J. Biochem. **140**, 349–357 (2006)
doi:10.1093/jb/mvj156

Thermal Unfolding Process of Dihydrolipoamide Dehydrogenase Studied by Fluorescence Spectroscopy

Etsuko Nishimoto[1], Yoichi Aso[2], Toshiaki Koga[3] and Shoji Yamashita[1,*]
[1]Institute of Biophysics and [2]Institute of Protein Chemical Engineering, Faculty of Agriculture, Graduate School of Kyushu University, Hakozaki, Fukuoka 812-8581; and [3]National Institute of Advanced Industrial Science and Technology, Tosu, Saga 841-0052

Received April 12, 2006; accepted June 6, 2006

The thermal unfolding pathway for dihydrolipoamide dehydrogenase (LipDH) isolated from *Bacillus stearothermophilus* was investigated focusing on the transient intermediate state characterized through time-resolved fluorescence studies. The decrease in ellipticity in the far UV region in the CD spectrum, the fluorescence spectral change of Trp-91 and FAD, and the thermal enzymatic inactivation curve consistently demonstrated that LipDH unfolded irreversibly on heat treatment at higher than 65°C. LipDH took a transient intermediate state during the thermal unfolding process which could refold back into the native state. In this state, the internal rotation of FAD was activated in the polypeptide cage and correspondingly LipDH showed a peculiar conformation. The transient intermediate state of LipDH characterized in time-resolved fluorescence depolarization studies showed very similar properties to the molten-globule state, which has been confirmed in many studies on protein folding.

Key words: *Bacillus stearothermophilus*, flavoprotein, hydrolipoamide dehydrogenase, protein unfolding, time-resolved fluorescence.

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; LipDH, dihydrolipoamide dehydrogenase; MG, molten-globule; NAD, nicotinamide adenine dinucleotide; PDC, pyruvate dehydrogenase multi-enzyme complex; PMSF, phenylmethylsulfonyl fluoride; 9-CA, 9-cyanoanthracene; TPP, thiamine diphosphate.

A variety of experimental methods such as CD, NMR, infrared and raman spectroscopy have been used to study protein stability and unfolding/folding. Among them, the steady state and time-resolved fluorescence spectroscopic method is one of the most powerful methods. A tryptophan residue gives a large Stokes-shift in the fluorescence spectrum because of the larger increase of the dipole moment in the excited singlet state in the polar circumstance. This peculiar photo-physical property of a tryptophan residue is a useful indicator for the conformational change of a protein. Furthermore, analysis of the fluorescence lifetime can provide detailed information on the interactions of a tryptophan residue with the surrounding amino acid residues in the polypeptide cage. While many fluorescence spectroscopic techniques such as resonance excitation energy transfer and solute quenching are also useful for detecting the conformational change of a protein, the rotational motion of a fluorescent molecule covalently or non-covalently bound to the protein can also be analyzed with higher time-resolution than 10^{-11} s using the fluorescence depolarization method. Combined with the fact that fluorescence is only emitted from an excited molecule according to the photo-selection rule, the fluorescence depolarization method provides a clearer physical image of the internal motions of proteins, which is essential for understanding the protein conformation during the unfolding/folding process, because the fluorescence anisotropy decay is defined as the correlation function used in the general description of relaxation phenomena. Some reports on the molecular dynamics of proteins using tryptophyl fluorescence have been published, and the validity and limitation of these methods have been discussed (*1–3*).

Dihydrolipoamide dehydrogenase (LipDH) is one component constituting the pyruvate dehydrogenase multi-enzyme complex (PDC), and catalyzes the oxidation of dihydrolipoic acid coupled to the reduction of NAD^+ as the last step of acetyl CoA synthesis (*4–6*). Studies on the conformational stability and unfolding/folding mechanism of LipDH are very important because efficient enzymatic activity of PDC is completed by the regulation and integration of pyruvate dehydrogenase and LipDH on the dihydrolipoamide acetyltransferase that acts as the core of this multi-enzyme complex. The structural stability and folding state of LipDH have a large effect on the key steps in carbohydrate metabolism. As shown in Fig. 1, LipDH is composed of two identical subunits, and each subunit contains one FAD as a prosthetic group and a single tryptophan residue, Trp-91. The locations of Trp-91 and FAD, and the fine structure surrounding them are so clear in the X-ray crystallographic structure that the fluorescence spectroscopic data on the conformation and molecular dynamics can be interpreted more quantitatively (*7*). Trp-91 is located at the contact face between two subunits, which is an essential domain for the enzymatic activity of LipDH. FAD is not only another intrinsic fluorophore reflecting the conformation state but also significantly contributes to the structural stabilization and folding

*To whom correspondence should be addressed. Tel/Fax: +81-92-642-4425, E-mail: yamashita@brs.kyushu-u.ac.jp

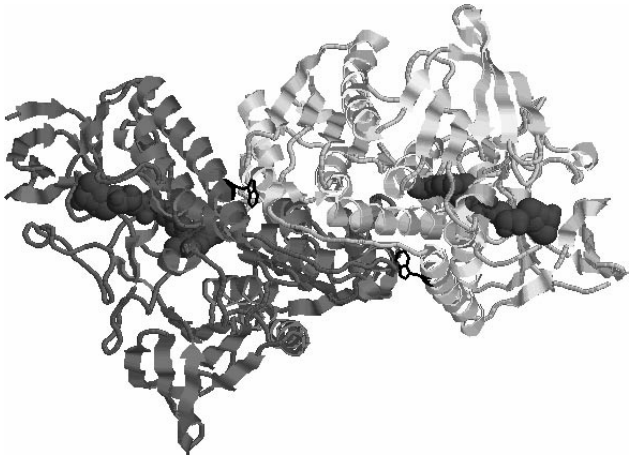

Fig. 1. **Ribbon representation of LipDH.** The structure was constructed from the coordinates from the Protein Data Bank file 1EBD (*10*). The Trp residue and FAD are shown as black sticks and dark-colored spheres, respectively.

of LipDH (*8*). The specific arrangement of these two fluorophores demonstrates that LipDH is a very interesting subject for studying the stability and unfolding/folding property of a protein by means of fluorescence spectroscopy.

The previous studies on protein unfolding/folding focused on specification of the partially folded state (molten-globule state) as the unfolding/folding intermediate. They provided valuable information for understanding the protein folding mechanism (*9–15*). But, the unfolding of a larger or multimeric protein is complicated and has been found to comprise multiphasic processes with the stabilization of a partially folded intermediate (*16–18*). LipDH is a dimeric protein and each subunit has four structural domains. It is expected that elucidation of the unfolding/folding process of LipDH and identification of the intermediate would shed light for a deeper understanding of protein stability and folding.

Based on the intuitive concept that protein structure is determined by the balance between a non-covalent attractive interaction and the collective destructive motion of the polypeptide elements, two potent approaches are considered for studying protein stability and the folding/unfolding mechanism. One is the reduction of the cohesive interaction by exposing the protein to a chemical denaturant or extreme acidic conditions. The other is enhancement of the thermal fluctuation by equilibrating the protein at higher temperatures. With the latter approach, the protein would be destabilized by the rigorous thermal agitation which biases the fluctuation of the peptide elements in such a way that the protein makes the transition from a compact structure to a random one. Although the thermal unfolding of a protein is expected to tell us more about protein stability, detailed studies on the mechanism are not as good as ones on the denaturant-induced unfolding.

In the present paper, we report the thermal unfolding of LipDH, focusing on the unfolding intermediate of LipDH characterized by steady-state and time-resolved fluorescence spectroscopy.

MATERIALS AND METHODS

Materials—Dihydrolipoamide dehydrogenase (LipDH) of *Bacillus stearothermophilus* was purchased from Seikagaku Kogyo Corp. (Tokyo, Japan). Dihydrolipoamide, thiamine diphosphate (TPP), and nicotinamide adenine dinucleotide (NAD^+) used for the measurement of enzyme activity were purchased from Sigma (St. Louis, MO, USA). They were of special grade and were used without further purification. A HiTrap Q column from GE Healthcare Bio-Science Corp. (Piscataway, NJ, USA) was used for purification of LipDH.

Purification of LipDH—Seven milligrams of LipDH was dissolved in 5 ml of 20 mM phosphate buffer (pH 7.0) including 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.15 mM phenylmethylsulfonyl fluoride (PMSF). For purification of LipDH, ion-exchange chromatography was performed. After application of the enzyme solution to the HiTrap Q column equilibrated with the phosphate buffer, LipDH was eluted with a gradient of NaCl (0.1–0.4 M). LipDH was obtained as the fraction centered at 0.2 M NaCl. It was dialyzed immediately against 20 mM phosphate buffer (pH 7.0). The purity of the obtained LipDH was examined by SDS polyacrylamide gel electrophoresis. The FAD of LipDH was quantitatively analyzed by the method of Whitby, and the stoichiometry of FAD as to the apo-protein was examined (*19*). For every spectroscopic measurement, the concentration of LipDH was adjusted to OD = 0.1 at 295 nm (0.17 mg/ml) using a Shimadzu UV-265 spectrophotometer (Tokyo, Japan). If not specified, the spectroscopic and enzyme activity measurements of LipDH were performed after thermal treatment at various temperatures for 30 min, followed by incubation at 30°C for 60 min. A thermo-regulated copper block was used for the heat treatment of LipDH in the phosphate buffer (20mM, pH 7.0).

Enzymatic Activity—After mixing 100 mM dihydrolipoamide prepared in ethanol with 20 mM phosphate buffer containing 1.05 mM $MgCl_2$, 0.21 mM TPP, and 2.8 mM NAD^+, the LipDH solution was added. The enzymatic activity of LipDH was evaluated by measuring the increase in the absorption at 340 nm caused by the reduction of NAD^+ to NADH. The enzymatic activity measurements were performed at 30°C after incubation for 60 min. at 30°C following thermal treatment at various temperatures.

CD and Steady State Fluorescence Spectra—The CD spectra of LipDH heat-treated at various temperatures were measured with a JASCO J-600 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1 mm optical path length. The steady state fluorescence spectra were measured with a HITACHI 850 fluorescence spectrophotometer (Tokyo, Japan). The excitation and emission bandpasses were 5 nm, respectively. Correction was made for the contribution from the undesired light scattering and the wavelength dependence of the instrumental response. Steady state fluorescence anisotropy was measured with two Glan-Taylor polarizers, one each just in front of and behind the cell holder. Fluorescence anisotropy was calculated based on its definition by measuring the vertical and horizontal fluorescence components against the vertical excitation. G-factors were given by the fluorescence intensity ratio of the vertical to horizontal components against the horizontal excitation.

Time-Resolved Fluorescence Measurements—The total fluorescence and fluorescence anisotropy decay measurements were performed with a multi-wavelength time-correlated single photon counting system Type C4708 (Hamamatsu Photonics, Japan). This system mainly comprised a combination of a monochromator and a streak scope that enables multi-wavelength photon counting with a time resolution of 5 ps and very high spectroscopic sensibility. The excitation pulse was obtained from a mode locked Ti:sapphire laser pumped by an argon ion laser Type 2080 (Spectra-Physics Inc., CA, USA). In order to set the excitation wavelength, a second or third harmonics generator GW-3FS (Spectra-Physics Inc., CA, USA) was used. The output laser pulse for excitation had a pulse-width narrower of than 1 ps, and its repetition was 1 MHz. When the total fluorescence decay was measured, the Glan-Taylor polarizer was set at 54.7° against the vertical excitation pulse. For the fluorescence anisotropy decay measurements, it was set vertical or horizontal against the vertical excitation. The fluorescence anisotropy decay was measured based on the definition

$$r(t) = \frac{I_{VV}(t) - GI_{VH}(t)}{I_{VV}(t) + 2GI_{VH}(t)}$$

where $I_{VV}(t)$, $I_{VH}(t)$, and G are the parallel and perpendicular fluorescence components, and the grating factor, respectively. G-factors for the measurements of FAD were conveniently determined as the integrated intensity ratio of the vertical to horizontal fluorescence components of 9-cyanoanthracene (9-CA) against the vertical excitation, because the fluorescence lifetime of 9-CA is so much longer that the effect of the polarization (the rotational correlation time) of 9-CA can be neglected. The fluorescence [$F(t)$] and fluorescence anisotropy [$r(t)$] decay kinetics are linear combinations of the exponential function,

$$F(t) = \sum \alpha_i \exp(-t/\tau_i)$$
$$r(t) = \sum \beta_i \exp(-t/\phi_i)$$

where τ_i and ϕ_i are the decay time and rotational correlation time of the i-th component, and α_i and β_i the corresponding amplitudes of the fluorescence and fluorescence anisotropy decay, respectively. They were analyzed with a nonlinear least-square algorithm to determine the decay parameters, α_i, τ_i, β_i, and ϕ_i (*20*). Adequacy of the analysis was judged by inspection of the plots of weighted residual and statistical parameters such as chi square (χ^2).

RESULTS

Dihydrolipoamide dehydrogenase (LipDH) isolated from the pyruvate dehydrogenase multi-enzyme complex of *B. stearothermophilus* exhibited higher thermal resistance (*21*). Curve a in Fig. 2 shows the residual enzymatic activity of LipDH after incubation at 30°C for 1 h following to thermal treatments at various temperatures. On treatment below 65°C, LipDH was not damaged at all or its activity was recovered on incubation at 30°C after thermal treatment. The onset temperature of the irreversible inactivation was 65°C and the activity was reduced more than

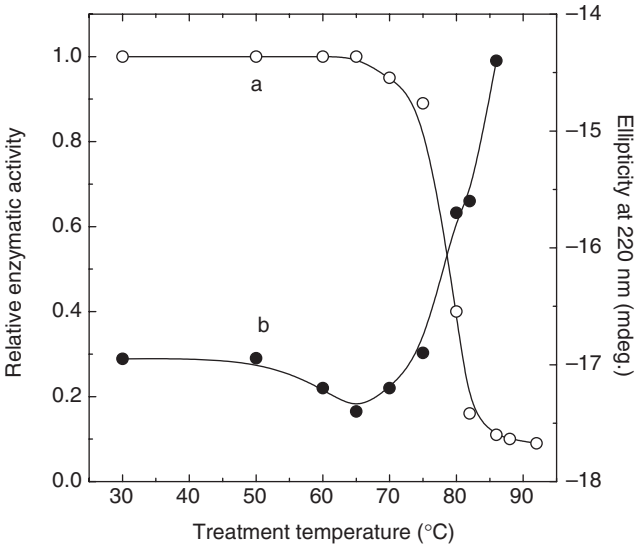

Fig. 2. **Effects of thermal treatment on the relative enzymatic activity (curve a) and ellipticity at 220 nm (curve b) of LipDH.** The enzymatic activities and CD were measured at 30°C after treatment at various temperatures, followed by incubation at 30°C for 60 min.

90% on treatment at 85°C. The inactivation curve was independent of the incubation time from 1 hour to 12 h after the thermal treatment.

Some proteins frequently undergo aggregation when they are denatured by heat treatment or a denaturant. The occurrence of protein aggregation is a serious cause of distortion in CD and fluorescence spectroscopic data. The static light scattering measurements at 540 nm were examined for LipDH thermally treated at various temperatures using a fluorescence spectrophotometer (data not shown). But an increase in the light scattering intensity was not found at protein concentrations below 0.1 mg/ml used in the present study.

The CD spectrum of the native LipDH showed a line shape with double minima at 208 nm and 220 nm, suggesting that the contents of beta-sheet and alpha-helix were 43% and 18%, respectively. When the CD spectrum of LipDH was measured after heat treatment at 30–85°C followed by incubation at 30°C, the line-shape of the spectrum was not so much changed, but the ellipticity at 220 nm started to decrease linearly at 65°C, reaching –14.5 mdeg. with treatment at 85°C (curve b in Fig. 2). If the enzyme is completely unfolded, the ellipticity would give smaller values. The present result suggests that the secondary structure is retained even at higher temperature or partially refolded back on incubation at 30°C following the thermal treatment. The temperature dependence curve of the enzymatic inactivation was almost the same as that of the CD spectral change. But it should be noted that LipDH showed the highest ellipticity when treated at 65°C followed by incubation at 30°C.

LipDH contains two major fluorophores, Trp-91 and flavin adenine dinucleotide (FAD). Figure 3 shows the fluorescence maximum wavelength (curve a) and intensity (curve b) of FAD in LipDH treated at various temperatures

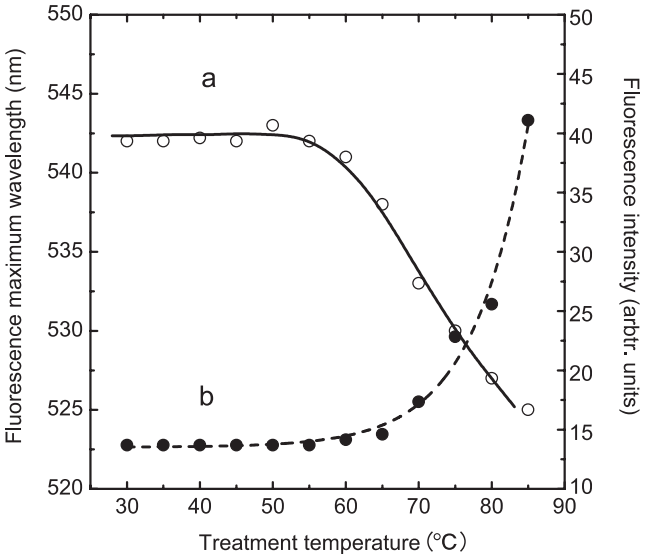

Fig. 3. **Effects of thermal treatment on the fluorescence maximum wavelength (curve a) and intensity (curve b) of FAD in LipDH.** The fluorescence spectra were measured at 30°C after thermal treatment for 30 min. and then incubation at 30°C for 60 min.

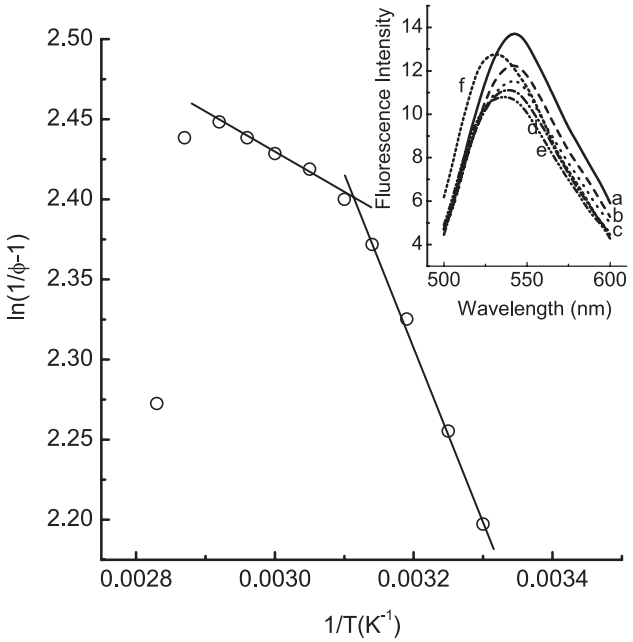

Fig. 4. **An Arrhenius plot for the thermal fluorescence quenching of FAD in LipDH.** The plot was based on $\ln(\frac{1}{\phi}-1) = \ln A - \frac{\Delta E}{kT}$, where ϕ is the fluorescence quantum yield experimentally determined by using quinine sulfate in 0.1 N H_2SO_4 as a fluorescence standard ($\phi = 0.546$) (*22*), and A is a constant related with the fluorescence radiative lifetime. ΔE, k, and T are the activation energy for the thermal quenching process, the Boltzmann constant, and the temperature in Kelvin, respectively. Insert, the temperature dependence of the fluorescence. Excitation wavelength, 480 nm. Curves a, b, c, d, e, and f were measured at 30, 40, 50, 60, 70, and 80°C, respectively.

and measured after incubation for 60 min. at 30°C. With treatment at lower temperatures, the fluorescence intensity was very weak and the maximum was observed at 542 nm. When the treatment temperature was higher than 65°C, the fluorescence maximum linearly shifted to the shorter wavelength side, reaching 525 nm at 85°C. Then, the fluorescence intensity was concomitantly enhanced three times.

The insert in Fig. 4 shows the fluorescence spectra of FAD in LipDH measured at 30–80°C. The fluorescence intensity decreased with increasing temperature from 30 to 70°C, but conversely it increased at higher than 70°C. Since the radiative transition probability is independent of temperature, the quenching rate constant is connected with temperature through the Arrhenius equation. In the case of simple thermal fluorescence quenching of FAD, Eq. 1 should be hold for the fluorescence quantum yield and temperature.

$$\ln\left(\frac{1}{\phi} - 1\right) = \ln A - \frac{\Delta E}{kT} \qquad (1)$$

Where ϕ is the fluorescence quantum yield experimentally determined by using quinine sulfate in 0.1 N H_2SO_4 as a fluorescence standard ($\phi = 0.546$) (*22*), and A is a constant related with the fluorescence radiative lifetime. ΔE, k, and T are the activation energy for the thermal quenching process, the Boltzmann constant, and the temperature in Kelvin, respectively. As shown in Fig. 4, the plots of $\ln(1/\phi - 1)$ against the reverse of temperature showed a linear line below 50°C, giving an activation energy of 2.2 kcal/mol $\approx$ 0.1 eV. The deviation from the linear line at higher than 50°C would suggest that some conformational change is induced around FAD.

Since the fluorescence quantum yield of FAD in native LipDH is low, FAD would be strongly interacting with the surrounding in the polypeptide cage. According to Bastiaens et al., the fluorescence of FAD is quenched by tyrosine residues in some flavoproteins (*23*). In fact, Tyr-186, -22 and -195 are located at positions 3.4, 14.0 and 14.0 Å apart from FAD, according to the X-ray crystallographic structure of LipDH (Fig. 1). The fluorescence decay kinetics of FAD in LipDH was described with a linear combination of triple exponentials, as shown in Table 1. Each decay component would be produced by the heterogeneous interactions between FAD and the tyrosine residues surrounding it. The corresponding decay times were shortened below 60°C and conversely increased at 70°C. These results also suggest that the conformational change induced in LipDH at 50–60°C is different from those at higher than 65°C.

The tryptophyl fluorescence of the native LipDH showed a maximum at 355 nm and its intensity was very weak. When LipDH was treated at temperatures higher than 65°C and incubated at 30°C for 60 min., the fluorescence intensity increased and the maximum wavelength shifted to the higher energy side, as shown in Fig. 5. In general, the fluorescence spectrum of a tryptophan residue shifts to the red side with a large conformational change such as protein unfolding. The peculiar spectral shift observed here suggests that the micro-environment surrounding Trp-91 is changed to be non-polar on the thermal treatment. In the native state, Trp-91 is located near the contact face of two LipDH subunits and is exposed to the outer face. Probably, Trp-91 is repacked into the new cage of

Table 1. **Fluorescence decay parameters of FAD in LipDH.**

Temperature (°C)	α_1	α_2	α_3	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{av} (ns)	χ^2
30	0.536	0.204	0.312	0.072	0.731	2.753	1.05	0.00011
40	0.566	0.268	0.238	0.093	0.746	2.704	0.89	0.00017
50	0.544	0.286	0.214	0.091	0.612	2.592	0.78	0.00010
60	0.402	0.349	0.238	0.074	0.521	2.272	0.75	0.00008
70	0.322	0.507	0.186	0.086	0.751	2.488	0.87	0.00007
80	0.173	0.741	0.106	0.170	0.992	3.240	1.10	0.00005
85	0.122	0.325	0.545	0.145	1.567	2.541	1.19	0.00006

The excitation and emission wavelengths were 480 nm and 540 nm, respectively. The fluorescence decay was given by $F(t)=\sum \alpha_i \exp(-t/\tau_i)$, where α_i and τ_i are the amplitude and corresponding decay time of the i-th component.

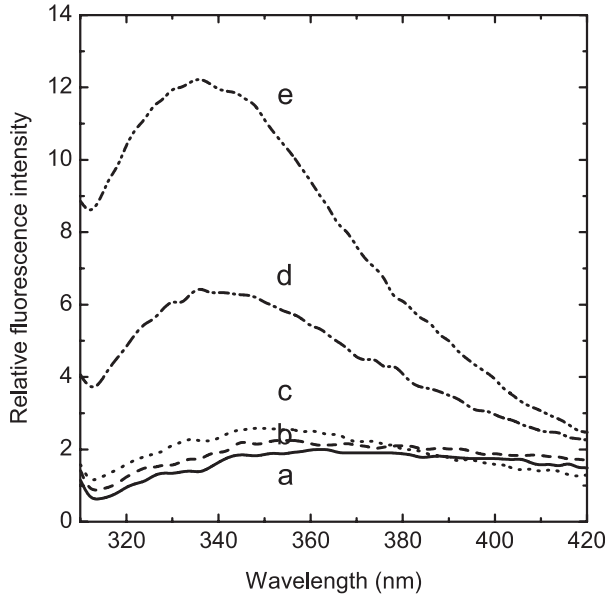

Fig. 5. **The irreversible fluorescence spectral change of Trp-91.** Excitation wavelength, 300 nm. Bandpass, Ex./Em. = 5 nm/5 nm. The spectra were measured at 30°C after heat treatment at 50°C (curve a), 60°C (curve b), 65°C (curve c), 75°C (curve d), and 80°C (curve e).

the peptide chain on incubation at 30°C after thermal treatment at higher than 65°C.

The absorption spectrum of FAD showed three maxima at 260 nm, 370 nm, and 450 nm in LipDH. The absorption band centered at 370 nm overlaps with the fluorescence emission band of Trp-91. Such spectral overlapping between the fluorescence and absorption is a determining factor for the resonance excitation energy transfer efficiency from Trp-91 to FAD. The extremely weak fluorescence of Trp-91 observed here would reveal that the excitation energy of Trp-91 would be efficiently transferred to FAD. Indeed Trp-91 is arranged near FAD and the center-to-center distance between these two chromophores is estimated to be 15 Å, according to the X-ray crystallographic structure (Fig. 1). In order to investigate the thermal effect on the conformation of LipDH, the excitation energy transfer between Trp-91 and FAD was investigated. The ratio of fluorescence intensity on excitation at 300 nm to that on excitation at 480 nm indicates the relative energy transfer efficiency (E) from Trp-91 to FAD, as given by the following equation.

$$I^{480} = \frac{k_f^{FAD}}{k_f^{FAD} + k_i^{FAD}}$$
$$I^{300} = \frac{k_{ET}}{k_f^{Trp} + k_i^{Trp} + k_{ET}} \cdot \frac{k_f^{FAD}}{k_f^{FAD} + k_i^{FAD}} \quad (2)$$
$$\frac{I^{300}}{I^{480}} = E$$

Where, I^{480} and I^{300} are the fluorescence intensity of FAD on excitation at 480 nm and 300 nm, respectively. The energy transfer, radiative transition and non-radiative transition rates are k_{ET}, k_f, and k_i, respectively. Figure 6 shows the temperature dependence of the excitation energy transfer efficiency (E) estimated using Eq. 2. Below 50°C, E was independent of the temperature, but it increased and then decreased to give a maximum value of 0.46 at 65°C. This result suggests that FAD comes closer and/or is oriented in parallel to Trp-91 to increase the energy transfer efficiency at 50–65°C. Conversely, their mutual arrangement is disturbed at higher than 65°C.

The steady state fluorescence anisotropy of FAD in the native LipDH was determined to be 0.34 at 30°C. Since the transition moments of absorption and fluorescence were parallel when FAD was excited at 480 nm and its fluorescence was measured at 540 nm, it is clear that the internal motion of FAD would be restricted in the cage of the LipDH polypeptide. As shown in Fig. 7, the fluorescence anisotropy started to decrease at 50°C and then rapidly decreased with increasing temperature (curve b). On the other hand, when the fluorescence anisotropy was measured at 30°C after thermal treatment at various temperatures, it showed a constant value, 0.34, until 60°C (curve a in Fig. 7). These results demonstrate that the change in the internal motion of FAD induced by thermal treatment below 60°C is reversible.

In order to determine the temperature dependence of the molecular dynamics of LipDH in more detail, the time-resolved fluorescence anisotropy of FAD was measured. Figure 8 shows the fluorescence anisotropy decay curves at 30, 60, and 80°C. All of them were described with double exponential kinetics, which are characterized by shorter (ϕ_S) and longer (ϕ_L) rotational correlation times and corresponding amplitudes (β_1, β_2).

$$r(t) = \beta_1 \exp(-t/\phi_S) + \beta_2 \exp(-t/\phi_L) \quad (3)$$

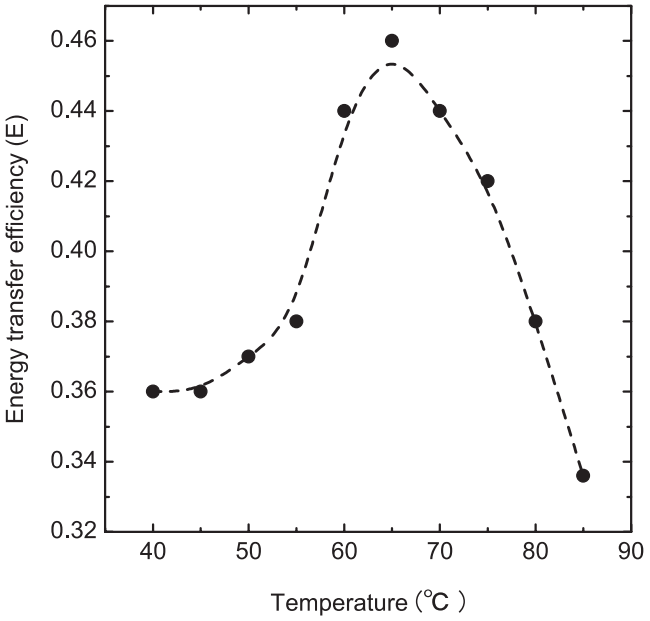

Fig. 6. **Temperature dependence of the energy transfer efficiency from Trp-91 to FAD.** *E* was given by I^{300}/I^{480}, where I^{480} and I^{300} are the fluorescence intensity of FAD excited at 480 nm and 300 nm, respectively.

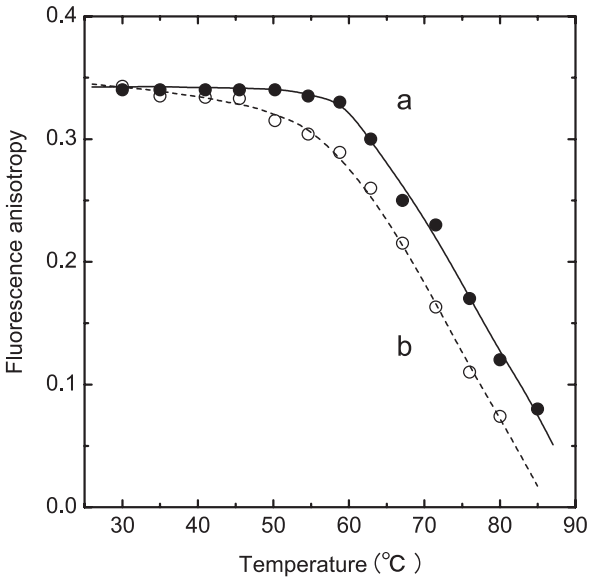

Fig. 7. **Temperature dependence of the steady-state fluorescence anisotropy of FAD in LipDH (curve b).** Excitation (Ex.)/emission (Em.) wavelength, 480 nm/540 nm. Bandpass, Ex./Em. = 5 nm/5 nm. Curve a, measured at 30°C for 30 min. after thermal treatment at the temperatures shown by the abscissa.

At 30°C, the fluorescence anisotropy decay was slow. At 60°C, the faster decay component contributed more to the fluorescence depolarization. As previously shown, the enzymatic activity decreased and the structure of LipDH changed irreversibly with the treatment at 80°C. The fluorescence anisotropy decay curve also changed drastically at this temperature. Within 300 ps after the excitation pulse, the fluorescence anisotropy rapidly decreased to 0.05 as the very fast decay component. The decay parameters of FAD in LipDH observed at various temperatures are plotted in Fig. 9. The rotational correlation time of the fast decay component of native LipDH was 8.7 ns and the corresponding amplitude was very small, 0.028. Although the rotational motion of FAD was not so activated by the increase in the temperature to 40–50°C, the amplitude was increased to 0.055. At temperatures higher than 50°C, the correlation time of the shorter decay component was further shortened from 7 ns to the sub-nanosecond range. At the same time, the corresponding amplitude remarkably increased. The rotational correlation time of free FAD was 0.22 ns in the buffer solution, which was almost consistent with that of the faster decay component of LipDH at temperatures higher than 80°C. Therefore, it is reasonable to consider that the faster decay component can be ascribed to the free rotation of FAD in the polypeptide cage of LipDH.

The rotational correlation times of the slower decay component were not so dependent on the temperature and they were estimated to be within 70–80 ns. This decay component doubtlessly corresponds to the fluorescence depolarization due to the entire rotation of LipDH. However, the estimated values for the longer rotational correlation time should include much uncertainty, since the average fluorescence lifetime of FAD in LipDH is shorter and is in the range of several nanoseconds. Regardless of this, the experimentally determined rotational correlation time is coincident with that calculated using the Einstein-Stokes law, because the ratios of the experimental correlation time to the theoretical one of many proteins are within 1.4–2.4 (*1*). LipDH is constructed of two subunits and its molecular weight is 110 kDa. Assuming that the specific volume and the hydration of LipDH are 0.73 ml/mg and 0.2, respectively, the rotational correlation time of LipDH is calculated to be about 42 ns. The enzymatic activity of LipDH decreased with thermal treatment at higher than 75°C. But no remarkable change was observed in the correlation time of the entire rotation of LipDH measured at the same temperature. It is also interesting to note that the internal motion of FAD changed preceding the large conformational change indicated by the CD spectrum, and the fluorescence properties of FAD and tryptophan residues.

DISCUSSION

The enzymatic activity of LipDH started to decrease on thermal treatment at temperatures higher than 65°C. This enzymatic inactivation corresponded to the reduction of the secondary structure contents. The enzymatic inactivation curve and the curve showing the CD spectral changes were not perfectly coincident with each other. The ellipticity of LipDH treated at 50–65°C and incubated at 30°C for 1 h exhibited a higher value, while the enzymatic activity recovered to the level of the native LipDH. It is probable that LipDH treated in this temperature range may refold back into a more compact form different from the native conformation.

Enzymatic inactivation frequently accompanies alterations of the tertiary structure. Although the fluorescence of 8-anilino-1-naphthalenesulfonic acid (ANS) is generally used as a useful indicator of the degree of protein unfolding

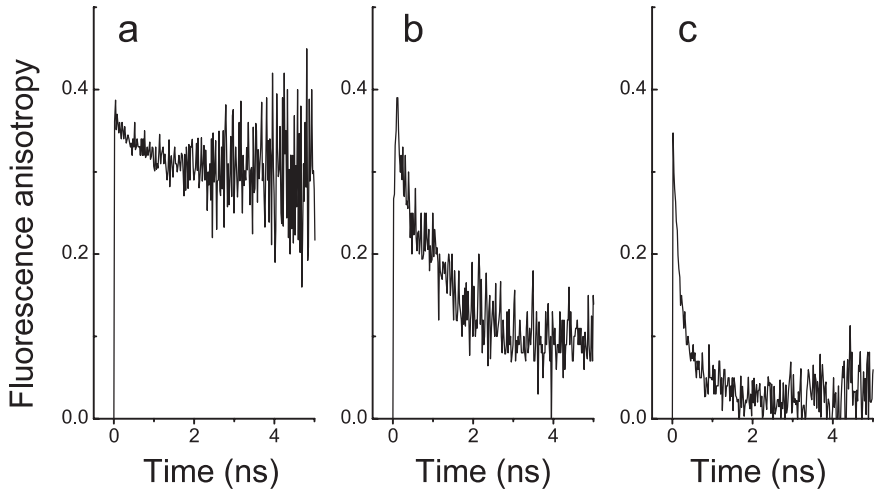

Fig. 8. **Fluorescence anisotropy decay curves of LipDH.** a, 30°C; b, 60°C; c, 80°C. Solid lines, the curves giving the best fits; the residuals are shown in lower panels. Excitation wavelength, 480 nm; emission wavelength, 540 nm.

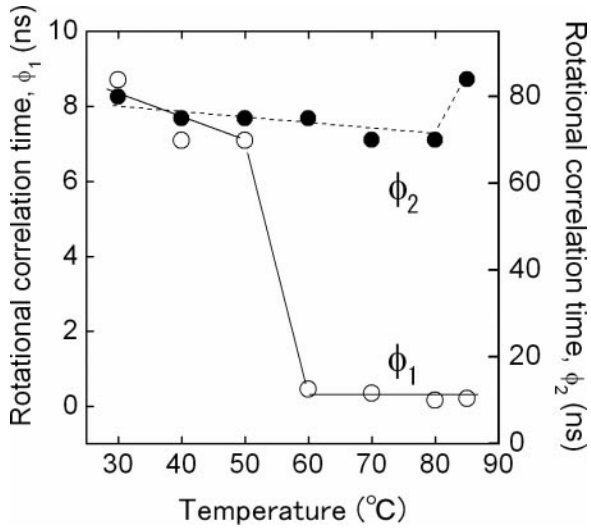

Fig. 9. **Temperature dependence of the rotational correlation times for the segmental rotation (ϕ_1) and entire rotation (ϕ_2) of LipDH.** The rotational correlation time was determined by non-linear curve fitting based on the equation $r(t) = \beta_1 \exp(-t/\phi_1)+\beta_2 \exp(-t/\phi_2)$, where β_1 and β_2 are the amplitudes of segmental and entire rotation, respectively.

(*24–26*), the fluorescence of FAD and Trp-91 revealed conformational changes on the thermal unfolding of LipDH. The fluorescence maximum of FAD shifted to the higher energy side and the intensity remarkably increased on thermal treatment at temperatures higher than 65°C, suggesting that the circumstances surrounding FAD became nonpolar and FAD was free from the quenching interaction with Tyr residues. The fluorescence spectral changes of Trp-91 treated at higher than 65°C showed that the polypeptide chain of LipDH would be mis-folded when it attempted to refold back into the native structure. These results consistently demonstrate that the irreversible unfolding of LipDH started at 65°C if the unfolding temperature is defined as the onset of the irreversible changes in the enzymatic activity, and the CD and fluorescence spectra of Trp-91 and FAD in LipDH. Mattevi et al. and van Berkel et al. proposed the dissociation of LipDH into two subunits as the thermal inactivation mechanism (*27*, *28*). But our results implied that the dimeric structure of LipDH was preserved and did not dissociate into the monomer even on treatment at 85°C, where the enzymatic activity of LipDH was decreased by more than 90%, because no prominent reduction in the entire rotational correlation time of LipDH corresponding to the dissociation was observed in the temperature range of 30–85°C. Probably, the large conformational change including the reduction of the secondary structure would be the main factor for the inactivation of LipDH. The fluorescence spectral shift of Trp-91 shows that a large conformational change would be induced around the contact face of the subunits, which is essential for the enzymatic activity of LipDH (Fig. 5).

At first sight, the thermal unfolding process leading to irreversible inactivation seems to comprise simple conversion from the native state to an unfolded state. But, more detailed analysis involving the energy transfer kinetics from Trp-91 to FAD, thermal fluorescence quenching and the fluorescence anisotropy demonstrated that the thermal unfolding of LipDH would proceed through a transient intermediate state. LipDH changes its own conformation such that Trp-91 and FAD would transiently become closer and/or mutually oriented in parallel at 50–65°C.

The time-resolved fluorescence depolarization studies substantiate more the thermal unfolding transient intermediate state of LipDH. FAD showed peculiar dynamical properties in this intermediate state. The fluorescence anisotropy decay kinetics of FAD in LipDH was described by double exponentials at any temperature investigated here. Each component was ascribed to the internal motion of FAD and the entire rotation of LipDH, respectively. This suggests that FAD thermally fluctuates according to the conformation of the polypeptide cage of LipDH. Using the general theory for a small fluorophore bound to a large molecule, the exponential function (Eq. 3) experimentally determined for the fluorescence anisotropy decay is replaced with Eq. 4,

$$r(t) = r_0\{f \exp(-t/\phi_F) + (1-f)\exp(-t/\phi_M)$$
$$f = \frac{\beta_1}{\beta_1+\beta_2}, \quad \frac{1}{\phi_1} = \frac{1}{\phi_F} + \frac{1}{\phi_M} \qquad (4)$$

The parameters, ϕ_F and ϕ_M, in Eq. 4 are the rotational correlation times of FAD and LipDH, respectively. f is

the degree of the motional freedom of FAD in the polypeptide cage of LipDH. Although the rotational correlation time of FAD in the native LipDH could be distinguished from the entire rotation of LipDH, it was much longer than that of the free FAD in the buffer solution. Indeed, the correlation time of the bound FAD in LipDH was estimated to be 8.7 ns. It is difficult now to specify definitely this rotational mode of FAD. But, as judged on comparison with other reports on protein dynamics, the rotational fluctuation of FAD may be caused by the structural tumbling of LipDH, where the motion of FAD would be synchronized with the fluctuation of the polypeptide elements of LipDH (*29*, *30*). At temperatures higher than 65°C, the shorter rotational correlation time of FAD in LipDH was shortened to the sub-nanosecond range, corresponding to that of the free rotation of FAD in the buffer solution. This result demonstrates that the interaction of FAD with its surroundings is weakened, resulting in the free rotation of FAD within the space created by the conformational change of LipDH.

The degree of the motional freedom, f, is connected with the order parameter (S^2) and the semi-cone angle (θ) of the motion of FAD using the amplitude (β) of the fluorescence anisotropy decay kinetics (*31*).

$$S^2 = 1\text{–}f = \left(\frac{1}{2}\cos\theta(\cos\theta + 1)\right)^2 \quad (5)$$

Figure 10 shows the temperature dependence of the order parameter and the motional freedom of FAD given by the semi-cone angle in LipDH. The order parameter of FAD in the native LipDH was 0.91 and the corresponding semi-cone angle was 14°. The motional freedom of FAD in LipDH critically increased to give $S^2 = 0.17$ and $\theta = 57°$C at 70°C. These changes concomitantly occurred with the shortening of the correlation time, suggesting that a large space which allows FAD almost free rotation would be induced in the polypeptide cage of LipDH. This free rotation of FAD with a large amplitude was one unique property of the transient intermediate state of LipDH during the thermal unfolding.

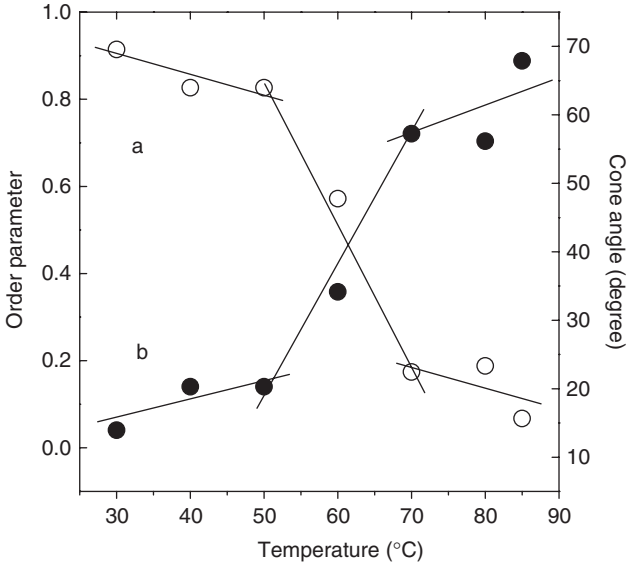

Fig. 10. **The order parameter (curve a) and cone angle (curve b) for the internal motion of FAD in LipDH.** The order parameter, S^2, was given by the equation $S^2 = 1\text{–}f = (\frac{1}{2}\cos\theta(\cos\theta + 1))^2$, where f is the degree of the motional freedom, and θ is the semi-cone angle of the motion of FAD using the amplitude (β) of the fluorescence anisotropy decay kinetics (*31*).

LipDH is constructed of two subunits and, furthermore, each subunit has some structural domains. Such a complex structure makes it difficult to elucidate the thermal unfolding/folding mechanism by means of a simple model that is successfully applicable to a single domain protein such as hen egg-white lysozyme. A transient intermediate state was confirmed here for the thermal unfolding of LipDH. The intermediate state showed similar properties to the molten-globule (MG) state, which has been confirmed in many studies on protein folding (*32*–*37*). The involvement of the MG state in the protein unfolding/folding process should be definitely recognized from the near UV CD spectrum. Unfortunately, in the present study, an apparent difference was not found in the CD spectrum at 250–300 nm between the thermally treated and non-treated LipDH. However, our results consistently demonstrate that LipDH transiently reach the intermediate state, which refolds back into a native-like state retaining the enzymatic activity, but the refolding of the LipDH thermally unfolded once is almost completely prohibited.

REFERENCES

1. Lakowicz, J.R. (1983) *Principles of Fluorescence Spectroscopy*, pp. 342–381, Plenum Press, New York
2. Tcherhasskaya, O., Ptitsyn, O.B., and Knutson, J.R. (2000) Nanosecond dynamics of tryptophans in different conformational states of apomyoglobin proteins. *Biochemistry* **39**, 1879–1889
3. Rischel, C., Thyberg, P., Righer, R., and Poulsen, F.M. (1996) Time-resolved fluorescence studies of the molten globule state of apomyoglobin. *J. Mol. Biol.* **257**, 877–885
4. Patel, M.S. and Harris, R.A. (1995) Mammalian alpha-keto acid dehydrogenase complexes: gene regulation and genetic defects. *FASEB J.* **9**, 1164–1172
5. Harris, R.A., Hawes, J.W., Popov, K.M., Zhao, Y., Shimomura, Y., Sato, J., Jaskiewicz, J., and Hurley, T.D. (1997) Studies on the regulation of the mitochondrial α-ketoacid dehydrogenase complexes and their kinases. *Adv. Enzyme Regul.* **37**, 271–293
6. Shepherd, B.S. and Hamares, G.G. (1977) Fluorescence energy transfer measurements in the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* with chemically modified lipoic acid. *Biochemistry* **16**, 5234–5241
7. Mande, S.S., Sarfaty, S., Allen, M.D., Perham, R.N., and Hol, W.G. (1996) Protein-protein interactions in the pyruvate dehydrogenase multienzyme complex: dihydrolipoamide dehydrogenase complexed with the binding domain of dihydrolipoamide acetyltransferase. *Structure* **4**, 277–286
8. Lindsay, H., Beaumont, E., Richards, S.D., Kelly, S.M., Sanderson, S.J., Price, N.C., and Lindsay, J.G. (2000) FAD insertion is essential for attaining the assembly competence of the dihydrolipoamide dehydrogenase (E3) monomer from *Escherichia coli*. *J. Biol. Chem.* **275**, 36665–36670
9. Jaenicke, R. (1987) Folding and association of proteins. *Prog. Biophys. Mol. Biol.* **49**, 117–237
10. Kim, P.S. and Baldwin, R.L. (1990) Intermediates in the folding reactions of small proteins. *Annu. Rev. Biochem.* **59**, 631–660
11. Ptitsyn, O.B. (1995) Molten globule and protein folding. *Adv. Protein Chem.* **47**, 83–229

12. Baldwin, R.L. and Rose, G.D. (1999) Is protein folding hierarchic? I. Local structure and peptide folding. *Trends Biochem.* **24**, 26–33
13. Chakraborty, S. and Peng, Z. (2000) Hierarchical unfolding of the lactalbumin molten globule: presence of a compact intermediate without a unique tertiary fold. *J. Mol. Biol.* **298**, 1–6
14. Sari, N., Alexander, P., Bryan, P.N., and Orban, J. (2000) Structure and dynamics of an acid-denatured protein G mutant. *Biochemistry* **39**, 965–977
15. Akhtar, M.S., Ahmad, A., and Bhakumi, V. (2002) Guanidinium chloride- and urea-induced unfolding of the dimeric enzyme glucose oxidase. *Biochemistry* **41**, 3819–3827
16. Jaenicke, R. (2000) Folding and association of oligomeric and multimeric proteins. *Adv. Protein Chem.* **53**, 329–401
17. Gokhale, R.S., Ray, S.S., Balaram, H., and Balaram, P. (1999) Unfolding of *Plasmodium falciparum* triosephosphate isomerase in urea and guanidinium chloride: Evidence for a novel disulfide exchange reaction in a covalently cross-linked mutant. *Biochemistry* **38**, 423–431
18. Hornby, J.A.T., Luo, J.K., Stevens, J.M., Wallace, L.A., Kaplan, W., Armstrong, R.N., and Dirr, H.A. (2000) Equilibrium folding of dimeric class μ glutathione transferases involves a stable monomeric intermediate. *Biochemistry* **39**, 12336–12344
19. Whitby, L.B. (1953) A new method for preparing flavin-adenine dinucleotide. *Biochem. J.* **54**, 437–442
20. McKinnon, A.E., Szabo, A.G., and Miller, D.R. (1977) The deconvolution of photoluminescence data. *J. Phys. Chem.* **81**, 1564–1570
21. Hiromasa, Y., Aso, Y., Yamashita, S.,and Meno, K. (2000) Thermal disintegration of the *Bacillus stearothermophilus* dihydrolipoamide dehydrogenase. *Biosci. Biotechnol. Biochem.* **64**, 1923–1929
22. Dawson, W.R. and Windsor, M.W. (1968) Fluorescence yields of aromatic compounds. *J. Phys. Chem.* **72**, 3251–3260
23. Bastiaens, P.I.H., van Hoek, A., Wolkers, W.F., Brochon, J., and Visser, A.J.W.G. (1992) Comparison of the dynamical structures of lipoamide dehydrogenase and glutathione reductase by time-resolved polarized flavin fluorescence. *Biochemistry* **31**, 7050–7060
24. Semisotnov, G.V., Rodionova, N.A., Kutyshenko, V.P., Event, B., Blank, J., and Ptitsyn, O.B. (1987) Sequential mechanism of refolding of carbonic anhydrase b. *FEBS Lett.* **224**, 9–13
25. Semisotnov, G.V., Rodionova, N.A., Razgulyaev, O.I., Uversky, V.N., Gripas, A.F., and Gilmanshin, R.I. (1991) Study of the molten globule intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymer* **31**, 119–128
26. Li, X., Lei, X-D., Cai, H., Sheng-Li, J.L., Wong, C.C., Tsou, C.L. (1998) Binding of a burst-phase intermediate formed in the folding of denatured D-glyceraldehyde-3-phosphate dehydrogenase by chaperonin 60 and 8-anilino-1-naphthalenesulphonic acid. *Biochem. J.* **331**, 505–511
27. Mattevi, A., Schierbeek, A.J., and Hol, W.G.J. (1991) Refined crystal structure of lipoamide dehydrogenase from *Azotobacter vinelandii* at 2.2 Å resolution. A comparison with the structure of glutathione reductase. *J. Mol. Biol.* **220**, 975–994
28. van Berkel, W.J.H., Benen, J.A.E., and Snoek, M.C. (1991) On the FAD-induced dimerization of apo-lipoamide dehydrogenase from *Azotobacter vinelandii* and *Pseudomonas fluorescens*. Kinetics of reconstitution. *Eur. J. Biochem.* **197**, 769–779
29. Nishimoto, E., Yamashita, S., Szabo, A.G., and Imoto, T. (1998) Internal motion of lysozyme studied by time-resolved fluorescence depolarization of tryptophan residues. *Biochemistry* **37**, 5599–5607
30. Ichye, T. and Karplus, M. (1983) Fluorescence depolarization of tryptophan residues in proteins: a molecular dynamics study. *Biochemistry* **22**, 2884–2893
31. Lipari, G. and Szabo, A. (1982) Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 2. Analysis of experimental results. *J. Am. Chem. Soc.* **104**, 4559–4570
32. Ptitsyn, O.B. (1973) Stages in the mechanism of self-organization of protein molecules. *Dokl. Acad. Nauk SSSR* **210**, 1213–1215
33. Jennings, P.A. and Wright, P.E. (1993) Formation of a molten globule intermediate early in the kinetic folding pathway of apomyoglobin. *Science* **262**, 892–896
34. Chamberlain, A.K., Handel, T.M., and Marquesee, S. (1996) Detection of rare partially folded molecules in equilibrium with the native conformation of RNaseH. *Natl. Struct. Biol.* **3**, 782–787
35. Mayr, E.M., Jaenicke, R., and Glockshuber, R. (1997) The domains in b-crystallin: identical fold-different stabilities. *J. Mol. Biol.* **269**, 260–269
36. Ayed, A. and Duckworth, H.W. (1999) A stable intermediate in the equilibrium unfolding of *Escherichia coli* citrate synthase. *Protein Sci.* **8**, 1116–1126
37. Koshiba, T., Yao, M., Kobashigawa, Y., Demura, M., Nakagawa, A., Tanaka, I., Kuwajima, K., and Nitta, K. (2000) Structure and thermodynamics of the extraordinarily stable molten globule state of canine milk lysozyme. *Biochemistry* **39**, 3248–3257