Protein Folding Pathways of Adenylate Kinase from *E. coli*: Hydrostatic Pressure and Stopped-Flow Studies†

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ABSTRACT: Adenylate kinase (AKe) from *E. coli* is a small, single-chain, monomeric enzyme with no tryptophan and a single cysteine residue. We have constructed six single-Trp mutants of AKe to facilitate optical studies of these proteins and to specifically examine the interrelationship between their structure, function, dynamics, and folding reactions. In this study, the effects of hydrostatic pressure on the folding reactions of AKe were studied. The native structure of AKe was transformed to a non-native, yet pressure stable, conformation by hydrostatic pressure of about 300 MPa. This pressure lability of AKe is rather low for a monomeric protein and presumably may be attributed to substantial conformational flexibility and a correspondingly large volume change. The refolding of AKe after pressure-induced denaturation was reversible under ambient conditions. At low temperature (near 0 °C), the refolding process of pressure-exposed AKe mutants displayed a significant hysteresis. The observation of a slow refolding rate in the 193 region and a faster folding rate around the active site (86, 41, 73 regions) leads us to suggest that in the folding process, priority is afforded to functional regions. The slow structural return of the 193 region apparently does not hinder the more rapid return of enzymatic activity of AKe. Circular dichroism studies on the pressure-denatured Y193W mutant show that the secondary structure (calculated from far-UV spectra) returned at a rapid rate, but the tertiary structure alignment (calculated from near-UV spectra) around the 193 region occurred more slowly at rates comparable to those detected by fluorescence intensity. Denaturation of AKe mutants by guanidine hydrochloride and subsequent refolding experiments were also consistent with a much slower refolding process around the 193 region than near the active site. Fast refolding kinetic traces were observed in F86W, S41W, and A73W mutants using a fluorescence detection stopped-flow rapid mixing device, while only a slow kinetic trace was observed for Y193W. The results suggest that the differences in regional folding rates of AKe are not derived from the specific denaturation methods, but rather are inherent in the structural organization of the protein.

Adenylate kinase (AKe)[1] from *E. coli* is a ubiquitous monomeric enzyme of 23.5 kDa that catalyzes the reaction: $Mg^{2+}ATP + AMP \leftrightarrow Mg^{2+}ADP + ADP$ (*1*). The cell uses this reaction to convert AMP to ADP (*2*), thereby regulating the adenine nucleotide levels in the cell. AKe is also involved in other reactions such as the biosynthesis of phospholipid (*3*). Recently, a new form of adenylate kinase has been found in murine cells that are up-regulated by p53, but its biological function remains unclear (*4*). The biological significance of AKe motivates our interest in examining the relationship between its conformational dynamics and function.

AKe is well suited for studies of protein folding. Its amino acid sequence is known, and the X-ray crystallographic structures of wild-type AKe in the absence or presence of substrates have been solved (*5*). Based on these crystal structures, it was shown that AKe undergoes large conformational changes upon substrate binding, indicating high structural flexibility. Subsequently, we demonstrated by fluorescence energy transfer methods that in solution AKe undergoes a large domain motion, resulting in shielding of the substrate binding site (*6*). These observations suggest that hydrostatic pressure may be used as a facile perturbant of AKe conformations.

Hydrostatic pressure is a thermodynamic variable useful for studying protein folding and measuring the associated volume changes. The effects of pressure upon the fluorescence of a number of proteins have been studied (*7*−*10*). Compared with chaotropic agents, pressure does not add additional components to the system. Denaturation is induced at constant temperature in the absence of chemical denaturants. One can describe the change in protein free energy as a function of the interatomic distances in the protein molecules which, in turn, are changed by variations in applied pressure (*11*). The use of pressure as a "reagent-less"

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[1] Abbreviations: AKe, adenylate kinase; GdnHCl, guanidine hydrochloride; CD, circular dichroism; Trp, tryptophan.

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denaturant is also advantageous from a methodological point of view: transition to standard conditions (renaturation) is achieved simply by releasing the pressure.

The fluorescence of a tryptophan (Trp) residue in a protein is very sensitive to the changes of its local environment caused by temperature, pH, polarity, and other perturbants. Measuring the changes in the Trp fluorescence spectrum is a sensitive means to study protein folding. Wild-type adenylate kinase is devoid of Trp. Using site-directed mutagenesis, we introduced single-Trp mutations into different regions of AKe (S41W, A73W, F86W, Y133W, F137W, and Y193W) without disrupting the overall structure or activity of the enzyme. The positions of mutation were selected to cover different regions of the protein: some at the active site, some on the surface, and some deeply buried (Figure 1). The fluorescence of the Trp mutants can be used to determine local conformational changes upon AKe folding. The stability of proteins is remarkably tolerant to amino acid substitutions. Mutations are often accommodated by minor adjustments of protein structure without a substantial decrease in overall stability (*12*). The single-Trp mutants of AKe have all been well characterized, confirming the general observation that each individual mutation at most only slightly altered the overall structure and enzymatic activity (*13*).

Experimental results from genetic engineering, peptide synthesis, 2D nuclear magnetic resonance (NMR), stopped-flow fluorescence spectroscopy, stopped-flow circular dichroism (CD) spectroscopy, pressure-jump, temperature-jump, and other experimental approaches have provided new insights for the folding of globular proteins; it is a complex series of events that can occur over the 8 orders of magnitude time range ($10^{-6}-10^{2}$ s). Some processes in protein folding, such as the formation of secondary structure, are completed on the sub-millisecond time scale (*14*, *15*), yet the overall structure requires minutes or even hours to fully recover in vitro. For example, a recent report on the folding of L9 indicates that regions of this multidomain ribosomal protein fold independently and with remarkably different rates. The ability for one domain to fold very rapidly is not functionally relevant, because both domains must be folded for the protein to bind RNA. However, these observations do suggest that the faster folding domain of L9 might play a role in protecting the slower folding domain from proteolysis (*16*).

Protein folding pathways have usually been studied from the perspective of secondary or tertiary structure evolution. However, it is possible that in some enzymes, the hierarchy of the folding pathway relates to the role a particular region plays in the enzyme's function. For example, a fast folding domain may protect a slower folding domain from proteolysis, or ongoing enzymatic activity in a fast folding region may be unaffected from the slower folding of a remote region.

In our study of the folding of the AKe wild-type and tryptophan mutants induced by high hydrostatic pressure, we initially sought to identify intermediate states in the unfolding and refolding pathway, and the effect of mutation on the stability of the protein. Distribution of tryptophan residues throughout different regions of AKe provided a spectroscopic reporter of conformational dynamics in that region. During this study, we observed that different regions of the protein reach their equilibrium state in significantly different time regimes after compression/decompression. Combining the results of enzymatic activity assays and some GdnHCl denaturation/renaturation experiments, we suggest that AKe's folding pathway might be related to its regional functionality. Apparently, the substrate binding regions of AKe fold faster than other regions. Once the native structure of the substrate binding regions is restored, the protein's enzymatic activity also recovers. The slow establishment of tertiary structure in the 193 region, which is remote from the active site, does not affect the protein's enzymatic activity. This may account for the regional folding rate hierarchy.

MATERIALS AND METHODS

Sources. Gel blue A affinity resin was purchased from Bio-Rad Laboratories.

Protein Purification. The wild-type AKe and its mutants were purified using a Gel Blue A affinity column as previously described (*6*).

Fluorescence Spectroscopy. Some fluorescence measurements were performed on an ISA SPEX Fluoromax-2. All fluorescence measurements under high pressure were carried out on an AB2 spectrofluorimeter (SLM Instruments Co.). This instrument was modified in the INSERM laboratory to measure fluorescence in the pressure range from 0.1 to 700 MPa through a thermostated pressure vessel similar to the one described in (*17*). Water was used as a pressure vector. The pressure was generated by a manual pump and monitored by a pressure gauge (type PR851C, Top Industry, France) coupled to an Infinity barometer (Newport Electronique, France). The samples were placed in a 0.6 mL cylindrically shaped quartz cuvette (5 mm path length) sealed with a polyethylene membrane (Dura-Seal, PolyLabo) and a rubber ring. Samples were allowed to equilibrate for 10 min before each round of data collection. Tryptophan fluorescence was excited at 295 nm (4 nm slit), and emission spectra were collected between 300 and 420 nm (4 nm slit). Each spectrum was the average of 8 accumulations. Fluorescence intensity spectra were corrected by subtracting the fluorescence spectra of the buffer at each pressure.

Circular Dichroism Spectroscopy. All circular dichroism (CD) measurements were carried out on a Jasco-720. The far-UV CD spectra (250−190 nm) were measured with 0.1 cm path length cuvette. The near-UV CD spectra (300−250 nm) were measured in a 5 cm path length cuvette. The temperature of the sample was kept constant with an exterior water-bath circulating system during the measurement. The low-temperature CD measurements were conducted at 5 °C, rather than 0.5 °C used for the high-pressure fluorescence data, due to technical difficulties.

Stopped-Flow Fluorescence Spectroscopy. AKe mutants were denatured by 2 M GdnHCl, and loaded into a stopped-flow fast mixing device (Bio-logic, SFM-3). Various final GdnHCl concentrations were achieved by mixing the denatured protein solution with buffer solution at different volume ratios. Due to technical difficulties, all experiments were carried out at 5 °C rather than at 0.5 °C used for the high-pressure fluorescence data. Samples were excited at 295 nm, and the total fluorescence intensity was collected as the function of time with a long-band cutoff filter (WG320).

Enzymatic Activity Assay. The activity of AKe was determined by a reverse, coupled enzyme assay as previously

described (*18*):

$$2\text{ADP} \xrightarrow{\text{AKe}} \text{ATP} + \text{AMP}$$

$$\text{glucose} \xrightarrow{\text{ATP,coupled-enzymes}} \xrightarrow{\text{NADP}} \text{6-phosphogluconate}$$

The ATP produced by AKe was used to phosphorylate glucose to give glucose 6-phosphate, which in turn was oxidized by NADP. The activity of AKe was determined by monitoring the amount of NADPH produced at 340 nm. The assays were conducted at room temperature to minimize the temperature effect on the coupled enzymes. Individual assay generally requires 3 min to complete, which was a relatively short time span compared with the slow overall folding of the protein.

Analysis of Fluorescence Spectra and Determination of Thermodynamic Parameters. The shift in the fluorescence spectrum caused by protein denaturation was quantified by the average energy of the emission: center of mass (cm^{-1}), which was defined by Weber and co-workers

$$\langle \nu \rangle = \frac{\sum \nu_I F_I}{\sum F_I} \quad (1)$$

where F_I stands for the fluorescence intensity emitted at wavenumber ν_I. The degree of denaturation is defined from the following equation (*19*):

$$\alpha_P = \left[1 + Q\frac{(\langle \nu \rangle_P - \langle \nu \rangle_U)}{(\langle \nu \rangle_N - \langle \nu \rangle_P)}\right]^{-1} \quad (2)$$

where Q is the ratio of the quantum yields of the native and denatured protein. A two-state transition with a constant reaction volume was assumed. The equilibrium constant at a given pressure (K_P) is

$$K_P = \frac{\alpha_P}{1 - \alpha_P} \quad (3)$$

The thermodynamic parameters of ΔG_0 (standard free energy for unfolding) and ΔV (volume change upon unfolding) were determined by eq 4.

$$-RT \ln K_P = \Delta G_0 + P\Delta V \quad (4)$$

The pressure of half-transition ($P_{1/2}$) is given by eq 5.

$$P_{1/2} = -\frac{\Delta G_0}{\Delta V} \quad (5)$$

RESULTS

Intrinsic Fluorescence. We have constructed six single-tryptophan mutants of AKe. The position of each substituted residue is marked in Figure 1, which is derived from the crystal structure of wild-type AKe. The details of the crystal structure of the various Trp mutants are unknown. The F137W and Y133W Trp residues are located in the variable loop that covers bound ATP. The F86W Trp mutation is located near the AMP binding site, and the S41W Trp residue is in the smaller loop that closes over AMP. The A73W Trp residue is also located near the AMP binding region. The Y193W Trp residue is in a strand of the core β-sheet distant from substrate binding sites. All the Trp mutants have enzymatic activity roughly comparable (within 10%) to the wild-type AKe except for F86W (within 50%). The mutation in F86W disrupts the substrate binding efficiency rather than the activity.

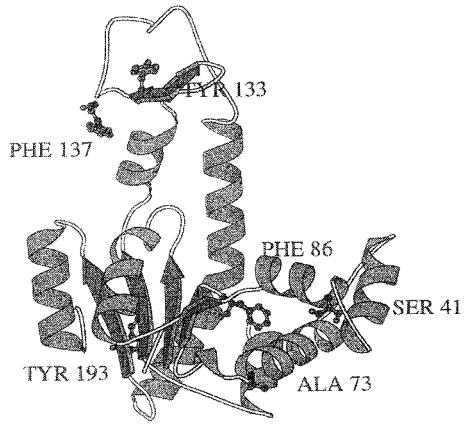

FIGURE 1: Ribbon representation of the X- ray crystal structure of adenylate kinase from *E. coli.* Each labeled residue is replaced by Trp (shown in ball and stick notation) to make a single-Trp mutant. The X-ray crystal structures of these single replacement mutants are unknown.

Wild-type AKe has seven tyrosine (Tyr) residues. The composite fluorescence from these residues shows a maximum at 301 nm. To avoid energy transfer between the Tyr and the Trp in the mutants, 295 nm was used as the excitation wavelength for the fluorescence studies. The emission maxima of adenylate kinase tryptophan mutants range from 326 nm for F86W and 355 nm for A73W, with Y193W, F137W, and S41W having intermediate values. The characteristics of the fluorescence spectra of these mutants are consistent with their local environments deduced from the X-ray crystal structure vis-à-vis solvent exposure.

Folding Reactions under High Hydrostatic Pressure. Compression of AKe by high hydrostatic pressure causes the enzyme to unfold, while decompression facilitates refolding. Figure 2 shows several representative examples of how the tryptophan fluorescence spectra of various AKe mutants change with hydrostatic pressure (at 30 and 0.5 °C). Upon compression, the spectrum of F86W was red-shifted, and the intensity was decreased; the spectrum of S41W showed no shift, yet the intensity was decreased; the spectrum of Y193W was red-shifted, and the intensity was increased. The fluorescent spectrum of mutant F137W showed no apparent changes with pressure perturbation (data not shown), perhaps because this residue is located in a flexible, disordered, and solvent-exposed environment under ambient conditions (see Figure 1). Wild-type AKe, which was monitored through emission of its seven tyrosine residues, also showed only minor spectral changes with compression. Figure 3 shows representative pressure (compression/decompression) curves for the percentage of fluorescence intensity change in S41W and Y193W at 30 °C. The unfolding curves are sigmoidal, suggesting a cooperative nature for the transition. The same sigmoidal pattern was also observed for pressure perturbation of AKe mutants at low temperature (0.5 °C). The high hydrostatic pressure denaturation curves for the Trp mutants of AKe showed the following general characteristics:

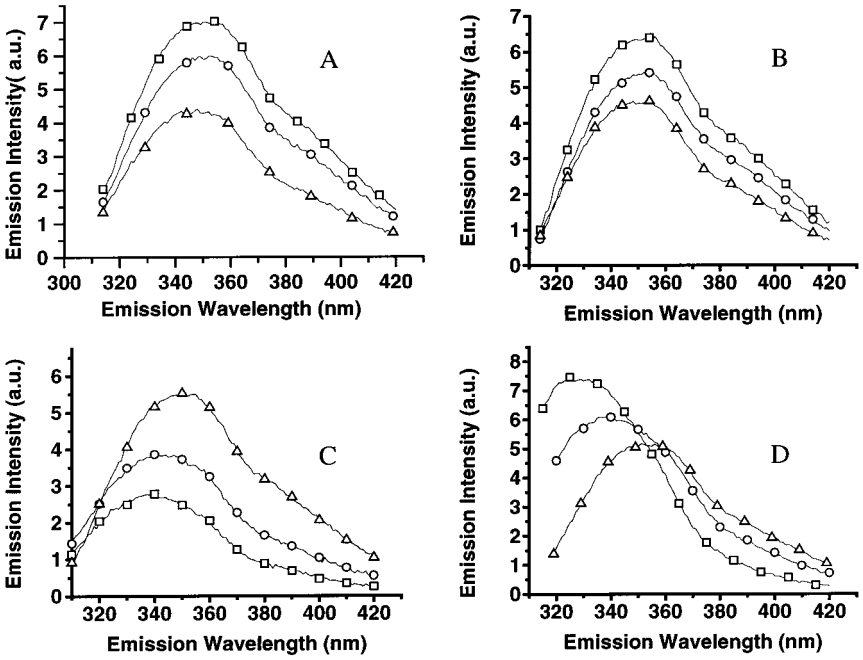

FIGURE 2: Emission spectra of AKe mutants upon compression. (A) S41W (30 °C); (B) S41W (0.5 °C); (C) Y193W (0.5 °C); (D) F86W (0.5 °C). AKe mutants at atmospheric pressure (□); AKe mutants at transition pressure (○); AKe mutants at 300 MPa (△).

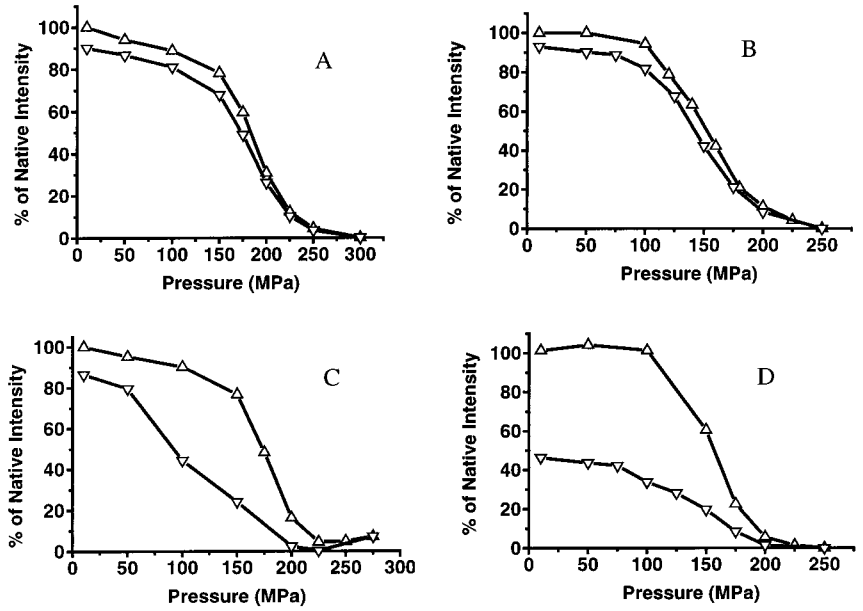

FIGURE 3: Unfolding (△) and refolding (▽) curves for (A,C) S41W and (B,D) Y193W induced by high hydrostatic pressure at 30 °C (A,B) and 0.5 °C (C,D).

•Between 0.1 and 130 MPa, the fluorescence spectra were largely invariant, suggesting minor changes, if any, in the protein structure. We assign structural stability to AKe in this pressure range.

•Between 130 and 250 MPa, there were large changes in the fluorescence spectra, indicative of large conformational changes in the protein.

•At 300 MPa, a plateau region was obtained, suggesting that AKe attains a non-native conformation, which is a pressure-stable state.

The unfolding curve for all the AKe mutants displayed an apparent two-state transition upon compression, suggesting that the entire protein was unfolded in a two-state process. Based on eqs 2−5, the calculated ΔG_0, ΔV, and $P_{1/2}$ are listed in Table 1.

Table 1: Thermodynamic Parameters of AKe Mutants at 0.5 and 30 °C

	S41W at temp (°C)		F86W at temp (°C)		Y193W at temp (°C)	
	0.5	30	0.5	30	0.5	30
$P_{1/2}$ (MPa)	173	185	187	200	156	152
ΔV (mL/mol)	−146	−112	−142	−96	−149	−110
ΔG (kcal/mol)	6.03	4.98	6.26	4.57	5.96	4.55

When the pressure was released to 0.1 MPa at the same pace of compression, AKe mutants showed little hysteresis in the return of the fluorescence spectra to the native species at 30 °C, and varied degree of hysteresis at 0.5 °C (Figure 3). Hysteresis is a delay of returning to the same structure at the corresponding condition. For example, at 0.5 °C, upon

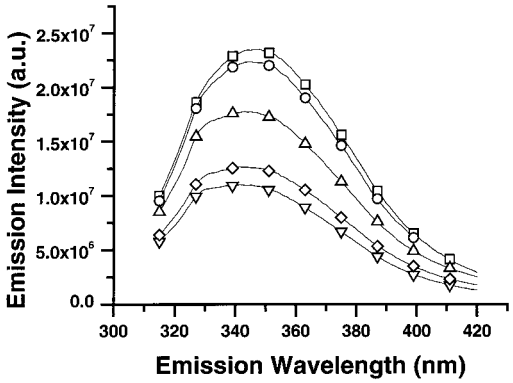

FIGURE 4: Spectra of Y193W were measured after high-pressure treatment with different incubation times. (□) 0 h after incubation, (○) 2 h after incubation, (△) 8 h after incubation, (◇) 24 h after incubation. Emission spectra of Y193W before high-pressure treatment (▽). The measurements and incubations were conducted at 0.5 °C.

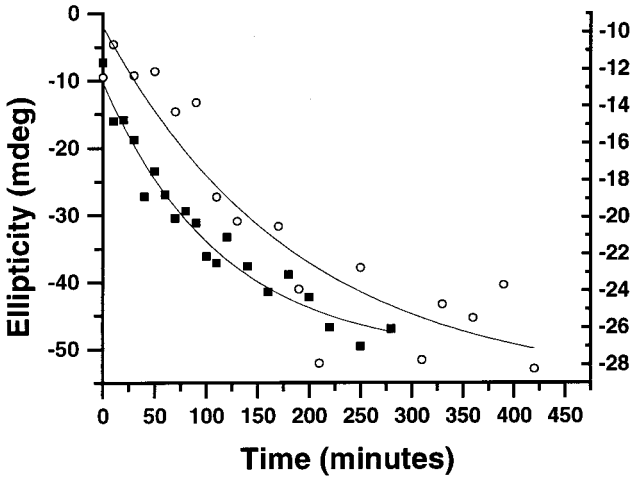

FIGURE 5: Folding of AKe mutants (■, F137W; ○, Y193W) at 5 °C after high-pressure treatment. The data points represent the sum of the change in ellipticity in the 260−285 nm region as a function of time.

decompression, mutant S41W displayed hysteresis at the intermediate pressure condition, yet returned to its native structure once the decompression was complete. While mutant Y193W displayed hysteresis not only at the intermediate pressure range but also at 0.1 MPa, only 50% of the fluorescence of native Y193W was recovered. F86W showed similar unfolding and refolding profiles to S41W (data not shown), suggesting the prompt return of the native regional structure of these unfolded mutants. Incubation of the Y193W mutant at atmospheric pressure and 0.5 °C for a longer period of time after pressure treatment resulted in the gradual recovery of its original spectrum. Figure 4 shows the emission spectra of Y193W before and after pressure treatment. It also shows the spectral recovery measured after different incubation times (2, 8, and 24 h). The slow recovery of the fluorescence spectrum to that of the fully folded conformation of mutant Y193W strongly suggests that the region in Y193W that was being tracked by tryptophan fluorescence was refolding at a much slower rate than other regions in AKe.

AKe is considered to be a monomeric enzyme in solution (*20*); however, the unexpectedly low $P_{1/2}$ observed for unfolding is more consistent with a multimeric protein dissociation. Additional experiments were performed to confirm that the changes in the fluorescence spectra of each AKe mutant upon compression/decompression were not due to dissociation of putative protein aggregates. Denaturation of a monomeric globular protein is a zero-order reaction. Its K_{eq} is protein concentration independent. On the other hand, protein dissociation is a multiple-order reaction, which results in concentration dependence for the K_{eq}. When the AKe mutants were denatured by pressure at different concentrations, no shift in the K_{eq} was observed, indicating a zero-order reaction (data not shown). Calculation indicates that if it were a dimer to monomer dissociation at 175 MPa, then a 5-fold concentration difference would shift the unfolding curve 0.2 MPa, which could be easily distinguished from the graph. The monomeric nature of AKe was also confirmed by gel experiments (data not shown).

The far-UV and near-UV circular dichroism (CD) spectra of the AKe mutants were measured before and after pressure treatment at 5 °C. The far-UV spectra (190−250 nm), which reflect the extent of secondary structures in a protein, returned to their native state values after decompression (data not shown). By contrast, the near-UV spectra (250−310 nm) of the AKe mutants all shared a slower recovery rate after decompression at 5 °C. Figure 5 showed the change in ellipticity as a function of time in the near-UV region of F137W and Y193W after the compression/decompression cycle at 5 °C. It takes hours for the protein to return to its native structure. The slow recovery of the near-UV protein CD spectra, which report on the orientation of aromatic residues and tertiary structure, suggests that in all the AKe mutants a longer time is necessary to establish tertiary contacts than to form secondary structure. This result is important, because it underscores the fact that unlike the distinguishing fluorescence data for Y193W, the CD spectral data show a commonality for all the AKe mutants. From the CD spectral data, one may infer that the secondary and tertiary structural contacts around residue 193 experience similar folding patterns whether the tyrosine residue found in the wild-type enzyme is substituted by tryptophan.

Folding Reactions Induced by GdnHCl. AKe mutants can be completely unfolded by 2 M GdnHCl and reversibly refolded upon dilution of the chaotropic agent. The kinetics of the refolding process can be divided into multiple phases: burst phase, fast phase, and slow phase as shown in Figure 6. Taking the fluorescence intensity for native F86W as 100%, the completely denatured protein (in 2 M GdnHCl) displayed 57% of its original intensity. When the GdnHCl concentration was jumped from 2 to 0.4 M at 5 °C, 23% of the original fluorescence intensity was recovered within the dead-time of mixing (burst phase, which is less than 5 ms). An additional 10−15% of the intensity was recovered in less than 5 s (fast phase). The remaining 5−10% of the intensity required a few minutes to recover (slow phase). Mutants S41W and A73W showed similar multiple phases in the refolding process (data not shown). However, Y193W displayed a substantially different refolding process. The completely denatured protein (in 2 M GdnHCl) displayed 250% of its original intensity. When the GdnHCl concentration was jumped from 2 to 0.2 M, a single-component kinetic trace was obtained with no apparent burst phase or fast phase (Figure 7). At 30 °C, the original intensity was recovered in 10 min; at 20 °C, the intensity was

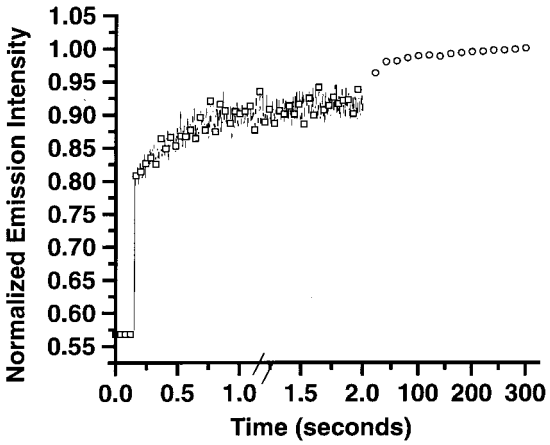

FIGURE 6: Refolding kinetics of F86W at 5 °C by diluting GdnHCl from 2 to 0.4 M. The horizontal line at the beginning of the curve indicates the fluorescence intensity of denatured F86W. The vertical line represents the burst phase, which cannot be measured by the instrument. The kinetic trace can be divided into three phases: burst phase <5 ms, fast phase <5 s, slow phase <300 s.

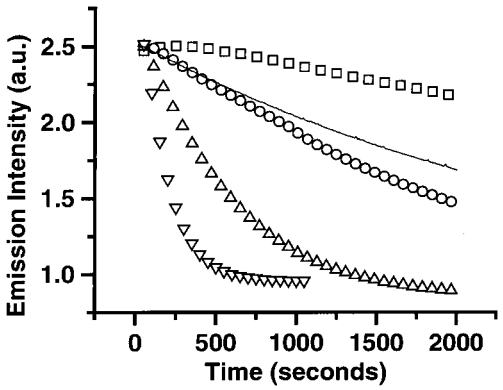

FIGURE 7: Refolding of Y193W by diluting GdnHCl from 2 to 0.2 M at different temperatures (□, 5 °C; ○, 10 °C; △, 20 °C; ▽, 30 °C). The solid line is the refolding trace of Y193W after high-pressure treatment at 10 °C.

Table 2: Specific Activity of AKe and Its Mutants before/after Pressure Treatment at 0.5 °C

	wild type	S41W	F86W	Y193W
native (μmol min^{-1} mg^{-1})	288 ± 30	289 ± 30	155 ± 20	288 ± 30
refolded (μmol min^{-1} mg^{-1})	262 ± 25	257 ± 25	148 ± 20	271 ± 25

recovered in less than 30 min. When the refolding reaction for Y193W was carried out at 5 °C, only a small portion of the intensity was recovered in 30 min, unlike the other mutants, which recovered almost all their intensity in 30 min. The folding trace of Y193W at 10 °C after high-pressure denaturation was plotted in Figure 7 for comparison. Clearly, the 193 region is folding at a much slower rate than the other regions or, alternatively, Y193W becomes trapped in a transient folding state.

Enzymatic Activity Assay. The activity assays were performed on all the refolded mutants and wild-type AKe proteins immediately after pressure treatment. The coupled enzyme assay requires about 3−4 min. The results showed that refolded protein after pressure denaturation (0.5 °C) regains most (85−95%) of its original activity as judged by specific activity (Table 2). Specific activity is defined as the amount (micromoles) of ADP that is converted to ATP by 1 mg of AKe in 1 min. Table 2 shows the specific activity of each mutant before and after pressure treatment at 0.5 °C. A control experiment was carried out to confirm that the protein did not gain additional native structure during the course of assay. No change was observed in the fluorescent spectra of the protein before and after the assay.

Table 3: Folding Kinetics ($t_{1/2}$) of Y193W and Other Mutants Measured by Fluorescence and Circular Dichroism

	Y193W	other mutants
far-UV CD 5 °C	within minutes (pressure)	within minutes (pressure)
near-UV CD 5 °C	>2 h (pressure)	>2 h (pressure)
fluorescence 0.5 °C	~8 h (pressure)	within minutes (pressure)
fluorescence 5 °C	>2 h (only slow phase, GdnHCl)	within minutes (has burst, fast, slow phase using stopped-flow, GdnHCl)
fluorescence 10 °C	~40 min (only slow phase, GdnHCl), ~40 min (pressure)	N/A
fluorescence 20 °C	~15 min (only slow phase, GdnHCl)	N/A
fluorescence 30 °C	~4 min (only slow phase, GdnHCl)	N/A

To further confirm the full recovery of the enzyme's function after pressure treatment, the K_m for ATP binding to the Y193W mutant was measured. The K_m for pressure-treated Y193W was 34 ± 5 μM, while the K_m for nonpressurized Y193W was 38 ± 3 μM.

DISCUSSION

High Hydrostatic Pressure as a Perturbation of Protein Structure. It has been recognized for some time that high hydrostatic pressure can disrupt interactions between protein regions (intra- and intermolecular contacts) by compression of void volumes and cavities (*19*, *21*). At low protein concentration, most proteins display complete folding reversibility upon the release of high pressure (*22*, *23*). The folding process of AKe mutants after compression was completely reversible at high temperature (30 °C). From these experiments, assuming that they occur under thermodynamic equilibrium, one can calculate the free energy and volume change of the reaction. The generally mild perturbation induced by pressure and the level of reversibility in the reaction make it a useful research tool in studying protein structure and dynamics. While earlier studies examined the thermodynamics of protein folding in response to pressure perturbation (*11*), more recent work has explored kinetic aspects with pressure jump studies (*22*, *24*). The volume changes associated with denaturation of globular proteins have generally ranged below −100 mL/mol. It is clear from the data in Table 1 that AKe showed a larger volume change and a $P_{1/2}$ significantly lower than that observed in other globular proteins. Small monomeric proteins are usually denatured in the pressure range of 400−800 MPa (*25*). Proteins with multiple subunits tend to dissociate at lower pressure. Control experiments eliminated the possibility that the changes in the fluorescent spectra of each AKe mutant upon compression/decompression were caused by protein aggregation. The low $P_{1/2}$ values of the AKe mutants indicate their conformational lability. A recently published paper on pressure denaturation of a 33 kDa protein from the spinach

photosystem II particle also showed a low $P_{1/2}$ (120 MPa) and a large volume change (in excess of −100 mL/mol) (*26*). In an earlier study (*6*), we measured domain motion in AKe of roughly 15 Å to shield a substrate binding region. This is a very large motion in such a relatively small enzyme, and it suggested that conformational lability was a structural hallmark of AKe. There are two possible sources for AKe's lability under pressure: (1) the enzyme is very unstable, which is reflected by a low $\Delta G°$ (Table 1), and/or (2) the enzyme has multiple void volumes, resulting in large volume changes (Table 1).

At 300 MPa, AKe and its mutants were at a non-native, pressure-stable state. Under those experimental conditions, we had no available methods for establishing the existence of any residual structures in that conformation of AKe. However, the large spectral red shift in the AKe fluorescence did suggest a certain degree of disruption in their secondary and tertiary structure. In general, a variety of approaches have supported the idea that the denatured conformation is not necessarily unique and that residual structure may remain in many unfolded proteins (*27*, *28*). This is an ongoing area of research, and no compelling theory has emerged to explain these observations.

The Refolding Process of AKe Mutants. AKe mutants were denatured with high hydrostatic pressure or GdnHCl at different temperatures, and the refolding processes were followed by fluorescence, far-UV, and near UV circular dichroism at different time scales. Chaotropic agents unfold proteins by migrating into the interior of the protein and forming hydrogen bonds to atoms in the backbone (*29*). Regardless of the different mechanisms involved in high-pressure and GdnHCl protein denaturation, we obtained very similar folding kinetics. At low temperature, the folding kinetics were relatively slow, which made them easy to follow. In the high-pressure denaturation experiments, the AKe mutants displayed variable levels of folding hysteresis at low temperature arising from folding retardation. The difference in the degree of hysteresis among the AKe mutants suggested varied regional folding rates.

Table 3 summarizes all the kinetic information on the folding rates of Y193W and the other mutants. The first column from the left lists all the detection methods and the temperature conditions. The next two columns present the approximate $t_{1/2}$ for Y193W and the other mutants. The denaturation methods are listed in parentheses next to the $t_{1/2}$. The major folding rate pattern is that Y193W folding is much slower than that of the other mutants regardless of detection or denaturation methods. All mutants have an increase in folding rate at higher temperature.

With fluorescence-detected, stopped-flow, rapid mixing, we were able to monitor the folding process of AKe mutants in the millisecond time scale. At 5 °C, when the AKe mutants were diluted from 2 to 0.4 M GdnHCl, several mutants (F86W, S41W, A73W) displayed multiple phases (burst, fast, and slow phase). Using fluorescence resonance energy transfer (FRET), the Haas group measured the intramolecular distance distribution during the folding of adenylate kinase on the millisecond time-scale (*30*). Their results showed that the mean of the apparent intramolecular distance distribution reduced to a value close to its magnitude in the native protein (within 2 ms, the dead-time of the instrument). However, the width of that distribution was rather large. Only at much longer times, did the width of the distribution recede to its value in the native conformation. The presence of multiple phases during the folding of AKe described by Haas et al. is consistent with our observations. However, we observed a different folding pattern in mutant Y193W. It only displayed a single slow phase in the refolding process. Even at elevated temperatures, Y193W still showed a single folding phase, which lasted minutes (Figure 7).

The fluorescence of tryptophan in a protein has been extensively studied as an intrinsic probe of protein structure and dynamics. The sensitivity of tryptophan's spectroscopic properties to the (solvent) microenvironment provides a tool to follow the regional and global conformational change of the protein as the protein unfolds, refolds, or interacts with a substrate. However, if the changes in protein structure occur far away from the reporter, the Trp spectrum may not fully reflect the conformational change of the particular region. The different folding kinetics found for the AKe mutants suggest that some regions of AKe (F86, S41, A73, etc.) return to their native state faster than other regions (Y193). The Trp in the Y193W mutant is located in the hydrophobic core of the protein, where one expects rapid folding. The Gruebele group observed that the collapse of the unfolded state of apomyoglobin to a compact state was completed in a few microseconds (*15*). The lack of a burst or a fast phase, as detected by fluorescence, in Y193W apparently contradicts this widely accepted view of protein folding. However, in Y193W, the fluorescence pattern is different from the other mutants. For example, its fluorescence intensity was greatly enhanced upon unfolding, which indicates that a strong quencher was located near the Trp in its native structure. It is possible that the 193 region was misfolded during the core formation (collapse). Such misfolding events have been observed in many proteins (*31*−*33*). In the misfolded state, if the Trp is kept away from the quencher, then the burst and fast phases of the refolding will not be associated with any change or only an insignificant change in Trp emission. In other words, the fluorescence intensity will not change during core collapse. The far-UV circular dichroism spectra of Y193W and other mutants measured before and after refolding indicated a fast and essentially full recovery of the protein's secondary structure. Only at the final stages of folding, when the protein is adjusting its tertiary structure, will the Trp in Y193W relocate closer to its quencher until it resumes the native structure. If this hypothesis is true, then the slow folding rate observed in Y193W is due to a conformational rearrangement. Similar observations have been reported by the Rinas group (*34*). They found that the very fast and early phases of refolding of hFGF-2 are not associated with any change in tryptophan emission.

Similar folding patterns were also observed in the folding of AKe mutants induced by high pressure. Due to instrumental limitations, we could not directly measure the folding rate of the AKe mutants after the compression/decompression cycle on a millisecond and second time scale. At 0.5 °C, all the AKe mutants, except mutant Y193W, returned to their native state after the compression/decompression cycle (minutes later). It took many hours for mutant Y193W to return to its native state based on its fluorescence spectra (Figure 4). At a higher temperature (e.g., 10 °C), it still took Y193W more than 1 h to recover its native fluorescence spectrum (Figure 7). The fluorescence refolding kinetics of

Y193W at 10 °C induced by high pressure and GdnHCl were equivalent. This result suggests that the folding kinetics of AKe mutants are independent of denaturation methods, but rather they are inherent to the structural organization of the protein. Furthermore, the folding rates of AKe differ from region to region.

There is a possibility that the slow return in Trp fluorescence of mutant Y193W is due to the Trp substitution. There have been findings that a single-site mutant can lead to a dramatic change in the refolding rate. Maki et al. found the folding kinetics of staphylococcal nuclease were remarkably affected by some proline mutations (*35*). A single mutation, Phe173 to Ala, induces a molten globule-like state in murine interleukin-6 (*36*). However, our mutation of 193 is a conserved residue substitution, which should have minimal effect on the local and overall protein structure. The stability and specific enzymatic activity for all the mutants were about the same. This result suggests that the slow folding of the 193 region is intrinsic to the protein rather than an artifactual effect of mutation. The near-UV circular dichroism spectra of Y193W and the other mutants after the compression/decompression cycle at 5 °C were measured to exclude such a possibility. The near-UV CD spectra mainly reflect the orientation of the Trp and Tyr residues in the protein. In AKe, five out of seven Tyr residues are located near the 193 region. Therefore, if the 193 region is indeed folding slower than other regions, we should be able to observe the same slow folding rate not only in mutant Y193W, but also among the rest of the AKe mutants. If it is only a Trp residue mispacking due to mutation, then we would only be able to observe the slow folding rate in Y193W, but not in any other AKe mutants. Our near-UV CD experimental results showed that a longer incubation time (hours) was needed for the full return of the spectra of all AKe mutants after compression (Figure 5). This result clearly demonstrated that the slow folding rate reflected the 193 regional folding rate, rather than single Trp misplacement.

A Link between AKe's Refolding Pathway and Its Regional Function. When a protein refolds from a denatured state, secondary structure and then tertiary structure arise. It is generally observed that a protein's enzymatic activity returns at a later stage of folding. However, this was not the case in the folding reaction of the AKe mutants. We have observed that the protein's enzymatic activity recovered before the complete recovery of the structure. Our conclusion is based on the fact that the fluorescence spectrum of the Y193W mutant after the compression/decompression cycle did not return to its native fluorescent spectrum; however, its enzymatic activity was 85−95% recovered (Table 2). At room temperature, the K_m of ATP binding to the Y193W mutant remained similar to that of native Y193W after compression/decompression, further confirming that the non-native structure in the 193 region did not affect AKe's enzymatic activity and its binding affinity for its substrate. The enzymatic activity assay usually takes 3 min to complete. One could argue that the non-native Y193W returned to its fully folded state in that 3 min. However, based on the folding kinetics of Y193W (Figure 7), we know that at ambient temperature the native structure of Y193W is fully recovered in about 30 min. Compression/decompression of Y193W in the presence of substrate analogue (AP5A, a nonhydrolyzable substrate analogue) did not accelerate the enzyme's return to its native conformation (data not shown). Consequently, we do not anticipate that substrate binding during the enzyme activity assay will accelerate AKe folding kinetics. In addition, if there is an increase in the enzyme's activity as a function of time during the assay, we should be able to see a nonlinear enzymatic activity curve. The linear enzymatic curve obtained in our experiment suggested a constant enzymatic activity of the measured protein.

Closer examination of the function of each region of AKe revealed that the Trp residues of F86W, S41W, and A73W mutants report on regions adjacent to the AMP or ATP binding sites, which could be considered as functionally important regions. The 193 region is remote from the active site and can be regarded as a noncritical region in terms of enzymatic activity. Perhaps this is why the slow return of native structure in the 193 region does not affect this mutant AKe's enzymatic activity. In other words, the recovery of AKe's enzymatic activity only correlates with the return of the structure at its active site. Based on our observations, the functionally important regions of AKe refold in the time scale of minutes or even faster, while the noncritical region requires a much longer time to regain a native structure. Since the slow return of structure to the noncritical region does not affect the protein's enzymatic activity, a slow folding rate is not functionally detrimental.

In summary, the protein folding process usually spans many orders of magnitude in time. The general sequence of protein folding involves formation of secondary and then tertiary structure followed by the restoration of enzymatic activity. In AKe, we observed an interesting pattern, in which the protein recovered a fully functional active site faster than the restoration of full tertiary contacts. For AKe, the incomplete folding of the 193 region did not diminish the protein's enzymatic activity once its active site structure was attained. This observation is in agreement with the concept that some structural changes (such as removing certain segments of the protein) do not necessarily change the protein's function as long as the functional site is conserved.

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