Volume 221, number 1, 115-118 FEB 05055 August 1987

Rapid formation of secondary structure framework in protein folding studied by stopped-flow circular dichroism

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Received 20 May 1987; revised version received 6 July 1987

Kinetic refolding reactions of ferricytochrome *c* and β-lactoglobulin have been studied by stopped-flow circular dichroism by monitoring rapid ellipticity changes of peptide backbone and side-chain chromophores. In both proteins, a transient intermediate accumulates within the dead time of stopped-flow mixing (18 ms), and the intermediate has an appreciable amount of secondary structure but possesses an unfolded tertiary structure. It is suggested that the rapid formation of a secondary structure framework in protein folding is a common property observed in a variety of globular proteins.

Ferricytochrome *c*; β-Lactoglobulin; Protein folding; Folding intermediate; Stopped-flow; Circular dichroism

1. INTRODUCTION

Recent studies of folding reactions of α-lactalbumin, lysozyme and carbonic anhydrase B have revealed the transient accumulation of a compact intermediate that has a native-like backbone structure [1,2]. Because these proteins show relatively slow folding reactions over a time longer than 10 s under the conditions used in those studies, the detection and characterization of the transient intermediate could be made by conventional CD measurements. Folding of other proteins, however, often occurs much faster in time ranges down to milliseconds, thus requiring a rapid reaction technique such as stopped-flow CD for dealing with the problem of protein folding [3].

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Abbreviations: Cyt *c*, cytochrome *c*; βLG, β-lactoglobulin; GdnHCl, guanidine hydrochloride; N, U, native and unfolded states

In the previous report [4], one of the present authors has described the construction of a slit-type mixer specially designed for stopped-flow X-ray scattering. Here, a similar but modified slit-type mixer has been employed for studying rapid CD changes associated with the refolding reactions of Cyt *c* and βLG. The structural patterns of Cyt *c* (all α) and βLG (anti-parallel β-sheets) are different from each other and from those of the three proteins mentioned above [5,6] and hence are suitable for investigating whether the existence of a structural intermediate during folding is a general rule in globular proteins.

2. MATERIALS AND METHODS

Ferricytochrome *c* (type VI from horse heart, purchased from Sigma) was further purified as described in [7]. Separation and purification of the variants A and B of βLG were performed as in [8]. Protein concentrations were determined spectrophotometrically using the extinction coefficients, $\epsilon_{409\ nm} = 1.06 \times 10^5\ M^{-1} \cdot cm^{-1}$ for Cyt *c* and

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00145793/87/$3.50

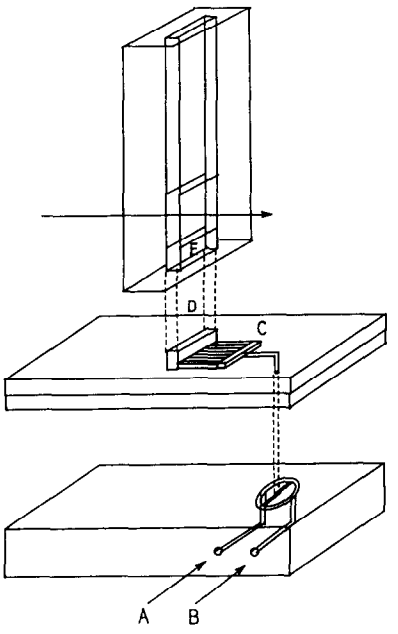

Fig.1. Diagrammatic representation of the modified slit-type mixer. Two solutions from inlets A and B are mixed in a double two-jet mixing part, divided into 8 flow lines at C and then expelled through a slit D (1 × 8 mm) to enter a flat observation cell E with laminar flow (see fig.2 of [4] for comparison).

$\epsilon_{278\ nm} = 1.77 \times 10^4\ M^{-1} \cdot cm^{-1}$ for βLG. CD values in the peptide and aromatic regions are expressed as mean residue molar ellipticities, and those in the Soret region as molar ellipticities of the heme group.

Equilibrium and kinetic CD measurements were carried out in a Jasco J-500A spectropolarimeter. The stopped-flow apparatus attached to the spectropolarimeter is largely similar to that presented previously [4] except for a modification made in the mixer to ensure more efficient mixing. Fig.1 shows a diagrammatic representation of the mixer used here. In refolding experiments, protein solution in the U state in concentrated GdnHCl was rapidly diluted with buffer solution with a volume ratio of 1:9–1:10. The optical path length of the observation cell was 1.0 mm for measurements in the Soret region of Cyt *c* and in the peptide region for the two proteins and 4.0 mm for aromatic CD of βLG. The dead time of stopped-flow mixing was 18 ms (1 mm cell) or 32 ms (4 mm cell) at a driving pressure of 4 atm.

3. RESULTS

3.1. *Equilibrium unfolding*

The equilibrium unfolding transitions of the two proteins were found to be reversible under our present conditions. The unfolding of Cyt *c* is essentially complete above 3.5 M GdnHCl and the protein is in the N state below 1.5 M GdnHCl, at both 4.5 and 25°C. βLG is in the U state above 4 M GdnHCl and in the N state below 1.5 M GdnHCl at 4.5°C; there was no difference detected between the two components of βLG. Fig.2 shows equilibrium CD spectra of Cyt *c* and βLG in the N and U states.

3.2. *Kinetics of folding and unfolding*

The refolding kinetics of Cyt *c* induced by a concentration jump of GdnHCl from 4 to 0.4 M were monitored by stopped-flow CD at 420 and 222.5 nm. The results at 25°C are shown in fig.3a,b, and similar results were also obtained at 4.5°C except that the apparent refolding rates observed were 10–20-fold lower at 4.5°C. The curve at 420 nm could be decomposed into three kinetic phases. The multiphasic kinetics of Cyt *c* refolding may be due, at least in part, to the presence of multiple unfolded species [9], and this itself is not necessarily a condition that satisfies the presence of a folding intermediate. The kinetically observed CD change, $\Delta\theta_{kinetic}$, during the time interval shown in fig.3a is -6.7×10^4 degree $\cdot$ cm^2 $\cdot$ dmol^{-1}, which is close to the value expected from the difference in ellipticity, $\Delta\theta_{equil.}$ ($= \theta_N - \theta_U$), between the N and U states (fig.2). However, at 222.5 nm (fig.3b), $\Delta\theta_{kinetic}$ (-2×10^3 degree $\cdot$ cm^2 $\cdot$ dmol^{-1}) is less than 20% of $\Delta\theta_{equil.}$, and most of the CD change associated with refolding occurs within the dead time of stopped-flow mixing at both 25 and 4.5°C. Such noncoincidence of the refolding curves measured at the different wavelengths demonstrates the existence of an early intermediate that is still unfolded when measured at 420 nm but has folded secondary structure as measured by CD at 222.5 nm [2].

Instantaneous formation of the secondary structure within the dead time was also observed in βLG folding. The kinetic refolding curves of βLG A measured at 293 and 219 nm are shown in fig.3c,d, the same results being obtained for βLG B. Also in this case, the theoretical fitting of the refolding

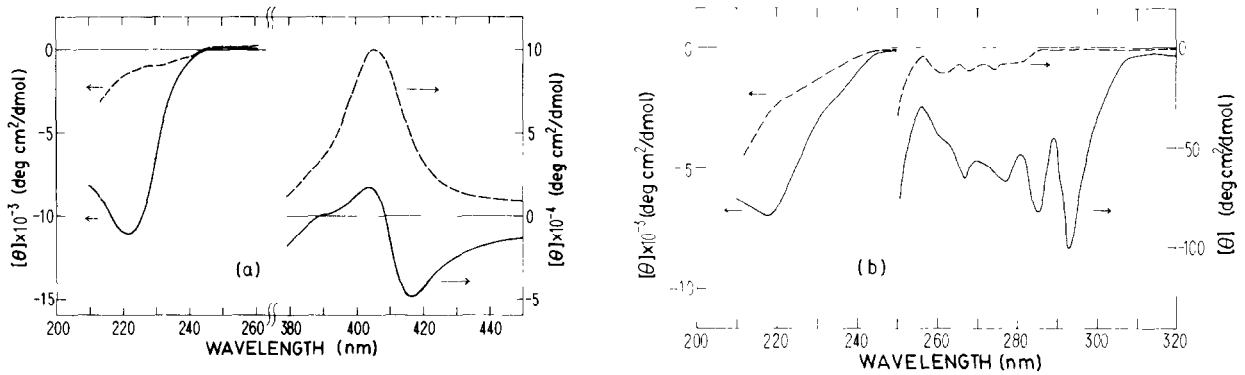

Fig.2. Equilibrium CD spectra. (a) Cyt *c* at pH 6.8–7.0 and 25°C (0.2 M NaCl-50 mM phosphate); and (b) βLG A at pH 3.2 and 4.5°C (0.1 M NaCl). (——) Spectra in the N state and (– – –) spectra in the U state in 4 M GdnHCl.

curve at 293 nm shows multiphasic kinetics (fig.3c). The CD value decreases with time as expected from the equilibrium CD spectra, and $\Delta\theta_{\text{kinetic}}$ observed in the first 505 s (-81.6 degree·cm^2·dmol^{-1}) is close to $\Delta\theta_{\text{equil.}}$. At 219 nm, however, the CD value increases with time ($\Delta\theta_{\text{kinetic}} = 3.2 \times 10^3$ degree·cm^2·dmol^{-1}) in spite of a negative value of $\Delta\theta_{\text{equil.}}$ (-4.0×10^3 degree·cm^2·dmol^{-1}). Therefore, at the beginning of the reaction, the intensity of the negative ellipticity is higher than that in the N state.

Unfolding kinetics induced by concentration jumps of GdnHCl from 0 to 3.6 M for Cyt *c* and from 0 to 4.0 M for βLG were investigated at 4.5°C at the wavelengths used in refolding experiments. In all cases, single-phase kinetics were observed, and the values of $\Delta\theta_{\text{kinetic}}$ were found to

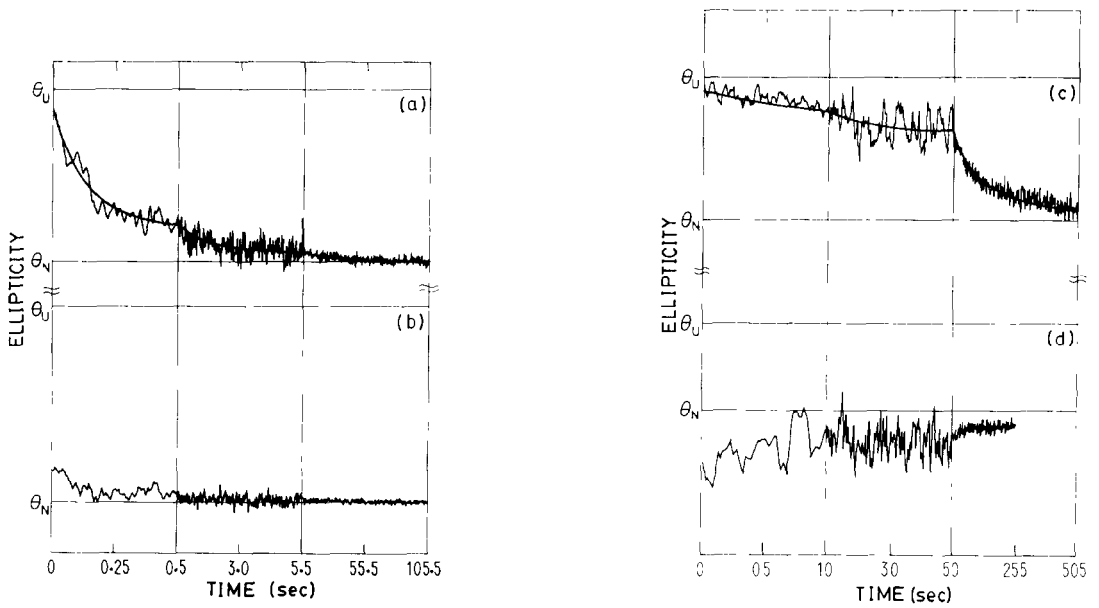

Fig.3. Kinetic refolding curves of Cyt *c* (pH 6.8–7.0, 25°C) and βLG (pH 3.2, 4.5°C) at 0.4 M GdnHCl. (a) 420 nm and (b) 222.5 nm for Cyt *c*, and (c) 293 nm and (d) 219 nm for βLG. θ_N and θ_U denote equilibrium CD values in the N and U states. The time axis in each figure is divided into three time domains. In (a,c), theoretical curves fitted with three exponential terms are also shown; the rate constants used in calculations were: (a) $k_1 = 3.75 \times 10^{-2}$, $k_2 = 8.65 \times 10^{-1}$, $k_3 = 8.36$ s^{-1}; (b) $k_1 = 2.33 \times 10^{-3}$, $k_2 = 3.46 \times 10^{-2}$, $k_3 = 1.25$ s^{-1}.

be very close to those of $\Delta\theta_{equil.}$, indicating that there is no kinetic intermediate in the unfolding reactions.

4. DISCUSSION

The present results demonstrate that the framework formation of a backbone structure during an early stage of folding is found in a variety of globular proteins with different structural patterns. Fine adjustment of the already folded structural segments may be the most difficult step in the folding reactions.

It is well known that the coil-helix transition of polypeptides is a very rapid process that can take place well within 10^{-5} s [10]. The analogy between instantaneous formation of a secondary structure in protein folding and the rapidity of the coil-helix transition of polypeptides has been discussed earlier [2]. However, the kinetics of β-structure formation are not well understood. Early work focused on poly(L-Lys) reported a slow process of β-structure formation [11], while a recent report on poly(L-Tyr) has demonstrated rapid formation of the β-structure with a lifetime of $\sim 10^{-2}$ s [12]. The present results indicate that the β-structure formation of βLG during folding is very rapid and complete within the stopped-flow dead time. It has been suggested theoretically that the rate of β-structure formation is sharply accelerated with an increase in β-structure stability [13].

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