**Research paper**

**α-Helical burst on the folding pathway of FHA domains from Rad53 and Ki67**

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**A R T I C L E   I N F O**

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**A B S T R A C T**

We investigated refolding processes of β-sheeted protein FHA domains (FHA1 domain of Rad53 and Ki67 FHA domain) by cryo-stopped-flow (SF) method combined with far-ultraviolet (far-UV) circular dichroism (CD, the average secondary structure content) and small angle X-ray scattering (SAXS, measuring the radius of gyration). In case of FHA1 domain of Rad53, no detectable time course was observed except the initial burst on its refolding process at 4°C, suggesting that the FHA1 domain of Rad53 was already refolded to its native state within the dead time of the SF apparatus and the rate of the refolding is too fast to be observed at this temperature. In contrast, there was an observable α-helical burst at $-15^\circ$C and $-20^\circ$C in the presence of 45% ethylene glycol (EGOH) by CD-SF. Besides, the radius of gyration (Rg) of the burst phase intermediate at $-20^\circ$C shows the intermediate is already compact, and the compaction process was accompanied with the decrease of α-helical content at the same temperature.

In case of Ki67 FHA domain, ellipticity change at 222 nm was observed on its refolding pathway at $-28^\circ$C in the presence of 45% EGOH and 2 mM DTT, indicating that Ki67 FHA domain also takes non-native α-helix-rich intermediate on its folding pathway. Time-resolved SAXS experiment was done. As the signal/noise ratio is low, we could not observe the time-dependent signal change through the time course. However, the initial Rg value was obtained as 18.2 ± 0.5 Å, which is much smaller than the unfolded Rg value (26.5 ± 1.2 Å), and is slightly larger than the native one (15.9 ± 1.8 Å).

These results suggest that Ki67 FHA domain also forms compact non-native α-helix-rich intermediate before refolding to its native β-structure on the refolding pathway.

These results are in good agreement with other β-proteins, such as bovine β-lactoglobulin (BLG), src SH3 domain proteins. It seems the α-helical burst phases appear on the folding pathway of β-sandwiched proteins.

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1. Introduction

Recently, we have reported the folding processes of α-helical-rich transient intermediate of src SH3 domain is rich in α-helix and compact globule state, a little bigger (19 Å) than its native state (15 Å), and much smaller than its denatured state (27 Å). The mutant, A45G, forms α-helix-rich intermediate at pH 3 even at equilibrium, and forms α-helix-rich folding transient intermediate from pH 3 to 6 [4]. Both equilibrium and transient intermediates...
are compact as the transient intermediate of the wild type src SH3 domain [4]. In addition to these β-proteins, α-helical bursts have also been observed in 6 proteins (horse heart apomyoglobin, Fyn SH3 domain, ubiquitin, lambda representer, hen egg lysozyme and bovine ribonuclease A) under various conditions [3]. The ellipticities at 222 nm (θ̂_{222}) of the burst phase intermediates of all investigated proteins are well correlated to the ellipticities at 222 nm (θ̂_{222}) calculated by program Helix2 [3].

These results seem to demonstrate that all proteins, including β-rich proteins such as β-lactoglobulin and SH3, takes transient α-helix-rich intermediate on their folding pathway. Thus, we have a very simple question; do β-structure proteins other than β-lactoglobulins and SH3 domains, also take the same folding processes, forming α-helix-rich transient intermediate on their folding pathways? If yes, are the ellipticities at 222 nm (θ̂_{222}) of the burst phase also well correlated to CD ellipticities at 222 nm (θ̂_{222}) calculated by Helix2? To answer these questions, we started investigating the folding processes of two FHA domains (FHA1 of Rad53 and Ki67), as other β-proteins.

FHA1 domain of Rad53 is composed 151 amino acid residues and consists of 11 β-strands; β1–β10 are anti-parallel, β3–β3’ are parallel β-strands, and 3 α-helices. The shape of FHA1 domain of Rad53 is a β-sandwich typed, which is formed by two large twisted anti-parallel β-sheets [10,11]. Ki67 FHA domain is composed 120 amino acid residues and consists of only β-sheets and no helices [12]. This protein is also a β-sandwich typed. Folding processes of β-sandwiched proteins such as both FHA domains have not been studied so far. It is then of interest to investigate folding processes of both proteins, even if the hypothesis described above does not hold in these proteins.

2. Materials and methods

2.1. Materials

We have studied FHA1 domain of Rad53 and Ki67 FHA domain. FHA1 domain of Rad53 was expressed in E. coli and purified as described [10]. Yeast Rad53, a check point protein that prevents cell division when DNA is damaged or incompletely replicated, contains two highly divergent FHA domains: FHA1 in the N-terminal region and FHA2 in the C-terminal region [10]. Human Ki67 protein is involved in the protein interaction network that drives cell division cycles, which contains a FHA domain [12]. Ki67 FHA domain was also expressed in E. coli and purified as described [12].

Guandine hydrochloride (GuHCl) is of ultra pure reagent grade from ICN Biomedicals Inc. (Lot No. 3814J). The concentration of GuHCl was calibrated by refractive index measurements [13,14] The temperature was controlled within ±1 °C by a temperature controller ULT-80 of NESLAB.

2.2. Stopped-flow apparatus

The stopped-flow device was constructed for special use of high viscosity and low temperature in collaboration with Unisoku Inc [1–4]. Its dead time was estimated to be c.a. 6 ms by the reduction of 2, 6-dichloroindophenol by ascorbic acid at 4 °C and −28 °C (data not shown) [1–4].

2.3. CD measurements at equilibrium

The samples of FHA1 domain of Rad53 were prepared in 50 mM phosphate buffer at pH 6.5 with various concentrations of GuHCl in the presence and absence of 45% EGOH, respectively. The concentration of the protein was 20–30 μM. CD measurements were performed at various temperatures with a spectropolarimeter specially designed by Unisoku Inc. Cuvettes of 1 mm path-length were used for all measurements.

2.4. Time-resolved refolding CD measurements

Time-resolved refolding experiments were done by diluting unfolded protein solution with the excess amount of buffer. Stopped-flow apparatus was used for quick dilution.

In the present case, one volume of FHA domain protein solution with 5 M GuHCl was mixed with 6 volumes of buffer, thus giving 0.7 M GuHCl after mixing. In case of FHA1 domain of Rad53, the protein was dissolved in 50 mM phosphate buffer at pH 6.5 with 5 M GuHCl in the presence and absence of 45% EGOH, and then the solution was diluted with 6 times refolding buffer (0 M GuHCl) so as to initiate the refolding. In case of Ki67 FHA domain, the protein was first dissolved in 50 mM phosphate buffer and 2 mM DTT at pH 7.5 with 5 M GuHCl in the presence of 45% EGOH and the solution was diluted with 6 times refolding buffer (0 M GuHCl) so as to initiate the refolding. In both cases, the final concentration of GuHCl was 0.7 M, and the final concentrations of FHA domain proteins were 10–30 μM.

The refolding process was monitored by CD at 222 ± 2 nm (θ̂_{222}) at each temperature. Measurements were repeated, and the time-dependent signals were accumulated to get a good signal/noise ratio. The averaged data were normalized to give molar ellipticity.

At each condition, in addition to the refolding experiments described above, two more experiments (initial and final) were always done; mixing the unfolded protein in the unfolded buffer with the same unfolded buffer. This gives us the initial CD level. After the refolding experiments, we left the solution long time at the same condition, and then measured CD. This gives us the final level. Usually, we measured the final level 20–30 min after the folding reaction initiated. In many cases, the first level of the refolding experiments differs from the initial level, indicating the existence of the burst phase. Also, in some cases, the last level (the averaged value of the last ten or thirty data points of the corresponding time frames) of the refolding did not coincide with the final level, indicating the existence of another phase between the last level and the final level.

2.5. SAXS measurements

Small angle X-ray solution scattering measurements (SAXS) were performed at beamline 15A of the Photon Factory at KEK (Tsukuba, Japan) [15,16]. The X-ray wavelength was λ = 1.504 Å with the CCD detector for both kinetic and static measurements [17,18].

Time-resolved SAXS measurements of FHA1 domain of Rad53 and Ki67 FHA domain were performed at −20 °C and −28 °C, respectively as described elsewhere [19,20]. As in case of CD, in addition of the refolding experiments, experiments for getting initial and final levels were done.

Helium gas was flowed in and around the observation cell in order to prevent frost from forming on the window. X-ray scattering data were taken both on protein solutions and on the corresponding buffers. The scattering data of the buffer were subtracted from those of the protein solution. X-ray scattering data were analyzed by Guinier approximation, assuming an exponential dependence of the scattering intensity on h², where h = 4πsinθ/λ, and θ is half of the scattering angle [21]. The linear Guinier region was selected to obtain a radius of gyration (Rg) and zero angle intensity (I₀), where the value of Rg*λh is ≤ 1.3.
Equilibrium experiments were performed using a static-flow cell at 4 °C for preventing radiation damage of the proteins [22]. Concentrations of both FHA domains were less than 140 μM.

3. Results

3.1. GuHCl-induced unfolding at equilibrium and the kinetic study of the refolding of FHA1 domain of Rad53 at 4 °C

We first investigated the GuHCl-induced unfolding of FHA1 domain of Rad53 at equilibrium by the measurements of far-UV CD at 4 °C. In Fig. 1, typical far-UV CD spectra at 0.50 M GuHCl and at 5.2 M GuHCl are shown. The spectrum at 5.2 M GuHCl is typical of the unfolded protein. GuHCl concentration dependence of ellipticities at 222 nm ([θ]_{222}) is shown in Fig. 2a. The CD value at 222 nm does not change below 1 M GuHCl, whereas it increased above 1 M GuHCl and reached the saturated value above 2 M GuHCl. The CD value again increased gradually above 4 M GuHCl, showing another saturated value above 6 M GuHCl. As the spectrum at 0.50 M GuHCl is the same with that at 0 M GuHCl, we speculate that the protein with GuHCl below 1 M takes the native conformation.

Next, we performed kinetic refolding experiments of FHA1 domain of Rad53 from the denatured state in 5 M GuHCl to the native state by performing the experiments at subzero temperature by using the SF apparatus. This suggests that the refolding process was too fast to be observed at this temperature.

From these results, it is likely that FHA1 domain of Rad53 was already refolded to its native state at 4 °C within the dead time of the SF apparatus. This suggests that the refolding process was too fast to be observed at this temperature.

Since detectable refolding process of FHA1 domain of Rad53 was not observed at 4 °C, we carried out kinetic refolding experiments at subzero temperatures. Previously, we reported that refolding rate of other proteins could slow down due to viscosity and temperature by performing the experiments at subzero temperatures [1,2,23–26]. We used EGOH as cryo-solvent in our previous studies [1,2,23–26].

EGOH does not affect significantly on the protein conformation [1,2,27]. We tested the effect of EGOH on FHA1 domain of Rad53 at 4 °C by means of far-UV CD. Results showed the CD values of FHA1 domain of Rad53 at 222 nm did not change at EGOH lower than 60%. Above 70% EGOH, far-UV CD spectra were dramatically changed. UV absorption spectra also changed due to light scattering (data not shown). This indicates that FHA1 domain of Rad53 showed aggregates above 70% EGOH. Thus, we concluded that FHA1 domain of Rad53 takes native β-structure in EGOH lower than 60%. Hereafter, we use 45% EGOH as a cryo-solvent as in cases of other proteins [1,2,23–26].

As it was reported above in case of 4 °C, we also investigated GuHCl-induced unfolding of FHA1 domain of Rad53 at −20 °C in the presence of 45% EGOH by far-UV CD. In Fig. 1, far-UV CD spectra

![Fig. 1. Far-UV CD spectra of FHA1 domain of Rad53 in 50 mM phosphate buffer at pH 6.5 with various concentrations of GuHCl. 0.50 M in the absence of 45% EGOH at 4 °C (black), 0.52 M in the presence of 45% EGOH at −20 °C (red), 5.2 M in the absence of 45% EGOH at 4 °C (blue), 4.9 M in the presence of 45% EGOH at −20 °C (green). In short wavelength region, the data are not shown due to the absorption of GuHCl impurities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).](image1)

![Fig. 2. [θ]_{222} dependence of unfolding transition of FHA1 domain of Rad53 on GuHCl concentration in 50 mM phosphate buffer at pH 6.5. (a) in the absence of 45% EGOH at 4 °C, (b) in the presence of 45% EGOH at −20 °C. A continuous line represents the theoretical curve based on equation (1). Broken lines indicate the initial and final values.](image2)
of 0.52 M GuHCl and 4.9 M GuHCl are presented in the presence of 45% EGOH at −20 °C. The spectrum at 0.52 M GuHCl at −20 °C is the same with that at 0.50 M GuHCl at 4 °C, and the spectrum at 4.9 M GuHCl at −20 °C is the same with that at 5.2 M GuHCl at 4 °C. The ellipticities at 222 nm ([θ]222) of FHA1 domain of Rad53 at −20 °C are plotted against GuHCl concentration in Fig. 2. The transition midpoint was slightly shifted to higher GuHCl concentration than the case in the absence of 45% EGOH at 4 °C (1.6 M–2.3 M). This indicates that FHA1 domain of Rad53 was more stabilized in the cryo-solvent. It is also found that ellipticities at 222 nm ([θ]222) did not change below 1.5 M GuHCl. In contrast, ellipticities at 222 nm ([θ]222) above 3 M GuHCl increased slightly with the increase of GuHCl concentration (Fig. 2b). Since the spectrum at 0.52 M GuHCl is the same with that at 0 M GuHCl as in the case at 4 °C in the absence of 45% EGOH, we speculate that the protein with GuHCl below 1.5 M takes the native conformation.

As FHA1 domain of Rad53 takes the native state below 1.5 M GuHCl and the unfolded state at 4.9 M GuHCl at −20 °C, we performed kinetic refolding experiments by CD-SF as in the case at 4 °C. The results are shown in Fig. 3b. There are three aspects in the kinetic refolding experiments. First, an α-helical burst phase appeared within the dead time of the SF device. This shows that FHA1 domain of Rad53 formed an α-helix-rich transient intermediate on its folding pathway. Second, refolding curve shows a detectable time course (black line). From a single exponential fitting curve (red line in Fig. 3b), the ellipticity at 222 nm ([θ]222) was changed from c. a. −9400 deg cm2 dmol−1 to c.a. −7500 deg cm2 dmol−1. This did not reach the final level within the time scale yet (Fig. 3b), showing that FHA1 domain of Rad53 was not refolded to its native state within 20 s in the presence of 45% EGOH at −20 °C. Third, the final level observed 30 min after the initiation of the refolding was c. a. −4000 deg cm2 dmol−1.

As the ellipticity of the native state is −5000 deg cm2 dmol−1 in the presence of 45% EGOH at −20 °C (Fig. 2b), the final level of the refolding is in good agreement with the ellipticity of the native state within experimental error.

We also performed kinetic refolding experiments in the presence of 45% EGOH at another temperature, −15 °C (Fig. 3c). There appeared also an observable α-helical burst phase within the dead time of the SF apparatus. The ellipticity at 222 nm ([θ]222) of the burst phase was c. a. −7900 deg cm2 dmol−1 (red line in Fig. 3c). This value is very similar with that of the last level of the observed time course (black line, −7500 deg cm2 dmol−1) at −20 °C (Fig. 3b). Besides, the ellipticity at 222 nm ([θ]222) of the last level of the refolding curve at −15 °C (−4800 deg cm2 dmol−1) was also very similar with the average value of the refolding curve in the absence of 45% EGOH at 4 °C (Fig. 3c and black line of Fig. 3b. −5200 deg cm2 dmol−1) and with the ellipticity of the native state in the equilibrium experiments (−5000 deg cm2 dmol−1, Fig. 2a and b). This indicates that FHA1 domain of Rad53 was refolded to its native state within the time scale in the kinetic refolding experiments at this condition.

3.3. SAXS experiments of FHA1 domain of Rad53

First, SAXS experiments of FHA1 domain of Rad53 were performed at equilibrium with the use of static-flow cell. As mentioned in Materials and methods, static-flow cells were used to prevent proteins from radiation damage [22].

Fig. 4 shows Guinier plots of the native and the unfolded states of FHA1 domain of Rad53 in the presence of 45% EGOH at 4 °C. From Guinier analysis, Rg values of the native and denatured states were obtained as 21.2 ± 0.4 Å and 35.5 ± 1.6 Å, respectively. Kratky plots of both native and denatured states were also obtained and shown in Fig. 5. The native state of FHA1 domain of
Rad53 shows obviously a peak (solid line in Fig. 5), whereas the denatured state of the protein has no peaks (dashed line in Fig. 5). These results indicate that the native conformation of FHA1 domain of Rad53 was compact and globule, and the unfolded state was not. These results are consistent with those obtained by CD experiments at equilibrium.

Next, we carried out kinetic refolding experiments in the presence of 45% EGOH at −20 °C by SF combined with SAXS in order to monitor the refolding process [28]. The protein in 5 M GuHCl was mixed with the buffer (6 times large volume), reaching 0.7 M GuHCl after mixing, which was monitored with X-ray scattering. Obtained Guinier plots at each time frames do not show straight lines even in the small angle region, indicating aggregation. So, we used a generalized Guinier formula with exponential terms as previous studies [25,28]. Fig. 6 shows time-resolved Rg change on FHA1 domain of Rad53 refolding by GuHCl-jump. Although the data in Fig. 6 is noisy, we could obtain refolding time course and a single exponential fitting curve of the refolding data (solid curve). There are two phases, a burst phase and an observable phase in the kinetic refolding experiments. The Rg of FHA1 domain of Rad53 was largely decreased from 35.5 Å to 25.0 Å within the dead time of the SF (=burst phase). Then, Rg of the refolding trace was decreased with a single exponential decay (=observable phase). The Rg of the burst phase was 25.0 Å. This value is a little larger than that of the native state (21.2 Å), and much smaller than the denatured state (35.5 Å). This suggests that α-helix-rich transient intermediate in the presence of 45% EGOH at −20 °C was compact and its size is slightly bigger than the native structure (21.2 Å) of FHA1 domain of Rad53. The rate of the refolding process was 0.42 s⁻¹. This value was very similar with that of the decrease of α-helical content process by CD-SF at the same conditions (0.25 s⁻¹, Figs. 3 and 6b). These results indicate that compactness process and structural change of the secondary structure of FHA1 domain of Rad53 were simultaneously performed. Furthermore, the Rg of the refolding process did not reach the native value (21.2 Å) within the time scale. This result is also corresponding to that of the kinetic refolding experiments by CD. It is considered that the α-helix-rich transient intermediate of the burst phase in the presence of 45% EGOH at −15 °C was also compact size and a little larger than the native structure of FHA1 domain of Rad53.

3.4. Refolding of Ki67 FHA domain

We have tried to observe refolding processes of another FHA domain, Ki67 FHA domain, by cryo-CD-SF method. Native topology of the protein has only β-sheets and no any helices [12].

First, we performed kinetic refolding experiments of Ki67 FHA domain in the absence of DTT with 45% EGOH at −20 °C. No detectable time course was observed. The kinetic refolding trace was overlapped with the final level (Fig. 7a and Table 3), indicating the protein was refolded within the dead time of the apparatus. As the unfolded protein solution was easy to aggregate, we added 2 mM DTT for the further experiments.

Next, we performed Ki67 FHA domain refolding experiments by GuHCl-jump in the presence of 45% EGOH and 2 mM DTT at −28 °C. The result is shown in Fig. 7b. As seen in the figure, a detectable time course was observed with the rate constant of 7.01 ± 1.69 s⁻¹. The last level of the refolding curve and the final level are in agreement within the experimental error. The absolute ellipticity at 222 nm was not so stable in this experiment due to the instability of the CD apparatus. We repeated the refolding experiments at the same condition several times. It is clear the rate limiting folding
Table 3. Native structure and GuHCl-induced unfolding of FHA1 domain of Rad53

<table>
<thead>
<tr>
<th>GuHCl concentration (M)</th>
<th>a-helix (%)</th>
<th>b-sheets (%)</th>
<th>Random coil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>15.0 ± 0.1</td>
<td>43.0 ± 0.2</td>
<td>42.0 ± 0.3</td>
</tr>
<tr>
<td>0.50</td>
<td>16.0 ± 0.2</td>
<td>43.0 ± 0.3</td>
<td>41.0 ± 0.2</td>
</tr>
<tr>
<td>1.00</td>
<td>17.0 ± 0.3</td>
<td>43.0 ± 0.4</td>
<td>40.0 ± 0.1</td>
</tr>
</tbody>
</table>

Fig. 8. Guinier plots of native (0 M GuHCl, circle) and denatured state (5 M GuHCl, triangle) of Ki67 FHA domain in 50 mM phosphate buffer at pH 7.5 in the presence of 45% EGOH and 2 mM DTT at 4 °C. The solid line is fitting line of native state and the dashed line was fitting line of denatured state. By these fitting. Rg values were 15.9 ± 1.8 Å (native state) and 26.5 ± 1.2 Å (unfolded state).

Fig. 9. Kinetic refolding of Ki67 FHA domain of time-resolved SAXS measurements in 50 mM phosphate buffer at pH 7.5 in the presence of 45% EGOH and 2 mM DTT at 28 °C by SF. The solid line is fitting line. Rg was 18.2 ± 0.5 Å from the fitting.
We analyzed GuHCl-induced unfolding transitions of the protein as the two-state (native and unfolded) model. At the two-state model, the two unfolding transitions were analyzed by equation (1), in which the observed value ($\theta_{obs}$) of the ellipticity at 222 nm is represented as a function of GuHCl concentration ($c$) [6]:

$$
\theta_{obs} = \frac{\theta_N + \theta_U \cdot \exp[-m(c_M - c)]}{1 + \exp[-m(c_M - c)]}
$$

where $c_M$ is the $c$ at the midpoint of unfolding transition, $m$ represents the cooperativity of the transition, and $\theta_N$ and $\theta_U$ are the ellipticity values in the pure N and U states, respectively. In general, $\theta_N$ and $\theta_U$ are also dependent on $c$, i.e., $\theta_N = \theta_1 + \theta_2 c$ and $\theta_U = \theta_3 + \theta_4 c$. The obtained thermodynamic parameters are that $m$ is $6.88 \pm 1.42 M^{-1}$, $c_M$ is $1.52 \pm 0.05 M$ in the absence of 45% EGOH at 4°C, and $m$ is $5.96 \pm 0.76 M^{-1}$, $c_M$ is $2.26 \pm 0.05 M$ in the presence of 45% EGOH at $-20°C$, respectively. The GuHCl concentration of the transition midpoint of the latter was 0.7 M higher than the former, and $m$ value of the latter is smaller than the former one. As described above (in Results section), this suggests that FHA1 domain of Rad53 was more stabilized by cryo-solvent as observed mutants of ubiquitin and lambda repressor [24,26].

4.2. Folding pathway of FHA1 domain of Rad53

We observed a non-native $\alpha$-helix-rich transient intermediate of FHA1 domain of Rad53 on its refolding pathway. However, the refolding profiles at 4°C, $-15°C$ and $-20°C$ are not easily understandable. At 4°C, only a burst phase observed. The ellipticities at 222 nm of the burst phase and the last level (the level 2s after initiation) are $-5200$ deg cm$^2$ dmol$^{-1}$, which is in good agreement with that measured at equilibrium ($-5000$ deg cm$^2$ dmol$^{-1}$). At $-15°C$, the ellipticity at 222 nm of the burst phase ($-7900$ deg cm$^2$ dmol$^{-1}$) is less than that at equilibrium ($-5000$ deg cm$^2$ dmol$^{-1}$). At $-20°C$, the ellipticity at 222 nm of the burst phase is $-9400$, much less than that of the burst phase at $-15°C$ ($-7900$ deg cm$^2$ dmol$^{-1}$), and in addition, the ellipticity at 222 nm of the last level (20 s after the initiation) is $-7500$ deg cm$^2$ dmol$^{-1}$, different from the final level at $-20°C$ ($-3800$ deg cm$^2$ dmol$^{-1}$). It is more easily overviewed in Fig. 10. In the figure, the ellipticities at 222 nm ($\theta_{222}$) of the burst phases and the last levels of refolding traces at each condition are summarized. As seen in the figure, the ellipticity of the last level of the refolding trace (triangle) at $-20°C$ and that of the burst phase (circle) at $-15°C$ is almost the same, and the ellipticity of the last level of the refolding trace (triangle) at $-15°C$ and that of the burst phase (circle) at $4°C$ are also nearly the same.

To reach an integrated view, all data of ellipticities at 222 nm at each condition are summarized in Tables 1 and 2. In the same tables, $R_g$ values at the same conditions are also summarized.

From Fig. 10 and Tables 1 and 2, the following scheme can be proposed.

$$(U) \rightarrow (I_1) \rightarrow (I_2) \rightarrow (N)$$

Unfolded random coil (U) was first rapidly refolded to a compact non-native $\alpha$-helix-rich transient intermediate ($I_1$). Next, the transient intermediate ($I_1$) was converted to the different compact non-native $\alpha$-helix-rich transient intermediate ($I_2$), which is more compact and less $\alpha$-helical content than the first transient intermediate ($I_1$). Finally, the second compact non-native $\alpha$-helix-rich transient intermediate ($I_2$) is reached to the native $\beta$-structure (N).

At $4°C$, we observed the rapid jump from U to N. Intermediates $I_1$ or $I_2$ were not observed. At $-15°C$, we observed $I_2$ as the burst, and the time course of the conversion from $I_2$ to N (only by CD). At $-20°C$, we observed $I_1$ as the burst, and the time course of the conversion from $I_1$ to $I_2$ (both by CD and SAXS). The conversion from $I_2$ to N is slower than 20 s and faster than 30 min.

The folding scheme proposed above indicates that there are at least two local minima and two barriers on FHA1 domain of Rad53 folding landscape. Taking into consideration that the refolding trace was not observed any detectable time course in the absence of 45% EGOH at $4°C$, the two barriers are relatively small.

We inspected whether the ellipticity at 222 nm ($\theta_{222}$) of the burst phase of FHA1 domain of Rad53 is well correlated to the calculated ellipticity at 222 nm ($\theta_{222}^{cal}$) by Helix2 based on helix-coil transition [3,31]. Fig. 11 shows the ellipticity at 222 nm ($\theta_{222}$) of the burst phase of the proteins versus the calculated ellipticity at 222 nm ($\theta_{222}^{cal}$) by Helix2. At present, we revised the ellipticities at 222 nm ($\theta_{222}^{cal}$), which are considered subtracting the value of random coil (640 deg cm$^2$ dmol$^{-1}$) [3,30]. Both experimental and calculated ellipticities of various proteins are tabulated with other data in Table 4 [3,31,32]. As seen in the figure, although FHA1 domain of Rad53 is slightly discrepant from the fitting, the correlation between experiments and calculations is still good. This implies that the early stages in folding of FHA1 domain of Rad53 is

<table>
<thead>
<tr>
<th>State</th>
<th>Temperature (°C)</th>
<th>$\theta_{222}$ (deg cm$^2$ dmol$^{-1}$)</th>
<th>$R_g$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>4</td>
<td>the initial level</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>-15</td>
<td>the initial level</td>
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<td>-20</td>
<td>the initial level</td>
<td>900</td>
</tr>
<tr>
<td>$I_1$</td>
<td>-20</td>
<td>burst</td>
<td>9400</td>
</tr>
<tr>
<td>$I_2$</td>
<td>-15</td>
<td>burst</td>
<td>7900</td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>the last level</td>
<td>7500</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>the final level</td>
<td>5200</td>
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<tr>
<td></td>
<td>-20</td>
<td>the final level</td>
<td>3800</td>
</tr>
</tbody>
</table>

Table 1
Ellipticities at 222 nm ($\theta_{222}$) and $R_g$ of native and unfolded FHA1 domain of Rad53 in equilibrium.

Table 2
Ellipticities at 222 nm ($\theta_{222}$) and $R_g$ of native, transient intermediates and unfolded FHA1 domain of Rad53 in kinetics, respectively.
dominant transient local secondary structure interaction, and the interaction is very rapid.

These phenomena (forming compact α-helix-rich transient intermediate a little bigger molecular size than the native state and quite smaller unfolded random coil) are observed in BLG, src SH3 domain and its mutant, A45G [1,3,4,8] Although the native structure of these proteins are rich in β-structure, they take compact α-helix-rich transient intermediate on their folding. Why? Chikenji reported that BLG shows a strong α-helix-rich transient intermediate in the folding by molecular dynamics studies [33]. He showed that the non-native contacts of BLG are related with the entropy effect [33].

Kim et al. reported that the kinetic refolding of ubiquitin by cryo-SF method with terahertz absorption as a probe [34] and found the terahertz signal finished very rapidly in 45% EGOH at subzero temperatures (−20 °C and −28 °C). This supports the existence of very rapid phase in ubiquitin folding. This rapid folding process would be corresponding to the appearance of α-helical burst phase which we confirmed in FHA domain proteins in the present paper.

Chekmarev et al. showed that α-helical hairpins collapses into a semi-compact globule very rapidly, and then performs a relatively slow search to its native state by computing simulation of lattice model [35]. The folding process is characterized by a single exponential term [35]. This study is only using α-helical hairpin. A series of our studies [1,2,3,23–26] demonstrate that α-helical burst phase appeared very rapidly irrespective of the native conformation (α-rich or β-rich). These experimental findings are well explained qualitatively by the rapid collapse reported by Chekmarev et al.

Table 3

<table>
<thead>
<tr>
<th>State</th>
<th>Temperature (°C)</th>
<th>[θ]222 (deg cm² dmol⁻¹)</th>
<th>Rg (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U 4</td>
<td>equilibrium</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
<td>I −20</td>
<td>the initial level</td>
<td>3000</td>
<td></td>
</tr>
<tr>
<td>N 4</td>
<td>−20 and −28</td>
<td>15.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−20 and −28</td>
<td>−5800</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−28</td>
<td>−5800</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−28</td>
<td>−1100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−28</td>
<td>−11300</td>
<td></td>
</tr>
</tbody>
</table>

a Absolute values may be shifted due to the instability of the CD apparatus at the measurements.

b We did kinetic experiments several times. This value is averaged value among all experiments.

Table 4

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH</th>
<th>The ellipticity at 222 nm ([θ]222°) of the α-helical burst phase and the ellipticity at 222 nm ([θ]222°) of the α-helix calculated by Helix2.</th>
</tr>
</thead>
<tbody>
<tr>
<td>horse heart apo Mb</td>
<td>6</td>
<td>−8360h</td>
</tr>
<tr>
<td>BLG</td>
<td>2</td>
<td>−11760k</td>
</tr>
<tr>
<td>ELG</td>
<td>4</td>
<td>−5860d</td>
</tr>
<tr>
<td>Fyn SH3</td>
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<td>−8060d</td>
</tr>
<tr>
<td>src SH3</td>
<td>6</td>
<td>−7560d</td>
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<tr>
<td>ubiquitin</td>
<td>5.9</td>
<td>−8360f</td>
</tr>
<tr>
<td>lambda repressor</td>
<td>7</td>
<td>−19360g</td>
</tr>
<tr>
<td>RNase A</td>
<td>8</td>
<td>−3960h</td>
</tr>
<tr>
<td>lysozyme</td>
<td>8</td>
<td>−10160l</td>
</tr>
<tr>
<td>FHA domain of Rad53</td>
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<td>−8770j</td>
</tr>
<tr>
<td>Ki67 FHA domain</td>
<td>7.5</td>
<td>−5160l</td>
</tr>
<tr>
<td>lambda repressor</td>
<td>7</td>
<td>−19160k</td>
</tr>
</tbody>
</table>

*Absolute values may be shifted due to the instability of the CD apparatus at the measurements.

References


Fig. 11. The calculated CD value at 222 nm by Helix2 ([θ]222°) versus the experimental CD value at 222 nm ([θ]222°) of the α-helical burst for 11 proteins. [θ]222° and [θ]203° are [θ]222° and [θ]203° respectively, [θ]222° is calculated according to the equation: − [θ(H)]° + (−4.500°(1 − 4(N) − 640) + 640, where [θ(H)]° is the fraction of helix calculated by Helix2 and N is the residue number. [θ]203° and [θ]222° are the ellipticities of random coil, which was set at 640 [30]. Filled circle is FHA domain of Rad53. Filled triangle is Ki67 FHA domain. Regression line was obtained between [θ]222° and [θ]203°. The correlation coefficient between [θ]222° and [θ]203° was estimated as r = 0.79, demonstrating that the α-helix in the burst is strongly correlated to the helical fraction formed in equilibrium with a short lifetime.

Fig. 12. Ellipticities at 222 nm ([θ]°) of the α-helical burst phase and the ellipticity at 222 nm ([θ]°) of the α-helix calculated by Helix2.


