PROTEIN FOLDING OBSERVED BY TIME-RESOLVED SYNCHROTRON X-RAY SCATTERING

A Feasibility Study

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ABSTRACT A solution to the "protein folding" problem, the successful prediction of tertiary and quaternary protein structure from amino acid or gene sequence, would be a major advance in biology and biotechnology. Knowledge of any intermediate structure between fully unwound and folded would aid folding calculations. The use of high intensity synchrotron x-rays from the SUNY X21 beamline at National Synchrotron Light Source has been investigated as a probe of structural changes during protein folding and unfolding in solution. A temperature jump apparatus was used to study thermally-induced folding and unfolding. Scattering of solutions of myoglobin in the angular range 2θ = 1–50 mrad. was measured during temperature jumps between 26 and 76°C. There are clear signs of time/temperature-dependent structural changes, in the small angle region, consistent with those from other equilibrium techniques. Analysis indicates that this experimental technique can be extended to the higher angle region where theoretical calculations indicate more detailed structural information, for example when alpha-helix formation, is present.

INTRODUCTION

One main goal of biology and biotechnology is to use man-made, or man-designed, proteins to improve on naturally occurring molecules in a wide variety of industrial, agricultural, and medical applications. Presently, the most usual approach is to perform site-specific mutagenesis on a naturally occurring molecule whose structure and mechanism of action is known. The effects of substituting or inserting one or two amino acids may be modeled theoretically, then tested experimentally. One disadvantage of this methodology is that it is restricted to improving upon processes which occur naturally. To design a protein from scratch for an arbitrary task, it is neccessary to be able to predict tertiary and quaternary protein structure from amino acid sequence data and to predict the molecule's activity from its structure. It is the former capability, the "protein folding problem," which is the concern of this study. A protein folding calculation starts with a randomly oriented polypeptide chain and seeks to predict tertiary structure. Usually, the only experimental structural evidence guiding the development of the algorithms used in these calculations is the crystallographic 3-D structure, i.e., the end point of the process. Any experimental evidence about intermediate structures on the folding pathway can help improve the calculations. The present study is to determine if a time-resolved solution x-ray scattering of proteins can be used as a source of such evidence.

The most illustrative uses of time-resolved scattering so far in the study of proteins in solution are the small-angle x-ray scattering studies of microtubule assembly induced by T-jump (Mandelkow et al., 1980; Bordas et al., 1983; Mandelkow et al., 1986) and of aspartate transcarbamylase dissociation by mercurials by stopped-flow techniques (Moody et al., 1980; Moody, 1986). In these studies high intensity synchrotron radiation SAXS cameras with linear position sensitive detectors and time-resolving readout electronics were used (Hendrix et al., 1979; Bordas et al., 1980). Measurements were made at low resolution, obtaining information at the 50 Å level at best. No information at higher scattering angles, where more detailed structural information is in principle present but count rates are lower, was obtained. The powerful synchrotron source was neccessary to provide the count rates from the weak-scattering protein systems. The present study, to the authors' knowledge, is the first experimental x-ray scattering result of a protein unfolding.

As a test case for these ideas, a temperature jump study of solutions of sperm whale myoglobin was chosen. The data for appropriate pH and temperature conditions for unfolding myoglobin is available. Myoglobin is readily obtainable in large quantities. The crystal structure is

known and the secondary structure is mostly alpha-helix. This last property will aid any data interpretation in the high angle region.

EXPERIMENTAL METHODS

Measurements were made at the SUNY X21 beamline at National Synchrotron Light Source (NSLS) (Phillips et al., 1986) which provides a photon flux of 3.5 x 10^{11}/s in a focal spot 0.9 x 0.6 mm (FWHM) at 1.54 Å with the NSLS ring operating at 2.5 GeV, 140 mA (Phillips et al., 1986; LeGrand et al., 1988). These present data were collected during single bunch operation of NSLS which lowered the stored current to 37–80 mA, with proportionately less x-ray flux. A linear position-sensitive detector, with positional resolution of 0.2 mm, and time-resolved MCA system (LeGrand et al., 1988) and x-ray scattering apparatus (Chu et al., 1985) were used for data acquisition. A collimation system, a pair of slits immediately upstream of the sample, was used to eliminate parasitic scattering from the beamline's focusing mirror. The detector was oriented vertically with a 1 mm wide slit in front. The minimum angle resolved is then limited by the vertical beam size and the sample-detector distance (set to 1 m), to ~1 mrad. The maximum angle is determined by the 50 mm active length of the detector to 50 mrad. An ion chamber upstream of the collimator was used to monitor the incident x-ray flux which was used to normalize scattered intensities to the falloff of flux as the ring current decays. The temperature jump apparatus consisted of a copper bar with a hole for the x-ray beam and with heating coils and cooling tubes attached. Copper sample holders with mylar windows were coated with heat transfer compound and clamped to the bar. Two thermocouples were attached to the holder, again with heat transfer compound. The T-jump was synchronized with data collection by a start pulse connected to relays which turned on or off the heater power supply and cooling water pump. The T-raise 26–76° was set to 9 min. and the T-drop to 5 min. The ion chamber, thermocouples, and the voltage on the monochromator feedback system (Phillips et al., 1986) were monitored during data runs.

With a powerful x-ray beam, as was used here, radiation damage to the sample is a concern. Before any T-jump work, irradiation studies on hen-egg-white lysozyme showed that it took ~50 min. before noticeable protein degradation effects occurred (LeGrand et al., 1988). The myoglobin samples were irradiated for similar times, but with less flux. The protein was purchased from Sigma Chemical Company, St. Louis MO (Lot number 036F0353). Individual samples were weighed dry and kept frozen until shortly before use. Samples were then dissolved at 18 mg/ml in sodium acetate buffer, pH 4.57, and used immediately. In the time available, four T-raise and five T-drop scans were obtained. One of the T-raise scans is shown in Fig. 1. Three of the four T-raise scans show the general pattern in the small angle region of a small first peak at ~65°C followed by a sharp rise to a maximum at 76°C. The fourth scan shows only the sharp rise, occuring immediately, and one of the T-drop experiments is similar. The other four T-drop scans show a pattern like the final pattern of the T-raise scans and unchanging through the scan.

RESULTS

A tentative interpretation of this data is (i) the high temperature maximum is unfolded and aggregated protein as the temperature corresponds to that for full unfolding for the buffer and pH used (Privalov et al., 1986), the scattering pattern is that of a higher molecular weight and/or radius of gyration and the effect is irreversible (ii) the transient 65°C peak in the T-raise data represents a partially unfolded state, with radius of gyration slightly

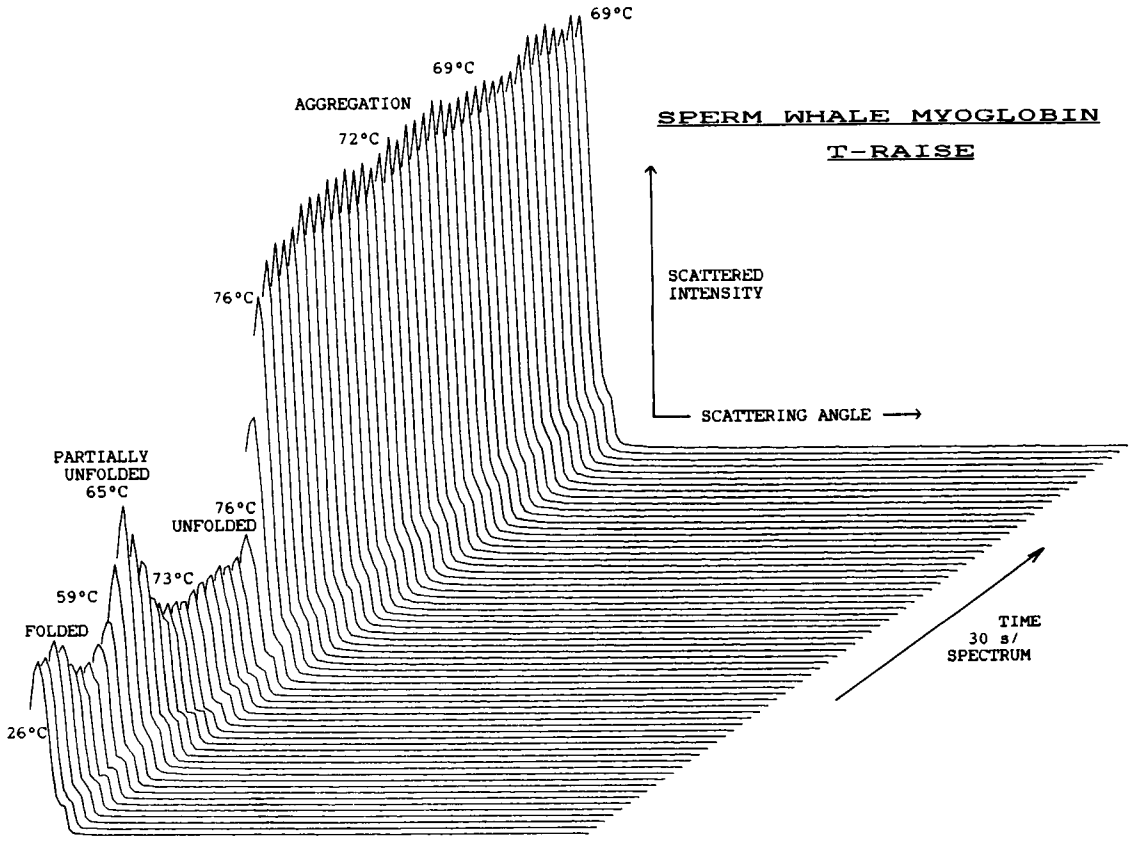

FIGURE 1 Time-resolved measurement of x-ray scattering of a solution of sperm whale myoglobin in the angular range 2θ = 1–50 mrad. as the temperature is raised.

larger than the intact protein. The first five patterns, as the temperature rises through 30°C, show some sign of the cold denaturation of myoglobin recently observed (Privalov et al., 1986).

Higher Angle Scattering

At best, the small-angle region can yield only the crudest of structural information, such as radius of gyration and deviation from spherical shape. Bordas and Mandelkow (1986) have discussed this in detail. To have impact on the protein-folding problem, greater details of structure formation are required. Fig. 2 shows a theoretical calculation of the scattering from an 8 residue alpha-helix and from a pair of such helices at various separations. The calculations used the Debye formula with angle-dependent atomic scattering factors. The absolute scale of scattering probability was fixed using formulae and values from the literature (Stuhrmann and Miller, 1978; Stuhrmann, 1980). There are clear differences in the patterns and other calculations show that helix length and angular orientation of two helices can be distinguished. However, the scattering probability at these angles for these relatively small particles is 10^{-4} that of myoglobin, which is itself 2 x 10^{-5}.

DISCUSSION

It is demonstrated that, with present high brightness synchrotron x-ray sources, changes in the small-angle x-ray scattering patterns of dilute protein solutions can be followed in a single pass experiment on the 10 s timescale. Averaging of passes would give proportionately better time resolution. Higher angle data, with detailed structural information is obtainable with a ten thousand-fold greater difficulty. An area, rather than a linear, detector and averaging of multiple passes should make possible such measurements. The availability of wiggler and undulator x-ray sources (See, for example, G. K. Shenoy and P. J. Viccaro, 1985), in combination with these experimental advances should allow work at a similar time resolution at high scattering angle as is possible now in the small angle region.

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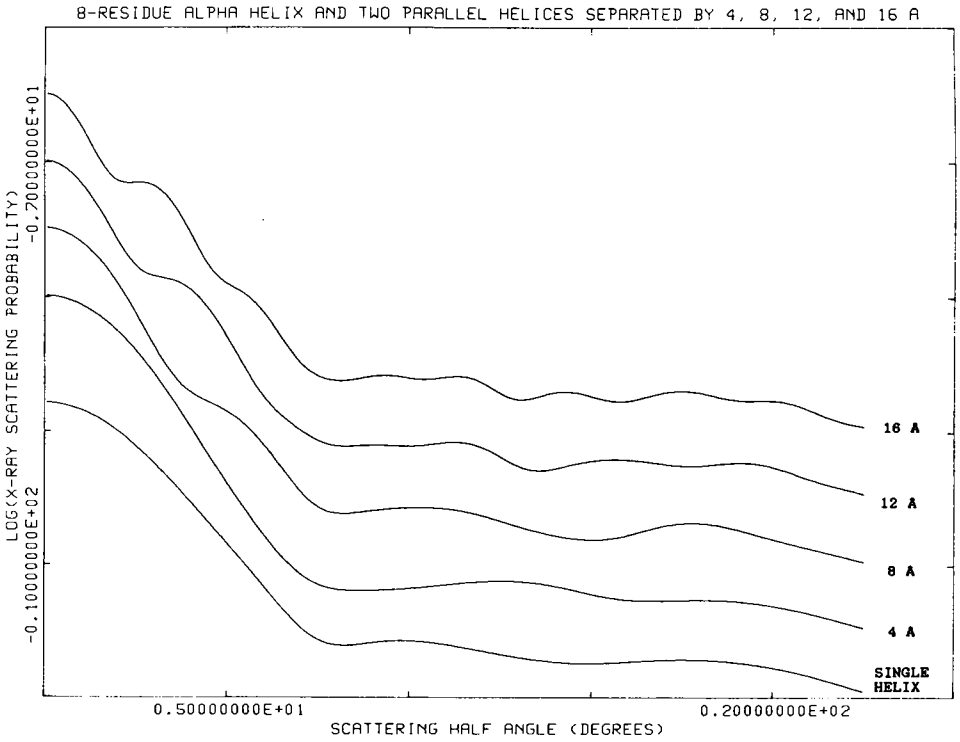

FIGURE 2 Theoretical scattering in 10 mMol aqueous solution of an 8-residue alpha-helix (*lowest curve*) and two parallel 8- residue alpha helices at separations (from next lowest upwards) of 4, 8, 12, and 16 Å. The curves have been separated vertically by constant increments of 0.5 for clarity. The second highest curve is on the correct scale.

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