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Compact thermally-denatured state of a staphylococcal nuclease mutant from resonance energy transfer measurements

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Abstract

Thermal denaturation of a staphylococcal nuclease mutant K78C, where lysine 78 is replaced by cysteine, was studied by circular dichroism (CD) and resonance energy transfer. CD spectra suggest that residual structures remain in the denatured state. Steady-state energy transfer from intrinsic tyrosines to a single and intrinsic tryptophan was measured at different temperatures. In the thermally-denatured state of K78C, there is still a substantial degree of energy transfer from tyrosine(s) to tryptophan, indicating residual structures in the denatured state. The cysteine residue in mutant K78C was labeled with a cysteine specific probe IAEDANS. Fluorescence decays of the tryptophan were measured to estimate distance distributions between Trp 140 and IAEDANS at position 78. Measurements were done as a function of temperature from 4°C (native) to 65°C (denatured) both with and without Ca^{2+} and inhibitor pdTp. Below 30°C, the apparent distance distribution of both the ligand-free nuclease and the enzyme with bound pdTp can be adequately described by a Gaussian model. Above 40°C, where the ligand-free nuclease but not the ternary complex begins to denature, two different populations are required to fit the data both with and without pdTp. One population has a compact structure and the other has an expanded structure. As temperature rises, the population of the expanded structure increases. At the highest temperature, the non-native compact structure is still the major form (60 to 70%). The overall thermally-denatured states of staphylococcal nuclease mutant K78C in the absence and presence of ligands are thus compact and heterogeneous.

Keywords: Staphylococcal nuclease; Resonance energy transfer; Compact denatured state; Circular dichroism; Fluorescence

1. Introduction

Structural characterization of native and denatured states of proteins in solution is important for the understanding of protein folding and stability. The native state of a protein is in a unique conformation with minimum free energy [1] though considerable structural fluctuations may occur in solution [2]. The structure of the denatured state of a protein, on the other hand, is much less defined. Conditions that can destabilize native proteins are so diverse that it is difficult to imagine, from a structural point of view, a unique denatured state for all proteins under different denaturing conditions. The denatured

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state of a protein is rarely an unfolded random coiled state except under extreme conditions [3,4]. This is especially the case in thermal denaturation [5]. Thus the cooperative breakdown of proteins from the native state to the denatured state leads only to partial unfolding of the protein structure. In other words, the denatured state of a protein in most cases may be viewed as a partially unfolded state. Since the denatured state may be structured, studies of the denatured state can provide useful information on protein folding/unfolding pathways.

In this work, we applied time-resolved resonance energy transfer methods [6,7] to characterize the thermally-denatured state of a staphylococcal nuclease mutant K78C, where lysine 78 is replaced by a cysteine. Wild type staphylococcal nuclease is a very small protein (16.8 kD) with a single chain lacking disulfide bonds and has been extensively studied. We show that (a) the thermally-denatured state of K78C is compact and (b) there is substantial structural heterogeneity in the denatured state. We reached these conclusions based on average distance and distance distribution analysis. Average distances were calculated from energy transfer efficiencies measured either from intrinsic tyrosines to an intrinsic tryptophan or from the tryptophan to an extrinsically labeled IAEDANS [1]. Distance distribution results were obtained from analysis of energy transfer from tryptophan to IAEDANS.

2. Materials and methods

2.1. Sample preparation

The preparations of staphylococcal nuclease mutants K78C and W140H (tryptophan 140 replaced by a histidine) and labeling procedures of the K78C mutant have been reported elsewhere [8]. The mutants were purified to about 99% and time-resolved fluorescence measurements showed no detectable fluorescent contaminants under ultraviolet light excitation, which confirmed the purity of the sample. Protein concentration was determined based on a molar absorption coefficient 15770 cm^{-1} M^{-1} for K78C at 280 nm. For resonance energy transfer measurements using tryptophan as a donor, K78C was labeled with IAEDANS as an acceptor. The percentage of labeling of the single thiol group-containing protein was about 100% both by absorption and by DTNB (5,5′-dithiolbis-2-nitrobenzoic acid) titration. The mutant K78C shows enzymatic activity comparable to that of the wild type nuclease and the labeled protein shows slightly lower activity. For the experiments with and without ligands calcium and 2′-deoxythymidine-3′,5′-diphosphate (pdTp), the protein was in 0.1 *M* Tris, 0.05 *M* NaCl, pH 7.8 at room temperature. The pH of the buffered solution was 7.2 at 60°C. Thus, at the highest temperature used in the experiment, the pH of the solution is not lowered by more than one unit, which is still well within the pH range where the stability of the nuclease remains unchanged.

2.2. Circular dichroism measurements

The CD spectrum of staphylococcal nuclease mutant K78C was measured on a Jasco J-710 spectropolarimeter. Ten millimeter rectangular cells were used in a thermostated cell holder. The protein was in 0.05 *M* sodium sulfate, 0.02 *M* sodium phosphate, 1 m*M* EDTA, and pH 7.0. About 0.5 m*M* dithiothreitol (DTT) was present to prevent thiol oxidation. We did not extend CD measurements below 200 nm due to the interference from DTT. For comparison of CD spectra at different temperatures, we collected spectra from 200 nm to 250 nm. For melting determination, we monitored the CD signal at 220 nm as a function of temperature.

2.3. Steady-state fluorescence measurements

To detect resonance energy transfer from intrinsic tyrosines to the single and intrinsic tryptophan, we measured the steady-state fluorescence intensities of K78C and W140H on an SLM 8000 photon counting spectrophotometer. The concen-

[1] IAEDANS: 5-((2-((iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid.

trations of the proteins were adjusted such that the absorbances due to tyrosines at 280 nm were about the same for K78C and W140H. The proteins were dissolved in 0.05 *M* sodium sulfate, 0.02 *M* sodium phosphate, and 1 m*M* EDTA, and pH 7.0. The fluorescence spectra were corrected for buffer background at the corresponding temperatures. The samples were excited at 280 nm to observe fluorescence from tyrosines in W140H and that from tyrosines as well as tryptophan 140 in K78C. When the samples were excited at 295 nm, only tryptophan fluorescence was observed.

2.4. Time-resolved fluorescence measurements

Fluorescence decay of tryptophan in the nuclease mutant K78C was measured by a time-correlated single photon counting apparatus as described before [9]. The light source was a Spectra Physics (SP 3000 series) picosecond synchronously pumped mode locked dye laser system. Excitation of the protein was set at 295 nm. The emission was detected around 350 nm with a polarizer set at the magic angle. The combination of excitation and emission wavelengths essentially eliminates the contribution from tyrosine fluorescence in the experiments. A Ludox solution was used to collect the instrument response. Photomultiplier tube color effect was corrected in reference to melatonin in water which has a single lifetime about 5.4 ns at room temperature. Temperature of the cuvette holder was controlled by a Neslab water bath. Duplicate or triplicate data were collected at each temperature. After the temperature measurements, the samples were checked by absorption. They were still fully labeled though there had been some changes for the sample that went through the highest temperature used in the measurements (65°C).

2.5. Data analysis

Analysis procedures for the fluorescence decays of donor tryptophan in the absence and presence of acceptor IAEDANS have been described elsewhere [10]. Here we present only a brief description. The analysis involved two steps. In the first step, we obtained photophysical parameters of the donor itself and in the second step, we obtained structural parameters. The decay of tryptophan fluorescence in the protein in the absence of acceptor was analyzed by a sum of exponentials,

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \tag{1}$$

where α_i and τ_i are the amplitude and lifetime of the ih component. $I(t)$ was then convoluted with the measured instrumental response and compared with the measured decay curve by a nonlinear least squares method. Reduced-χ^2, plots of weighted residuals, and autocorrelation of the residuals were used to judge the quality of the fits. Parameters that are temperature dependent were properly taken into account.

The apparent distance distribution between donor and acceptor is modeled by $p(r)$. The fluorescence decay of donor in the presence of acceptor can be written as [27]

$$I(t) = \sum_k a_k \int p_k(r) \sum_i \alpha_i \times \exp\left[-\frac{t}{\tau_i}\left(1 + \left(\frac{R_0}{r}\right)^6\right)\right] \mathrm{d}r \tag{2}$$

where a_k is the relative concentration of the kth population and $\int p_k(r)\,\mathrm{d}r = 1.0$. R_0 is the Förster transfer distance, which was determined from the emission of the donor and the absorption of the acceptor. The random average of the orientational factor $\kappa^2 = 2/3$ was used in the calculation of R_0. The diffusion between donor and acceptor (mainly due to the acceptor IAEDANS) was not included in the analysis. At higher temperatures, this assumption may be partially invalid. Since staphylococcal nuclease still possesses substantial residual structures at temperatures much higher than those employed here as shown in CD spectra [11] and structured CD is present in the mutant in the denatured state (see Section 3), large scale translational diffusion between donor and acceptor is unlikely to occur on the time scale of two to three nanoseconds. Some local and limited diffusion may occur and they tend to

narrow down the width of the distribution. A change in the average donor–acceptor distance of a few angstroms due to diffusion does not affect our conclusions regarding the number of populations and the compactness of the denatured states.

If a population of distances is beyond the energy transfer distance, then we can only estimate its concentration but not its distance between donor and acceptor. In this case, we have (for two populations)

$$I(t) = A\int p(r)\sum_i \alpha_i \exp\left[-\frac{t}{\tau_i}\left(1+\left(\frac{R_0}{r}\right)^6\right)\right] dr + B\sum_i \alpha_i \exp(-t/\tau_i) \quad (3)$$

where A and B are related to the relative concentration of each distance population. If a protein is not 100% labeled, the fluorescence from the unlabeled donor will contribute to the second term, thus complicating the determination of the second distribution. We can use this equation to determine the relative concentration of each population since our samples were fully labeled. We note that eqs. (2) and (3) contain both structural information in distance distribution and thermodynamic information in concentration of each population.

We used a Gaussian form [26] to approximate the shape of distance distribution between donor tryptophan and acceptor IAEDANS

$$p(r) = \frac{1}{\sqrt{2\pi}\sigma}\exp\left[-\frac{1}{2}\left(\frac{r-\bar{r}}{\sigma}\right)^2\right] \quad (4)$$

where $\bar{r}$ is the average distance and σ is related to the full width at half maximum height (FWHM) by FWHM = 2.355σ. Both $\bar{r}$ and σ are adjustable parameters in fitting the donor decay data. The quality of data fitting was also judged by reduced-χ^2, plot of residuals, and autocorrelation of the residuals. It should be noted that the distance distribution obtained this way is an "apparent" one that contains contributions from real distance heterogeneity, orientation factor, and the linker arm [12].

The fluorescence decays of the donor–acceptor pair were also analyzed by multiexponentials as in eq. (1). The amplitudes and lifetimes were used to compute the average lifetime of the donor–acceptor pair $\langle\tau_{DA}\rangle$. With the average lifetime of the donor-only $\langle\tau_D\rangle$ we calculated the average energy transfer efficiency $\bar{E}$ by

$$\bar{E} = 1 - \langle\tau_{DA}\rangle/\langle\tau_D\rangle \quad (5)$$

and the average distance (without distribution analysis) by

$$\bar{r} = R_0\left(\frac{1-\bar{E}}{\bar{E}}\right)^{1/6} \quad (6)$$

3. Results

3.1. Presence of residual structure in the thermally-denatured state of unlabeled K78C

We measured CD spectra of K78C under native and denaturing temperature to compare the contents of secondary structure in the protein. Figure 1 shows the spectra at 20, 40 and 60°C, respectively. The melting point of this mutant as monitored by CD at 220 nm is about 51°C and agrees with that from fluorescence measurement. At 60°C, where the protein is denatured, the CD spectrum is far from that of a random coiled state in the native buffer [13]. Residual structures are thus present in the thermally-denatured state of K78C. Due to the large difference in the shape of

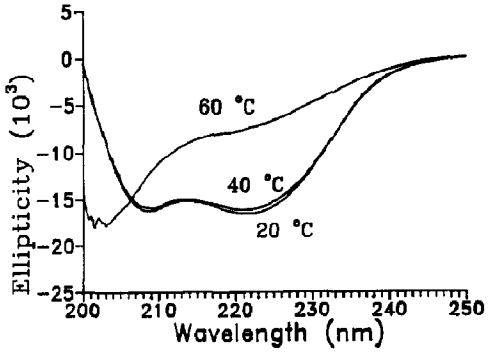

Fig. 1. CD spectra of staphylococcal nuclease mutant K78C at three temperatures (units in deg·cm^2/decimole). The protein is denatured at 60°C.

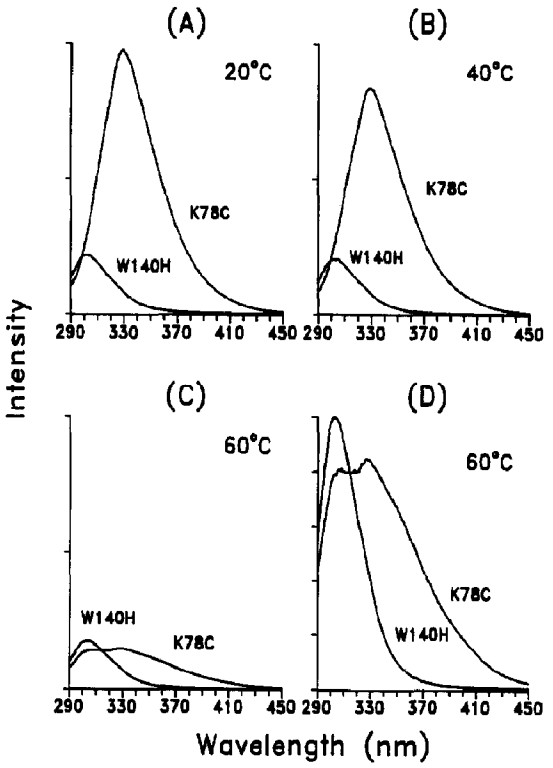

Fig. 2. Thermal denaturation of W140H and K78C as monitored by steady-state fluorescence. Excitation was at 280 nm. In (A), (B), and (C), the scales are the same. In (D) intensities were normalized to W140H peak. The absorbances due to tyrosines at 280 nm were adjusted to be the same for W140H and K78C.

random coiled state in staphylococcal nuclease and that from other proteins as pointed out by Shortle and Meeker [13], exact quantitation of the contents of residual structures in the denatured state is difficult. Nonetheless, the presence of residual structures implies that the thermally-denatured state of the protein is not fully extended and is likely to be compact.

3.2. Energy transfer from tyrosine to tryptophan in the unlabeled K78C

Figure 2 shows the steady-state fluorescence spectra of K78C and W140H in their native and denatured states. The samples were excited at 280 nm. There are seven tyrosines and one tryptophan in the wild type nuclease [14]. In W140H, the tryptophan is replaced by a histidine so that only fluorescence from tyrosines can be observed. In K78C both tyrosines and tryptophan 140 fluoresce when excited at 280 nm, while only tryptophan fluorescence can be detected with 295 nm excitation. In the native state, the fluorescence of K78C above 310 nm is primarily due to tryptophan (Fig. 2A). Although there are seven tyrosines in the nucleases, their total fluorescence intensity is weak as shown by that of W140H (Fig. 2A). Various factors in protein structure are responsible for the low intensity [15,16]. Compared to W140H, further quenching of tyrosine fluorescence in K78C is likely due to resonance energy transfer from tyrosines to tryptophan in the native state. At 60°C which is above the the melting points (50 to 53°C) of the proteins, the nucleases are in the denatured states.

The fluorescence of tryptophan in K78C is greatly reduced but the peak of the tryptophan emission (about 330 nm) is still close to that in the native state, where tryptophan 140 is buried. A fully solvent-exposed tryptophan should have an fluorescence emission peaked at about 350 nm (excitation at 295 nm leads to the same 330 nm peak position). Thus, there is some degree of clustering around tryptophan 140 in the thermally-denatured state. The intensity of tyrosine fluorescence in the denatured K78C is less than half of that in the denatured W140H. Thus a large degree of resonance energy transfer between tyrosine(s) and tryptophan 140 still occurs in the denatured state of K78C. We obtained similar results with the wild type nuclease. Since the Förster distance between tyrosine and tryptophan is about 10 to 15 Å [17], the observed energy transfer indicates a close distance between tyrosine(s) and tryptophan (< 20 Å) though it is not clear which tyrosines of the seven contribute to the transfer. There are 22 amino acid residues between tryptophan 140 and the nearest tyrosine (corresponding to an average distance of about 36 Å in a random coiled state). The results imply the existence of a partially-compact denatured state of K78C. By labeling cysteine 78, which is on the opposite side of tryptophan 140 in the protein, we obtain additional evidence that the overall thermally-denatured state of K78C is relatively compact.

3.3. Thermal denaturation of labeled K78C in the absence of ligands

The nuclease mutant K78C was labeled with IAEDANS for the study of the denatured state at high temperature. As we have shown elsewhere [8], K78C with an extrinsic probe but not an acceptor from tryptophan fluorescence showed similar stability as that of the unlabeled K78C. The overall dimension of the protein in the denatured state was estimated by resonance energy transfer measurements. Tryptophan fluorescence decays of K78C were measured at various temperatures in the absence and presence of acceptor and were analyzed by multiexponential with eq. (1) to obtain average lifetimes, and by distance distribution function with eqs. (2)–(4) to obtain distance distribution information. From the average lifetimes we then calculated the average transfer efficiencies and therefore average distances from eqs. (5)–(6). The results are shown in Table 1. At or below room temperature, the average distance is about 23 Å, which agrees with that from X-ray data of the wild type nuclease. As the protein is denatured, the average distance remains about the same. Thus the thermally-denatured state of K78C is compact. Next we study the heterogeneity of the denatured state by distance distribution analysis.

The tryptophan decay of K78C in the absence of acceptor was analyzed by two exponential decays from 4°C to 40°C, while at 50°C and 60°C, three exponentials were required to fit the decay data. The decay curve of tryptophan in the labeled protein was analyzed by one Gaussian apparent distance distribution from 4°C to 20°C. A typical fit is shown in Fig. 3. One population is adequate for the representation of the data. At and above 30°C, a two population model was required to fit the decay data. At 30°C, a two-population fit was slightly better than one population fit judged from reduced-χ^2 and autocorrelation. One population has an average distance of about 20 Å and the other has an average distance between the donor and acceptor of more than 40 Å. Since the second population is at a distance at which energy transfer is negligible, we can only

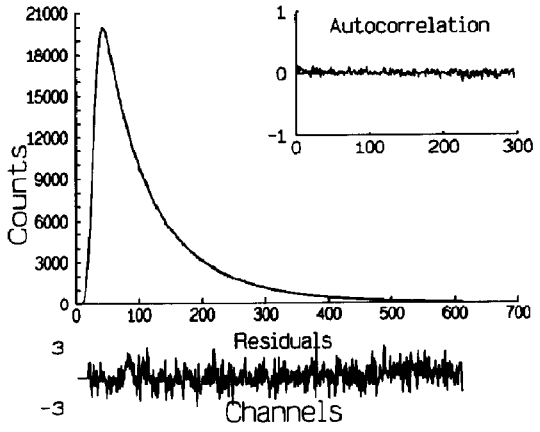

Fig. 3. Measured and fitted fluorescence decays of tryptophan in the IAEDANS-labeled K78C mutant. The decay data were collected at $T = 20$°C in 0.1 M Tris, 0.05 M NaCl, pH 7.8. The fit was performed using eq. (2) with one Gaussian distance distribution between Trp 140 and IAEDANS at 78. No Ca^{2+} and pdTp were present. Protein concentration was about 25 μg/ml. The fit to the decay of ternary complex was similar (not shown).

Table 1

Averaged parameters [a] at different temperatures

T (°C)	No pdTp(Ca^{2+})					pdTp(Ca^{2+})				
	$\langle\tau_D\rangle$ (ns)	$\langle\tau_{DA}\rangle$ (ns)	E%	R_0 (Å)	$\bar{r}$ (Å)	$\langle\tau_D\rangle$ (ns)	$\langle\tau_{DA}\rangle$ (ns)	E%	R_0 (Å)	$\bar{r}$ (Å)
4	5.67	3.05	46	22.8	23.4	5.60	2.91	48	22.8	23.1
20	5.19	2.89	44	22.8	23.7	5.02	2.81	44	22.8	23.7
30	4.72	2.85	40	21.4	22.9	4.65	2.73	41	21.4	22.7
40	3.96	2.50	37	20.0	21.9	4.17	2.56	36	21.4	23.6
51	2.46	1.66	33	18.6	20.9	3.77	2.38	37	20.0	21.9
57	–	–	–	–	–	3.45	2.25	35	19.6	21.7
65	–	–	–	–	–	2.49	2.04	18	18.6	23.9

[a] All average lifetimes were calcaluated from $\langle\tau\rangle = \Sigma_i \alpha_i \tau_i$.

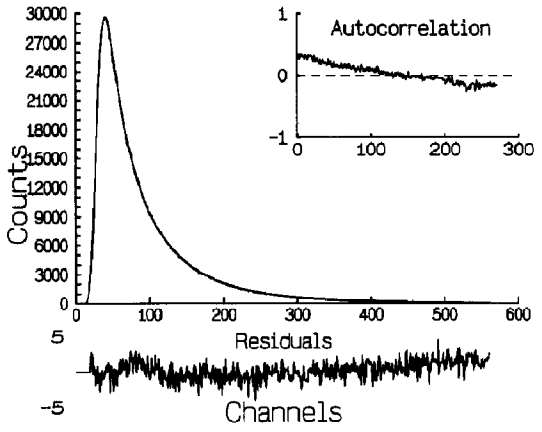

Fig. 4. Measured and fitted fluorescence decay of tryptophan in the labeled K78C at 51°C where the protein is denatured. One Gaussian fit to the decay using eq. (2). Reduced-$\chi^2 = 1.8$. Average distance 19 Å and half width 19 Å. The fit was poor as the weighted residuals and the autocorrelation of the residuals show systematic deviations. Two-population fit (not shown) using eq. (3) led to satisfactory results similar to that in Fig.3. Reduced-$\chi^2 = 1.3$. One population with an average distance of about 18.8 Å and half width of about 4.4 Å. Its relative concentration was about 70%. The second population with a large distance (more than 40 Å) had a relative concentration about 30%.

obtain an approximate estimate of its relative concentration based on eq. (3). We also tested the data at 20°C with eq. (3). The percentage of the second population was less than 5%, with no improvement in the goodness of the fit. Because of the large half width of the apparent distance distribution in the native state, this fraction may be due to some degree of compensation in the analysis step rather than due to the protein itself. In other words, the protein can be considered to consist of one population at 20°C or lower within the uncertainties of the experiments.

Figure 4 shows that at 51°C the one-population fit is inadequate and a two-population fit is required. The fraction of the population with a short average distance is about 0.7 (70%) and that of the population at a large distance 0.3 (30%). At 60°C which is far above the denaturing temperature, the fluorescence intensity was greatly reduced and the signal level was poor. From the decay curve we still obtained two populations, one with an average distance about 18 Å and another beyond 40 Å. Multiple conformations have been observed in other systems such as glycopeptides [27].

To determine whether an irreversible photochemical reaction of the donor or acceptor at high temperature could explain the results, the sample was heated to 51°C and then cooled down to 20°C and was examined. We recovered 92% of the native population. Only 8% was in a conformation with a large distance compared to 30% at higher temperature. We checked the reversibility of the protein denaturation with and without acceptor by heating the sample to 60°C and storing it overnight at 4°C. In the unlabeled (and renatured) protein, the two lifetimes of tryptophan at 20°C were identical to those before thermal denaturation. The average distance between the donor and acceptor in the labeled protein was about 24 Å and the half width was about the same. Within the uncertainties associated with the donor–acceptor pair in the protein, both the average distance and half width can be regarded as the same before the thermal denaturation and after the renaturation. Thus the protein denaturation is essentially reversible in our measurements (10 to 50 μg/ml range in concentration).

We term the population with an average distance between 20 and 30 Å a compact form in either the native or denatured states and the population with an average distance of more than 40 Å an expanded form. Figure 5 shows the temperature dependence of the average distance and half width of the compact form. We have discussed elsewhere [8] possible sources of the large half width of distance distribution in the native protein. Tryptophan orientation, the acceptor linker arm and protein conformational heterogeneity in the native state all seem to contribute and it is difficult to separate them for this donor–acceptor pair. This does not alter our results regarding the heterogeneity in the denatured state. As temperature rises, the half width of the distance distribution decreases probably due to increasing local rotational and translational motions of the donor and acceptor. The average distance is essentially comparable with that at low temperature where the protein is in its native state. We note that the average distance plus the reduction in the half width at high tem-

perature only exceeds the dimension of the native protein by a few angstroms and therefore faster local diffusion (as shown in the decrease in half width) is not the cause of change in the size of the protein. Thus the overall thermally-denatured state is compact even though there is considerable heterogeneity.

Figure 6 shows the temperature dependence of the population of the expanded form. As temperature increases, the fraction of the expanded form rises. At 51°C for K78C, the expanded form is about 30%, a considerable amount. Since the solution temperature is at the structural transition point, we might expect that the compact form is native and the expanded form is denatured. This is not the case. Although there is a large difference between the compact and expanded forms in terms of their average distance between Trp 140 and IAEDANS at position 78, thermodynamically the difference is small. If we plot the ratio of the two forms as a function of temperature, we then obtain a rough estimate of the enthalpy and entropy between the two. Going from the compact form to the expanded one,

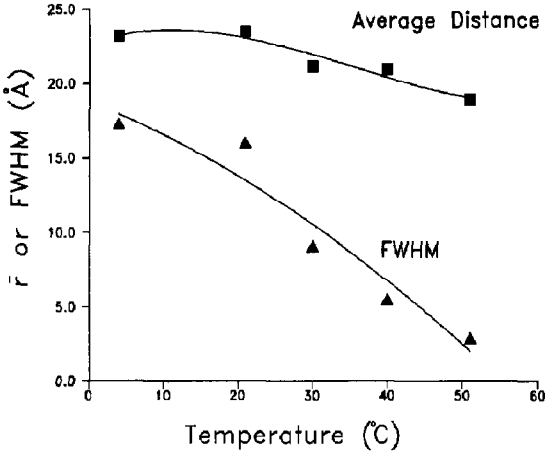

Fig. 5. Average donor–acceptor distance and half width of the compact form from native state to denatured state of the ligand-free nuclease as a function of temperature. Below and at 20°C, the data were obtained by one population fit. At and above 30°C, two population fit was performed. All values were the average of two or more independent measurements. The uncertainties of average distance were about 2 to 3 Å and those of half widths 2 to 3 Å at low temperature and 1 to 2 Å at high temperature. The concentration of the expanded form is shown in Fig. 6.

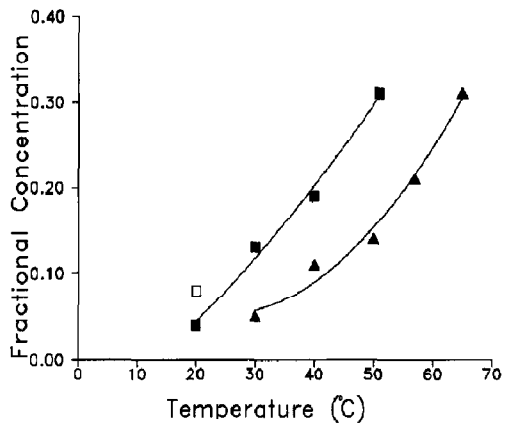

Fig. 6. Relative concentration of the expanded form as a function of temperature. Filled squares: ligand-free nuclease. One open square at 20°C was the data obtained after recooling from 51°C. Filled triangles: ternary complex. The fractions were obtained using eq. (3) with a two population fit. The concentrations of the ligands in the ternary complex were: $CaCl_2$ 20 m*M* and pdTp 2 m*M*. Uncertainties associated with the estimation were about 5% or the value of the lowest data point in the curve for the nuclease with and with ligands.

$\Delta H = 10.2$ kcal/mol, $\Delta S = 29.9$ cal/mol · K. These are much smaller than the typical enthalpy and entropy of denaturation of the nuclease [18]. Thus we cannot assign one as the native state and another as the denatured state. Above the melting point they are both denatured. As a result, there are two denatured states. The free energy difference between the two denatured states is close to zero because the positive enthalpy from the compact to expanded denatured state is compensated by a positive entropy term.

We note that the two forms of the denatured state are resolved by nanosecond fluorescent probes. This indicates that the conversion between the two occurs on a slower time scale, i.e. on sub-microsecond or longer. Whether they can be resolved by techniques of slower time scale remains to be seen.

3.4. Thermal denaturation of labeled K78C in a ternary complex

When complexed with Ca^{2+} and pdTp, staphylococcal nuclease is denatured at a higher temperature [14]. At 65°C, the protein is essentially in its denatured state. As in the case of ligand-free

nuclease, the tryptophan decays of the labeled K78C in the ternary complex were analyzed in two ways. Average lifetimes and average distances were obtained from eq. (6). The results are shown in Table 1. Again, the average distance at or below room temperatures agrees with that of the wild type nuclease from X-ray data. Similarly in the denatured state, the average distance is comparable to that in the native state in the ternary complex. Thus the thermally-denatured state of the ternary complex is compact. The heterogeneity of the denatured state is studied by distance distribution analysis.

The tryptophan decay in the ternary complex in the absence of acceptor can be fitted to two exponential decays up to 57°C but at 65°C, three exponentials are needed to fit the data. The distance distribution between Trp 140 and IAEDANS at position 78 can be fitted by one population from 4°C to 30°C, about 10°C higher than that of ligand-free nuclease. At 40°C, two populations are required to fit the decay data. Similar to the case of ligand-free nuclease, we used eq. (3) to estimate the fraction of the expanded form. The results are shown in Fig. 6. The temperature dependence of the concentration of the expanded structure in the ternary complex is similar to that of the ligand-free nuclease, with the population shifted to a higher temperature due to ligand binding. In analogy to the case of ligand-free nuclease, we define an equilibrium between the compact and expanded form. The enthalpy and entropy associated with the conversion to the expanded form are 10.9 kcal/mol and 30.5 cal/mol · K. These values are comparable with those of the ligand-free nuclease. This again shows that there are two denatured states.

We note that both the tryptophan and the acceptor IAEDANS are away from the binding site of calcium and pdTp. The shift of expanded form to higher temperature in the presence of ligands indicates that the appearance of the two distance distributions at high temperature is not due to some unknown reversible photoreaction of the probes themselves. Thus, together with the reversibility check in the case of the ligand-free nuclease, we rule out the possibility of either reversible or irreversible photoreaction of probes at high temperature that might contribute to the observed results here.

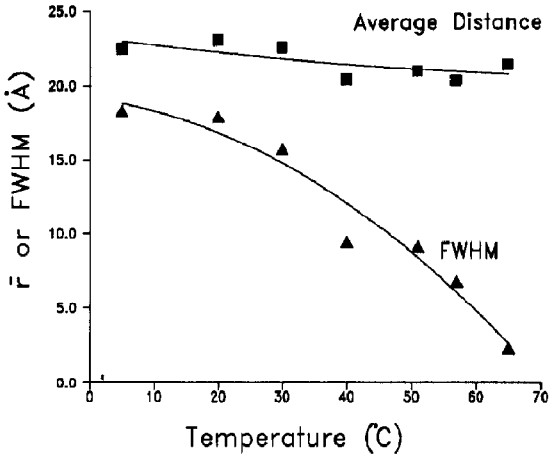

Fig. 7. Average donor–acceptor distance and half width of the compact form from native state to denatured state of the ternary complex as a function of temperature. Below and at 30°C, the data were obtained by one population fit using eq. (2). At and above 40°C, two population fit using eq. (3) was performed. All values were the average of two or more independent measurements. The uncertainties of average distance and of half widths were similar to those in Fig. 5. The concentration of the expanded form was shown in Fig. 6.

The average distance and half width of the compact form of the ternary complex shows similar temperature dependence as those of the ligand-free nuclease. As temperature increases, the average distance decreases mildly and the half width decreases. The results are shown in Fig. 7. Note again (cf. Fig. 5) that the average distance plus the reduction in the half width is only slightly larger than the dimension of the native protein. In addition, the average distances of the denatured state at the highest temperatures are comparable with and without ligands bound in spite of a temperature difference of more than 10°C. This in turn implies that large scale translational diffusion between donor and acceptor is unlikely when the protein is not fully extended. Thus the size of the thermally-denatured state is again comparable with that of the native protein.

4. Discussion

In this work we demonstrated the compact thermally-denatured state and the non-random residual structures in the denatured state of staphylococcal nuclease mutant K78C by CD measurements and distance estimation using intrinsic residues and an extrinsic label in resonance energy transfer measurements. We also measured the heterogeneity of the denatured state. The two thermally-denatured states of K78C found by energy transfer distance distribution analysis as a function of temperature demonstrates the complexities and heterogeneity of protein denaturation. In terms of size, the compact denatured state with an average donor–acceptor distance of about 21 Å is not larger than that of the native state. The expanded denatured state is less defined in this experiment. Even though its donor–acceptor distance is quite large, the expanded form may not necessarily be much larger than the compact form because the entropy difference between the two is quite small as estimated above and we only measured a distance between two residues. Since the compact denatured state is the major population, the overall physical characteristics of the denatured state of the protein are its compactness, which is far from a random coiled state. The compactness of the denatured state implies that there are some residual structures that are either transiently or permanently present as shown in CD spectra.

Evidence of a structured denatured state of staphylococcal nuclease also exists from results by others [11,13,19]. Substantial secondary structure of the mutants still persists at temperatures far above the total breakdown in structure of the wild type nuclease as detected by circular dichroism [11]. Based on CD and gel filtration studies of large nuclease fragments which are in denatured states under native conditions of the wild type, Shortle and Meeker [13] concluded that staphylococcal nuclease can exist in two denatured states, one compact and another expanded. Our results are consistent with theirs. It is possible that different mutants may have different amounts of one denatured form or another and that different solution conditions may change the properties of the denatured states.

Although the denatured states of the nuclease are basically compact by either thermal or GuHCl denaturation, they are different from the usual molten globule state [20,21] in the following ways. (1) The denatured state of K78C is quite heterogeneous in structural dimension as monitored by the distance distributions in energy transfer measurements. (2) Ligands such as calcium have quite a large effect on the denaturation of proteins that show molten globule state transition by GuHCl such as α-lactalbumin [22], the binding of Ca^{2+} and pdTp to K78C nuclease seems only to shift the thermal transition temperature without altering the multistate behavior of the protein. (3) We found no enhancement of TNS fluorescence when the dye is present in K78C solution either in the native state or the denatured state of the protein by GuHCl.

In the ligand-free nuclease K78C, the fluorescence decay of tryptophan at 40°C in the absence of acceptor can still be analyzed by two exponentials (the lifetimes are thermally quenched) as in the case at lower temperatures. This implies that the tertiary structure around tryptophan 140 is still intact (similar case applied to the ternary complex at higher temperature due to ligand stabilization). On the other hand, from energy transfer measurements, there is a substantial fraction (0.2) of the protein with a donor and acceptor distance more than doubled that of the native state. Thus it is likely that for this fraction of the protein, some parts of its tertiary structures are collapsed while the part around tryptophan is still native like.

The fluorescence spectrum of Trp 140 in the thermally-denatured K78C shows almost no red shift. This implies that tryptophan 140 immediately after the thermal denaturation is still in a hydrophobic environment as it was in the native state. This can be achieved by clustering some residues around the tryptophan. Clustering is possible since thermal effects near the denaturation temperature of this protein may have disrupted only part of the hydrophobic cores without stretching the peptide chain into a random

coil. By analogy, other residues may also form clusters. Clustering was also observed by NMR techniques [23,24]. Thermally-denatured state of other proteins such as ribonuclease A was also found to be compact [25]. These observations again suggest that the structures of thermally-denatured states of proteins are non-random.

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