Volume 223, number 2, 327–329 FEB 05246 November 1987

An early intermediate of refolding α-lactalbumin forms within 20 ms

Rudolf I. Gilmanshin and Oleg B. Ptitsyn

Institute of Protein Research, Academy of Sciences of the USSR, 142292 Pushchino, Moscow Region, USSR

Received 25 August 1987

The kinetics of α-lactalbumin refolding were studied by the stopped-flow method with the registration of CD and intrinsic fluorescence at several wavelengths. It was shown that the early kinetic intermediate forms during the dead-time of the experiment (20 ms). This intermediate has a considerable amount of secondary structure and unpolar clusters in its molecular structure but has no rigid tertiary structure.

α-Lactalbumin; Protein folding; Stopped-flow kinetic intermediate; Circular dichroism; Fluorescence

1. INTRODUCTION

The study of kinetic intermediates in protein refolding is important for understanding of protein self-organization [1]. It was shown that the refolding of several proteins goes through a compact [2,3] intermediate state with a pronounced secondary structure [2,4–6]. It was shown that this early intermediate forms during the dead-time of the experiment which in these experiments was no less than 2 s. Our goal was to obtain a more realistic upper estimation of the formation time of this intermediate.

To this end one of the authors (R.I.G.) has elaborated the stopped-flow attachment which permits one to register different optical parameters and to work with concentrated solutions of strong denaturants [7]. This attachment is used here in the study of the refolding of α-lactalbumin from the completely unfolded state by high concentrations of urea. The changes of secondary and tertiary structures were monitored by CD in far and near UV regions. The information on the solvation of tryptophan residues was obtained from fluorescence intensity measurements at different wavelengths. The preliminary results were published in [8].

Correspondence (present) address: R.I. Gilmanshin, Research Computing Center, Academy of Sciences of the USSR, 142292 Pushchino, Moscow Region, USSR

Abbreviations: BαLA, bovine α-lactalbumin; CD, circular dichroism; UV, ultraviolet; λ_{max}, position of the maximum of fluorescence spectrum; N and U, native and unfolded states of a protein

2. MATERIALS AND METHODS

BαLA, isolated as in [9], was a kind gift from Dr V.E. Bychkova. Its concentration was determined by the extinction coefficient $E^{1\%}_{1\,cm,280\,nm} = 20.9$ [10]. The experiments were carried out in different buffers at pH 7.5–8.0 containing 0.1 mM of $CaCl_2$. CD was measured by a Jasco-41A dichrograph slightly modified for kinetic experiments. Fluorescence was measured using the equipment described in [11] at an excitation wavelength of 285 nm.

Kinetic experiments were carried out with the aid of a stopped-flow attachment [7]. A flexible con-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

nection between the flow cell block and driving block was realized in this device. Moreover the external dimensions of the flow cell block are just the same as that of a standard spectrophotometer cell. These permit us to use the stopped-flow attachment in combination with conventional optical devices. The internal dimensions of a flow cell with a square cross-section are 3 × 3 × 5 mm. The block design allows the measurements of both the transmitted light and at right angles to the transmitted light. Special valves in the flow cell block cut off the reagent solution channels immediately after the flow stop. This leads to the diminishing of artefacts due to the density difference of reagent solutions [12]. The dead-time of the attachment was 5 ms. The circuit time in the CD measurement was limited by signal-to-noise ratio; therefore, in this case only the processes with half-times exceeding 20 ms were recorded reliably. Acquisition and treatment of kinetic curves were made as described in [13].

To determine λ_{max} we measured the fluorescence intensities $I(t)$ at two wavelengths (320 and 370 nm) which are at different sides from the spectrum maximum. The intensity ratio (parameter $A = I_{320}/I_{370}$) was used as a measure of λ_{max} displacement. The correspondence of A and λ_{max} was established by the comparison of equilibrium denaturation curves of BαLA tested using these parameters.

All experiments were carried out at 20°C.

3. RESULTS AND EXPERIMENTS

We have studied the refolding of BαLA from the unfolded state by 8.0 M urea. Fig.1 presents in a normalized form the kinetic curves of this process monitored by the changes of CD in far (226 nm) and near (272 nm) UV regions. The considerable change of ellipticity in the far UV region within the dead-time and the absence of change in the near UV region suggests the formation within less than 20 ms of the kinetic intermediate with a pronounced secondary structure but without a rigid tertiary structure. This result shows that the formation time of the secondary structure from an unordered protein chain is at least two orders of magnitude smaller than it follows from earlier data [5] where the dead-time was as large as 2 s.

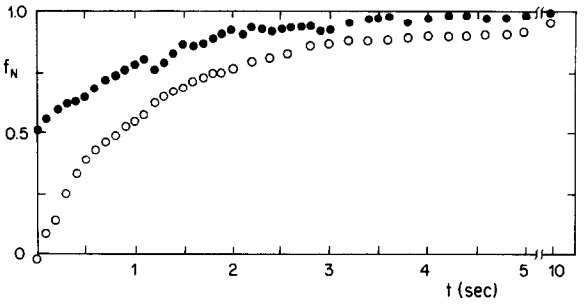

Fig.1. Kinetic curves of the refolding of bovine α-lactalbumin monitored by the changes of CD at 226 (—●—) and 272 (—○—) nm. The share of the native state $f_N = ([\theta]_t - [\theta]_U)/([\theta]_N - [\theta]_U)$ where $[\theta]_N$ and $[\theta]_U$ are the ellipticity values for the native and unfolded states and $[\theta]_t$ is the ellipticity value at the given time. The curves were obtained by averaging 10 experiments.

The measurements of the refolding kinetics of BαLA by the fluorescence intensity at 320 and 370 nm have shown that the parameter $A = I_{320}/I_{370}$ (see above) at dead-time reaches a value $A(t_d) = 0.74 \pm 0.04$ as compared with $A_U = 0.55 \pm 0.02$ for the U state. It corresponds to the blue shift of λ_{max} by $\approx$10 nm during the dead-time which shows that the environment of tryptophan residues became less polar. This result may reflect the appearance of unpolar clusters in the side chains.

The changes of fluorescence intensity and near UV ellipticity after the dead-time are biphasic and correspond to the processes with rate constants $k_1 = 0.9 \pm 0.2\ s^{-1}$ and $k_2 = 0.1 \pm 0.02\ s^{-1}$. The curve $[\theta]_{226}(t)$ has a small amplitude of the high noise level and can be approximated by a monophasic process with $k = 0.8 \pm 0.2\ s^{-1}$. The manifestation of the same process in the kinetic CD curves in the near and far UV regions may be explained by the contribution of aromatic groups in the far UV region [10]. However we think [14] that the more natural explanation connects the observed changes of CD in the far UV region with the completion of the secondary structure formation. In fact our refolding conditions (4.5 M urea, 20°C) differ more from the native ones than those used in [5] (0.3 M guanidine hydrochloride, 4.5°C). Therefore it is reasonable to assume that the kinetic intermediate observed in this paper is less ordered that that described in [5] because of the more stabilizing conditions.

ACKNOWLEDGEMENTS

The authors are indebted to Drs A.V. Finkelstein, G.V. Semisotnov, E.I. Shakhnovich and S.Yu. Venyaminov for fruitful discussions of the results and also to Dr V.E. Bychkova for the kindly provided sample of Bα-LA.

REFERENCES

[1] Kim, P.S. and Baldwin, R.L. (1982) Annu. Rev. Biochem. 51, 459–489.
[2] Dolgikh, D.A., Kolomietz, A.P., Bolotina, I.A. and Ptitsyn, O.B. (1984) FEBS Lett. 165, 88–92.
[3] Beasty, A.M. and Matthews, C.R. (1985) Biochemistry 24, 3547–3553.
[4] Matthews, C.R. and Crisanti, M.M. (1981) Biochemistry 20, 784–792.
[5] Kuwajima, K., Hiraoka, Y., Ikeguchi, M. and Sugai, S. (1985) Biochemistry 24, 874–880.
[6] Ikeguchi, M., Kuwajima, K., Mitani, M. and Sugai, S. (1986) Biochemistry 25, 6965–6972.
[7] Gilmanshin, R.I. (1987) Biofizika (USSR) 32, in press.
[8] Gilmanshin, R.I., Ptitsyn, O.B. and Semisotnov, G.V. (1986) Preprint (USSR), Pushchino, ONTI/NCBI.
[9] Armstrong, J.M., Hopper, K.E., McKenzie, H.A. and Murphy, W.H. (1970) Biochim. Biophys. Acta 214, 419–426.
[10] Dolgikh, D.A., Abaturov, L.V., Bolotina, I.A., Brazhnikov, E.V., Bushuev, V.N., Byckkova, V.E., Gilmanshin, R.I., Lebedev, Yu.O., Semisotnov, G.V., Tiktopulo, E.I. and Ptitsyn, O.B. (1985) Eur. Biophys. J. 13, 109–121.
[11] Semisotnov, G.V., Zikherman, K.K., Kasatkin, S.B., Ptitsyn, O.B. and Anufrieva, E.V. (1981) Biopolymers, 20, 2287–2309.
[12] Hiromi, K. (1979) Kinetics of Fast Reactions, John Wiley, New York.
[13] Gilmanshin, R.I. (1985) Biofizika (USSR) 30, 581–587.
[14] Gilmanshin, R.I., Ptitsyn, O.B. and Semisotnov, G.V. (1987) Biofizika (USSR) 32, in press.