (folded) structures. This requires the identification and characterization of intermediates in the protein folding process on all relevant timescales, from picoseconds to seconds. The short timescale events in protein folding have been entirely unknown. Prior to this work, state-of-the-art experimental approaches were limited to milliseconds or longer, when much of the folding process is already over. The gap between theory and experiment is enormous: current theoretical and computational methods cannot realistically model folding processes with lifetimes longer than one nanosecond. Our unique approach was to employ laser pump-probe techniques that combine novel methods of laser flash photolysis with time-resolved vibrational spectroscopic probes of protein transients. In this scheme, a short (picosecond to nanosecond) laser photolysis pulse was used to produce an instantaneous pH or temperature jump, thereby initiating a protein folding or unfolding reaction. Structure-specific, time-resolved vibrational probes were then used to identify and characterize protein folding intermediates.

1. Background and Research Objectives

Proteins consist of a chain of amino acid residues covalently linked together by the peptide bond. The sequence of amino acid residues defines the exact nature of the protein, its structure, and function. In solution, proteins are found in compact dense structures. It is generally believed that most if not all proteins fold into their native compact structures in

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ways that are determined by primary sequence and the interactions between the component residues with themselves and with their surroundings. The molecular details of the folding process are under intense investigation, both theoretically and experimentally. At present, kinetic measurements of protein folding, or the reverse situation of protein unfolding, are performed by using stop-flow measurements techniques. The reversibly 'denatured' unfolded protein is rapidly mixed together with a solution to reverse the denaturing conditions. For example, high concentrations of urea or guanidine hydrochloride will unfold a protein and their rapid dilution will often initiate refolding. Of more interest to this project, extremes of pH (either high or low) will also unfold proteins, and neutralization will refold them reversibly in certain cases. In addition, proteins fold and unfold as a function of temperature. The time constant of these kinetic studies is the mixing time of the stop flow apparatus, generally around one millisecond.

Some intermediates are observed in the folding process with time constants on the millisecond to minute time scale. However, considerable changes in structure during the folding process occur in shorter times. These events have not previously been monitored, and a central goal of our work was to investigate and establish methods to observe early intermediates.

We have studied the folding/unfolding steps of small synthetic peptides, ribonuclease A, and apomyoglobin. Our approach was to employ novel laser temperature jump techniques to initiate protein unfolding/folding and time-resolved vibrational spectroscopy to follow the time-course of these reactions. The advantages of time-resolved vibrational measurements as structure-specific probes of chemical dynamics are well established. The structural specificity of vibrational spectroscopy derives from the connection of molecular vibrations to specific structures that determine the frequency, intensity and linewidths of the absorptions. In principle, infrared (IR) spectroscopy yields information on every discrete structure and interaction in the protein, because virtually all of the normal vibrations of the protein are infrared active. The amide vibrations are sensitive to secondary structure, while higher order structures can in some cases be probed via the vibrations of amino acid side chains.

2. Importance to LANL's Science and Technology Base and National R&D Needs

The Department of Energy's Office of Health and Environmental Research (OHER) has instituted a major initiative in structural biology in which two interrelated components of the initiative were developed in parallel. The initiative seeks i) to build up the unique facilities of the national laboratories as structural biology centers for external and internal

users and ii) to strengthen the in-house structural biology research programs to address the missions of the DOE. Stated missions are the structural problems associated with the Human Genome Project, with DNA damage and repair and the application of unique capabilities to important biological problems. This work enhances LANL's ability to respond to this OHER initiative. First, the work applies LANL's unique laser/protein dynamics facilities to an important problem in structural biology, in collaboration with an external (academic) user. Second, it directly addresses one of the stated DOE missions since one of the major structural problems associated with the Human Genome Project is the development of algorithms for the prediction, from primary structure (amino acid sequence), of the folding pathways of proteins to their native three-dimensional structures. This work complements ongoing LANL programs in protein dynamics. In addition, other Laboratory programs in biosciences benefit from both the molecular understanding of protein folding and the technology developed by this work.

3. Scientific Approach and Results

3.1. General Methodology

A number of small proteins show early transients in their folding/unfolding kinetics. We chose to begin our investigations by studying the kinetics of ribonuclease A and apomyoglobin, including peptide fragments which serve as models for the folding of small protein regions. Our main approach to the problems at issue was to employ laser pump-probe techniques on all relevant timescales (picoseconds to seconds). Protein folding/unfolding reactions were initiated using laser pumping schemes including laser-induced temperature jump. Time-resolved infrared techniques were used to identify and characterize the protein folding intermediates and their dynamics.

3.2. Laser Pump/Probe Methods

(a) *The Laser-Induced Temperature Jump (T jump)*. Our laser systems produce millijoule energy pulses throughout the IR range. In order to rapidly heat a protein in solution, a laser pulse at a wavelength where water has absorption is the 'pump' pulse in the T-jump experiments. The laser energy is absorbed by the water and the temperature of the volume of water in the laser beam is rapidly (on the sub-picosecond time scale) increased. Studies have shown that the temperature is transmitted across proteins on the 1-5 picosecond time scale. The water bands at ca. 3600 cm^{-1} are ideal for this purpose, partly because they are strong absorption bands and partly because proteins do not absorb at this wavelength. The laser 'pump' pulse was tuned to this band at a point where the absorption coefficient was

such that about 10 percent of the IR light was absorbed to insure uniform heating of the sample.

(b) *Time Resolved Infrared Spectroscopy (TRIRS)*. Three TRIRS approaches were used. The first was real-time TRIRS, a single-wavelength technique developed in our laboratories for obtaining infrared transients on time scales from tens of nanoseconds to seconds. For survey TRIR spectra on timescales of 1 μ s and longer, we have developed another less time consuming technique, time-resolved step-scan Fourier-transform infrared spectroscopy (TRS²-FTIRS) in collaboration with Richard Palmer at Duke University. The general method requires initiating a transient event (e.g. photolytically) and recording the temporal evolution of the IR signal at each point in the interferogram. The mirror is held in a fixed position while the event (photolysis) is repeated a sufficient number of times to obtain an adequate signal-to-noise ratio, then stepped to the next position (the number of positions determined by the resolution and free spectral range required). Processing of the data involves sorting by time in order to produce an interferogram for each time, which are subsequently Fourier transformed to yield a set of time resolved spectra.

c) *Results*. Laser-induced temperature jump followed by time-resolved infrared probe of reaction dynamics was used to study the temporal evolution of polypeptide structure during protein folding and unfolding. Reactions were initiated in times of 50 ps or longer by T-jumps of tens of degrees, obtained by laser excitation of water overtone absorbances. Observation of the Amide I transient absorbances reveal melting lifetimes of helices unconstrained by tertiary structure to be ca. 100 ns in a model 21-residue peptide and ca. 30 ns in "molten globule" apomyoglobin. No other processes are observed in these systems over the timescale 50 ps to 2 ms. Equilibrium data suggest the corresponding helix formation lifetimes to be ca. 10 and 1 ns, respectively. In "native" apomyoglobin two helix melting lifetimes were observed and we infer that a third occurs on a time scale inaccessible to our experiment (> 1 ms). The shorter observed lifetime, as in the molten globule, is ca. 30 ns. The longer lifetime is ca. 70 μ s. We suggest that the slower process is helix melting that is rate-limited by the unfolding of tertiary structure. Equilibrium data suggest a lifetime of ca. 1 μ s for the development of these tertiary folds.

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