Early events of protein folding Studied by cryo-stopped-flow Method

Hiroshi Kihara, Jinsong Li, Yoshikata Matsumura, Masaji Shinjo and Xianju Jin

Kansai Medical University, Department of Physics, 18-89 Uyama-Higashi, Hirakata 573-1136, Japan

Early events of protein folding are the fast process started from 10 ns to ms region. They have been investigated by laser T-jump method exclusively[1]. However, laser T-jump method is not so well suited to probes such as **circular dichroism (CD)** and **x-ray scattering (XS)**, where the former gives information of **secondary structure** and the latter gives information on the **global conformation**. We have been applying stopped-flow (SF) method combined with CD and XS. The drawback of the system is its time limitation due to mixing. We then started to develop SF system combined with CD and XS at low temperatures.

There are several problems to be sort out for utilizing the system at low temperatures. Firstly, at low temperatures, we need to add antifreeze such as ethylene glycol. This requires us to have a thorough mixing device, as viscosity is high. To achieve this requirement, we developed a special mixer combined 1:6 mixing and 4-jet mixer (Fig.1). This allows us to investigate protein folding with 6 ms dead time at –28C.

We have applied SF to oligopeptides of α-helix (C17 and AK16), bovine and equine β-lactoglobulins, ubiquitin, SH3 domain protein, apomyoglobin and lambda repressor.

α-Helices were formed very rapidly within the dead time of SFapparatus even at –55C. It was also the case on apomyoglobin (-20C) and pseudo wild-typed lambda repressor (-28C). Laser T-jump indicated the initial folding events of apomyoglobin[1] and pseudo wild-typed lambda repressor[2] were at a few to 30 μs. This indicates that the folding process could not be slowed down to ms region even at –28C.

It is well known that α-helical burst appeared at the early stage of folding of bovine and equine β-lactoglobulins. In case of bovine **β-lactoglobulin,** we found the α-helical burst occurred with two steps at –28C; one within the dead time of SF apparatus, and the other at the observable time range[3]. Kratky plot indicates the first intermediate already takes partially folded conformation. This process, however, could not be monitored in case of equine β-lactoglobulin.

In case of **ubiquitin**, there also observed an α-helical burst phase prior to the two-step transition, though native state of ubiquitin takes β-conformation dominantly[4].

We are then more interested in investigating folding process of β-rich proteins, and chose **src SH3 domain** protein as the next candidate of the study on protein folding. To be surprised, this protein also showed a large α-helical burst at its folding pathway. It is also the case in fyn SH3. The burst was investigated by stopped-flow x-ray solution scattering. The burst phase was already compact in terms of radius of gyration and the peak of Kratky plot.

To be interested, amplitudes of α-helical burst in all proteins investigated are proportional to the fraction of α-helices predicted by the simplest helix-coil transition. This strongly suggests that the first intermediate of the folding is the collapsed α-helices whose helical content is proportional to α-helical fraction predicted by helix-coil transition theory; unstable helices are collapsed and stabilized to form the folding core, which is then converted to either stable α-helix rich conformation or the stable β-sheet-rich conformation.

It should be noted that the present study demonstrates the importance of the x-ray solution scattering for the folding problem, specially at low temperatures.

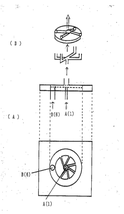


Fig. 1. Mixer

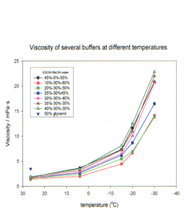
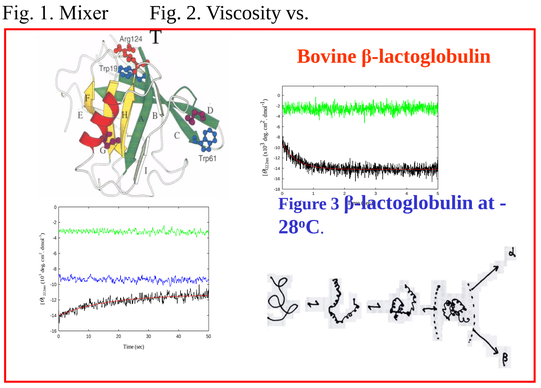
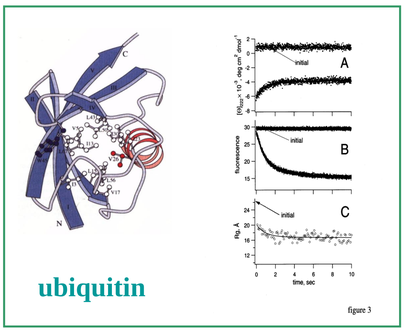
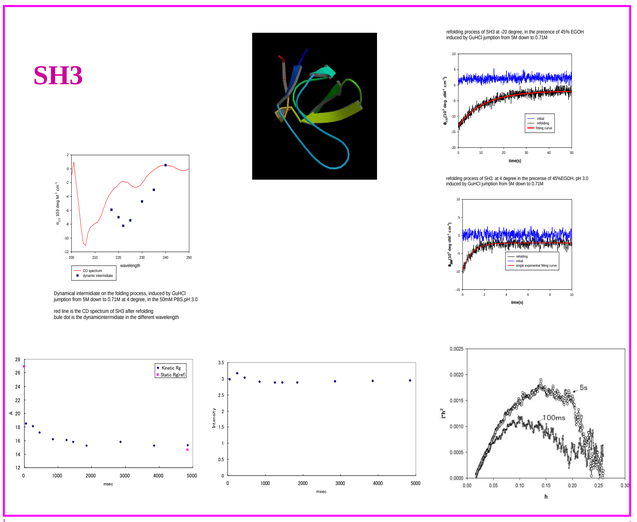
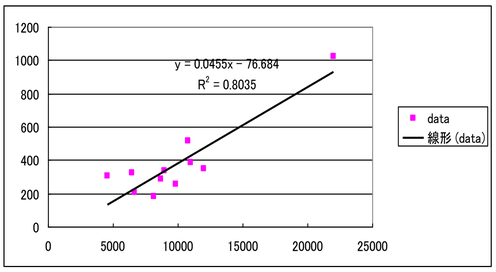


Fig. 2. Viscosity vs.









CD (cal) vs. CD (burst)

Reference

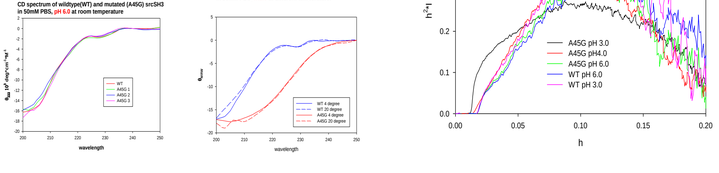
[1] Ballew RM, et al., Proc Natl Acad Sci U S A.**93** (1996) 5759-5764

[2] Yang WY, *et al*., Nature. **423** (2003) 193-197/ Biophys. J. **87** (2004) 596-608

[3] Qin Z, *et al*.FEBS Lett. **507** (2001) 299-302

[4] Qin, Z. *et al*. Phys. Chem. B **106**(2002) 13040-13046

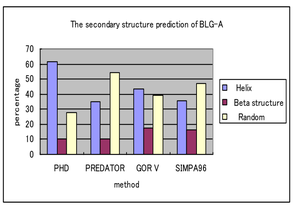
T-jump	
Initial collapse	10 ns
Alpha-helix formation	20 ns – 200 ns
Beta hairpin formation	a few us
Three-bundle helix	a few us
Myoglobin	a several us, ms

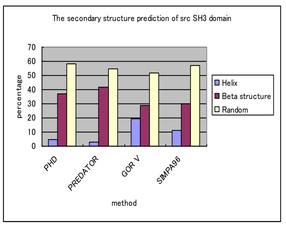


Equilibrium and Transient Intermediates of a mutant of src SH3 (A45G) are the same?

Xianju Jin[1], Jinsong Li[1], Masaji Shinjo[1], Yoshitaka Matsumura[1], Masami Ichikawa, and [O]Hiroshi Kihara[1]
•Dept of Physics, Kansai Medical Univ.

An α-helix-rich intermediate (burst phase) was found and studied on the folding pathway of a β-structure protein src SH3 domain. The α-helical content of the kinetic intermediate or transient intermediate (TI) was c. a. − 9,000 (deg.cm2.dmol−1) (CD value) and radius of gyration (Rg) of the T
I was 18.5 A. Judging from Kratky plot, the TI is compact [1]. A single mutant at 45 position of src SH3 domain from Ala to Gly (A45G) took a helix-rich intermediate at pH 3 in equilibrium (EI). The ellipticity of EI at 222 nm was c.a. − 10,000 (deg.cm2.dmol−1), while Rg was estimated to be 19.1 A.
From these results, it seems that TI and EI resemble with each other[2]. We, then, did refolding experiment of A45G at pH3 by means of x-ray scattering, which show no time dependent pathway, which demonstrates that TI was formed within the dead time of the apparatus, and showed no changes afterwards. This demonstrates that the short-lived TI is the same with EI in terms of alpha-helix content and radius of gyration. This result will be discussed with the results of pH-jump experiment from pH3 to pH6.

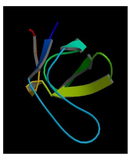




Conclusion

1. α-helical burst (TI) appeared on the folding pathway of src SH3.
2. The amplitude of α-helical burst and the predicted α-helical fraction by Helix2 are well correlated, which suggests the α-helical burst was formed by collapse of α-helices transiently formed.
3. A mutant, A45G showed an equilibrium intermediate (EI) at pH 3.
4. As far as Rg and CD are concerned, TI and EI are the same.
5. TI showed pH dependence.

wt src SH3



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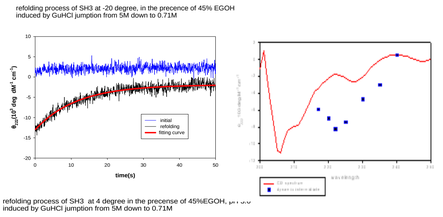
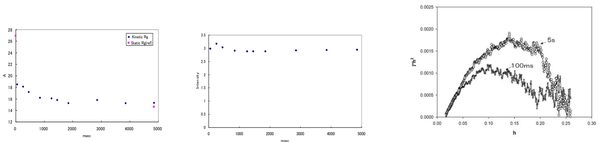
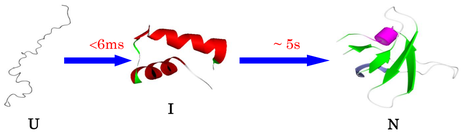






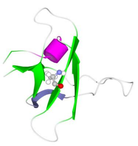
U; unfolded state, I; intermediate, N; native state.

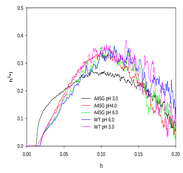
Folding scheme I

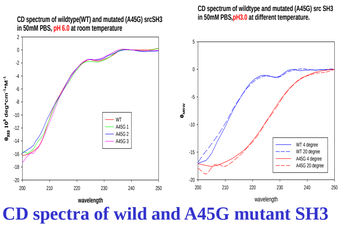
Table 1. *Rg* of wt SH3 and A45G at different pH

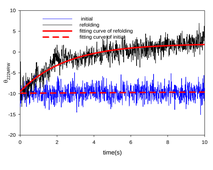
Protein	Condition	*Rg* from Guinier plot (Å)	*Rg* from Kratky plot (Å)[17]
A45G	pH 6.0	15.4	13.8
A45G	pH 4.0	17.3	16.2
A45G	pH 3.0	19.1	19.5
A45G	5M GuHCl	28	
WT	pH 6.0	15.1	14.6
WT	pH 4.0	14.8	15.2
WT	pH 3.0	15.3	14.7
WT	5M GuHCl	27	

A45G





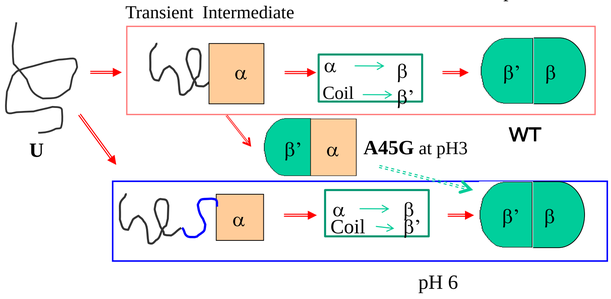




CD spectra of wild and A45G mutant SH3

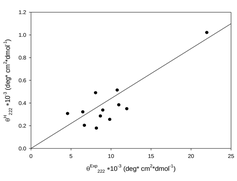
Table 2. Kinetically observed refolding CD-burst amplitude of WT SH3 and A45G at different conditions
* Observed CD signal of kinetic intermediate (deg*cm^2*dmol^{-1})

protein	temperature	condition	θ_{222}(deg*cm^2*dmol^{-1})	k_{obs} (s^{-1})
WT	20C	refolding	-6700*	14.7
	4C	refolding	-6500*	5.5
A45G	4C	refolding	-6200*	0.61
	4C	pH-jump	-10300	0.39



Folding scheme II

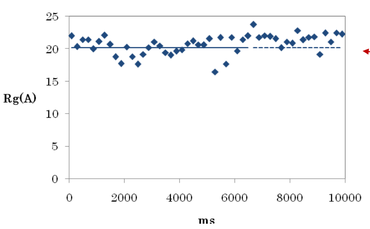
The calculated value of ellipticity at 222 nm by Helix2 (θ^H) vs the experimental value of ellipticity at 222 nm (θ^{Exp}) of the α-helical burst for 9 proteins.



CD (cal) vs. CD (burst)

θ^H is calculated according to the equation,

θ= f(H)/100* (-42500*(1-4/N)-640),
where f(H) is the fraction of helix calculated by Helix 2, and N is the residual number. Correlation coefficient betweenθ^H andθ^{Exp} . was estimated as r=0.83, demonstrating that α-helix in the burst is strongly correlated to the helical fraction formed in equilibrium with short time life time.



20.2 A

Averaged Rg of A45G at pH 3 and at 4C.

References
[1] Li *et al*. (2007) Biochemistry, 46, 5072-5082.
[2] Li *et al*. (2007) JMB, 372, 747-755.