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Fluorescence lifetime studies with staphylococcal nuclease and its site-directed mutant

Test of the hypothesis that proline isomerism is the basis for nonexponential decays

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ABSTRACT Using frequency domain methods, the fluorescence decay of Trp-140 in staphylococcal nuclease and its site-directed mutant (Pro-117→Gly) has been examined. Based on nuclear magnetic resonance (NMR) studies (Evans, P. A., C. M. Dobson, R. A. Kautz, G. Hatfull, and R. O. Fox. 1987. *Nature [Lond.]*. 329:266–268), it is believed that nuclease exists in two macroscopic, native conformations and that the slow interconversion of these conformations is controlled by the *cis*→*trans* isomerization of Pro-117. The above mutant shows only one native conformation in NMR experiments. To test the hypothesis that the biexponential fluorescence decay of Trp-140 of nuclease can also be related to the existence of these conformational states of the protein, we have compared the decay patterns of the wild type and mutant. Essentially no difference was observed, which indicates that there is some other basis for the nonexponential decay of Trp-140. We have used global nonlinear least squares analysis to link the fit of data at several temperatures.

INTRODUCTION

The fluorescence of tryptophanyl (trp) residues in proteins is often found to be nonexponential (1, 2). The single trp residues of a few proteins (ribonuclease T_1 (3–5), apoazurin (6, 7), whiting parvalbumin (8), and aspariginase (Eftink, M. R., unpublished observations), appear to decay as a monoexponential under certain conditions, but for other single trp-containing proteins, a double, triple, or distribution of lifetimes is needed to fit fluorescence decay data. This dispersion in the fluorescence decay is generally believed to be the result of two factors: (*a*) the existence of multiple, ground-state conformational states of the protein, and (*b*) the occurrence of various excited state reactions, such as dipolar relaxation of groups adjacent to the excited indole groups on a time scale in competition with fluorescence decay (i.e., nanoseconds). It is very difficult to determine which of these factors is the cause of the nonexponentiallity of the decay of a particular trp residue, but, in either case, if an assignment could be made it would provide useful information about the conformational dynamics and/or variability of a protein. Here we present work, with a site-directed mutant of *Staphylococcus aureus* nuclease, which is aimed at identifying these underlying factors.

Several workers (2, 9–11) have reported that the fluorescence of a single trp residue (Trp-140) of *Staph* nuclease decays as a double exponential. Recently Fox et al. (12) have performed nuclear magnetic resonance, (NMR) studies which indicate the existence of two distinct conformational states of nuclease in solution. The $H^{\epsilon 1}$ proton resonances of the four histidine residues are well resolved and assigned and each $H^{\epsilon 1}$ resonance is found to exist as a major (~90%) and minor (~10%) signal. The ratio of the minor to major peaks increases slightly with increasing temperature. The existence of the dual resonances has been interpreted as indicating the presence of two native conformational states of the proteins which interconvert on the time scale of >1 s.

Evans et al. (13) proposed that the existence of the slowly interconverting forms is due to the *cis*→*trans* isomerization of a particular proline residue, Pro-117. This proline is the only one which is observed to be in the *cis* orientation in the crystal structure of the nuclease–Ca^{+2}–thymidine-3′-5′-diphosphate complex. To confirm this interpretation, Evans et al. used site-directed mutagenesis to prepare a nuclease mutant in which Pro-117 is replaced by Gly. As anticipated, the minor His resonances were found to be absent in this mutant, indicating that only a single, dominant conformation exists. Thus the NMR studies with the wild-type and mutant forms of nuclease strongly support the hypothesis that the *cis*→*trans* isomerization of Pro-117 is responsible for the existence of multiple conformations of the wild type.

Here we have investigated the possibility that the apparent biexponential fluorescence decay of Trp-140 of nuclease can also be attributed to the existence of the conformational states due to the isomerization of Pro-117. Different workers (2, 9–11) have reported different ratios of the pre-exponential terms, α, for the fluorescence decay times of nuclease. This, no doubt, is due both to differences in experimental conditions and sample purity,

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but is also due to the inherent difficulty of analyzing nonexponential decays. Some of the reported α values are not too different from the ~1:10 ratio found for His NMR resonances so it seems possible that the *cis*→*trans* conformational states may be the basis for the biexponential decay of Trp-140. This trp residue is located at the end of an α-helix region and Pro-117 is located in a reverse turn at the top of this same α-helix region. Thus it is quite reasonable to propose that the *cis* and *trans* orientations about Pro-117 (which would slowly interconvert on both the NMR and fluorescence time scales) would result in two different fluorescence states of Trp-140. The fluorescence decays of wild-type nuclease and the Pro-117→Gly mutant (referred to as PG117) are here compared. If proline isomerism is the molecular cause of the biexponential decay of Trp-140 in nuclease, then we predict that the decay of PG117 should be closer to a single exponential.

MATERIALS AND METHODS

The wild-type nuclease was obtained by expression of the recombinant nuclease gene in *Escherichia coli*. The gene was cloned from *S. aureus* Foggi strain and was introduced by the vector pAS1 (R. O. Fox, unpublished observations). The PG117 mutant was prepared in similar manner through oligonucleotide-directed mutagenesis (Kautz, R. A., and R. O. Fox, manuscript to be published). These proteins contain the additional NH_2-terminal sequence fMet-Asp-Pro-Thr-Val-Tyr-Ser, which is cleaved from the mature protein in *S. aureus*. The proteins were purified on a phosphocellulose column, developed with a linear gradient of 0.3 M ammonium acetate, pH 6, to 1.0 M ammonium acetate, pH 8. The proteins are >95% pure and possess the expected enzymatic activity. For fluorescence measurements, the freeze-dried protein was dissolved in a buffer containing 0.025 M Tris-HCl, pH 7.0, 0.1 m NaCl. The samples were passed through a millipore filter before being studied.

Fluorescence lifetime measurements were performed with a SLM 4800 C (SLM Instruments, Inc. Urbana, IL) phase/modulation fluorometer, equipped with a multi-frequency light modulator (1–200 MHz) from ISS, Inc. (Champaign, IL), and a 300 W xenon lamp. A 10-nm-wide interference filter (Melles Griot, Irvine, CA), centered at 290 mn, was used for excitation and the emission was observed through a 7-60 bandpass filter (Corning Glassworks, Corning, NY). See references 4, 14, and 14 for further details. The thermo-jacketed cell holder was maintained at the temperatures indicated in Table 1 (temperatures measured in the cell). The compound, *p*-terphenyl, was used to give a reference fluorescence lifetime of 1.0 ns. We confirmed that this reference lifetime does not change more than ±5% over the temperature range studied.

Phase and modulation measurements on the protein samples (having an absorbance of ~0.1 at 290 nm) were made at ~15 frequencies at each temperature, and measurements were made at 11 temperatures from 1.6° to 66°C.

This temperature dependence study was performed to determine if any differences, between wild-type and mutant nuclease, are temperature dependent and to also enable us to perform a global nonlinear least squares analysis of multiple data sets (16–18). With such a global analysis, data sets are linked together via the Arrhenius relationship for each decay time (i.e., $1/\tau_i = A_i \exp(-E_{a,i}/RT)$, where $E_{a,i}$ and A_i are the activation energy and frequency factor for decay rate $1/\tau_i$). By simultaneously analyzing several linked data sets, the fitting parameter (i.e., for a biexponential decay law) are obtained with greater confidence. We have performed such global analyses using the program GLOBAL written by J. M. Beechem, University of Illinois, Laboratory for Fluorescence Dynamics.

TABLE 1 Fluorescence decay fitting parameters for PG117 nuclease mutant*

Temperature	τ_1	τ_2	α_1	χ^2
°C		*ns*		
1.6	5.62	—	1.0	9.40
	1.69	6.04	0.051	1.19
9.6	5.36	—	1.0	11.32
	1.69	5.83	0.064	0.63
20	5.03	—	1.0	9.95
	1.07	5.31	0.035	1.00
25	4.72	—	1.0	19.25
	1.42	5.26	0.078	1.49
30	4.56	—	1.0	14.8
	1.67	5.13	0.097	0.71
40	4.17	—	1.0	17.17
	1.93	4.95	0.175	1.05
45	3.76	—	1.0	35.5
	1.07	4.36	0.108	0.72
50.3	3.62	—	1.0	28.5
	1.71	4.60	0.221	1.66
55	2.78	—	1.0	98.5
	1.17	4.19	0.307	3.59
60	1.49	—	1.0	158.2
	0.89	4.19	0.654	22.87
66	1.781	—	1.0	116.1
	0.83	4×10^6	0.965	21.26

*Conditions: pH 7.0, 0.1 M NaCl, 0.025 M Tris-HCl; excitation at 290 nm, emission observed through a filter centered at 350 nm. A triple exponential fit resulted in an improvement in the χ^2 for data at 55–66°. In calculating χ^2, errors of 0.5 and 0.005 are used for the phase angle and relative modulation, respectively.

RESULTS AND DICSUSSION

In Fig. 1 is shown the frequency domain response data for the PG117 mutant at temperatures of 1.6°, 20°, 40°, 50°, 55° and 60°C. (Data collected were at pH 7.0, in order to compare with other fluorescence work with this protein. We have collected similar temperature dependence data at pH 5, where the NMR work was done. Results at low pH agree with those at pH 7.0) Monoexponential and biexponential fits for these data are shown in Table 1. Similar data and fits were obtained for the wild-type

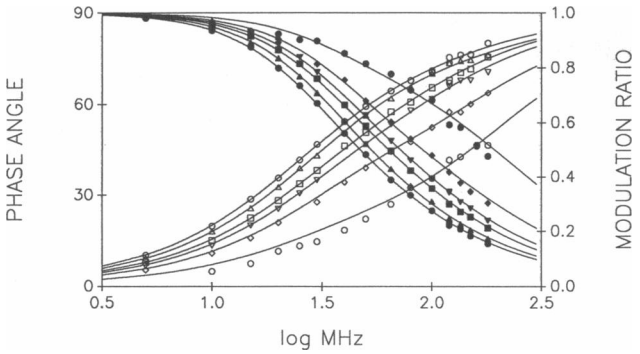

FIGURE 1. Multi-frequency phase (*open symbols*) and modulation data (*solid symbols*) for the fluorescence of the PG117 mutant of *Staph* nuclease at 1.6°, 20°, 40°, 50°, 55°, and 66°C (for the pairs of curves going from left to right). The lines through the data are best double exponential fits (see Table 1). Excitation was at 290 nm (10-nm bandpass interference filter); emisson was observed through a Corning 7-60 filter centered at 350 nm.

enzyme. For both proteins (at low temperatures), the biexponential decay is clearly superior (lower χ^2). At temperatures above 50°C, the biexponential fits become poor and decay times drop significantly; this is due to the thermal denaturation of the proteins.

It is difficult to compare the fitted τ_i and α_i for the two protein forms at any temperature. As an alternative fitting procedure, we have fitted the data to a continuous (Lorentzian) distribution of decay times. Such distribution fits yield χ^2 values that are comparable to those for a biexponential fit and generally it will be difficult to distinguish between these two models (19, 20). Here we present the distribution fits, in Fig. 2, as a more convenient way to directly compare fits to data for the two forms of the protein. As can be seen, the central lifetime, $\bar{\tau}$, is found to be virtually the same and to show the same temperature dependence for the wild type and mutant, at least up to 45°C. The width of the distribution, Δ, also shows a similar pattern for the two proteins, up to 50°C. Above this temperature the patterns deviate. Thermal denaturation of the wild-type nuclease occurs at ~54°C (21); the PG117 mutant denatures at a few degrees higher temperature (Fox, R. O., and R. A. Kautz, manuscript to be published). The drop in $\bar{\tau}$ and increase in Δ apparently are in response to the thermal unfolding of the protein. The distribution width appears to be largest at the midpoint of the unfolding transition and then appears to become smaller. This is expected if fluorescence contributions occur from a mixture of folded and unfolded forms at the midpoint temperature. (We note that, at or above the unfolding temperature, the frequency response data become very anomalous and we are not able to fit such data well with any model).

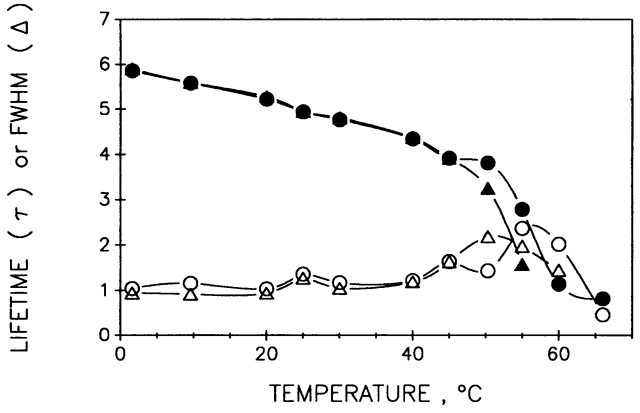

FIGURE 2. Temperature dependence of the central lifetime, $\bar{\tau}$, (*solid symbols*), and full-width-half-max, Δ (*open symbols*), for a Lorentzian distribution fit to the multi-frequency phase and modulation data for the PG117 mutant (●, ○) and wild-type ▲, △) nuclease.

Global analysis of the data in the range of 1.6°–40°C was performed for the two proteins to a biexponential decay model. We have done this both by (*a*) linking the α_i to be the same at each temperature, or by (*b*) allowing α_i to float at each temperature. Results of these global analyses are shown in Table 2. Nearly identical $E_{a,i}$, A_i, and α_i are found for each protein. Allowing the α_i to float does not result in much improvement in χ^2 and the parameters recovered when the α_i are linked are preferred.

The $E_{a,i}$ and A_i values correspond to $\tau_1 = 2.62$ ns and $\tau_2 = 5.80$ ns (and $\alpha_1 = 0.176$) at 20°C, for the wild-type protein. For the PG117 mutant, the values are $\tau_1 = 2.26$ ns and $\tau_2 = 5.69$ ns (and $\alpha_1 = 0.141$) at 20°C. Thus we find remarkably similar biexponential decay parameters for the wild-type and mutant proteins. We note that these τ_i and α_i at 20°C are in excellent agreement with recently reported values for nuclease by Brand and co-workers (10) and additional data to be published from his laboratory. We have also recently repeated our 20°C measurements of the wild type and PG117 using the 2-GHz phase fluorometer (23) in the laboratory of J. R. Lakowicz, University of Maryland Medical School. We obtained biexponential fits of $\tau_1 = 2.79$ ns, $\tau_2 = 6.09$ ns, $\alpha_1 = 0.312$ for the wild type and $\tau_1 = 2.51$ ns, $\tau_2 = 6.04$ ns, and $\alpha_1 = 0.313$ for the PG117 mutant (excitation at 295 nm, emission through a WG320 cutoff filter). We believe that these results agree quite well with Tables 1 and 2.

These fluorescence decay parameters do not support the hypothesis that the biexponential decay of nuclease is related to the *cis*→*trans* isomers of Pro-117. The biexponential pattern persists in the PG117 mutant. The non-exponential decay of Trp-140 must be explained in

TABLE 2 **Global fitting parameters for the wild-type and PG117 mutant of nuclease***

Sample	$E_{a,1}$	A_1	$E_{a,2}$	A_2	α_1	χ^2
	kcal/mol	ns^{-1}	kcal/mol	ns^{-1}		
Wild-type nuclease						
α_i-linked	2.14	15.22	1.00	0.978	0.176	2.09
α_i-varied	2.16	14.83	1.01	0.971	0.210, 0.195 0.170, 0.215 0.201, 0.231	1.58
PG mutant						
α_i-linked	2.08	16.02	1.00	0.986	0.141	2.25
α_i-varied	2.14	15.13	1.01	0.975	0.209, 0.193 0.172, 0.203 0.198, 0.224	2.00

*Data for six temperatures from 1.6 to 40°C linked via the Arrhenius relationship, $1/\tau_i = A_i \exp(-E_{a,i}/RT)$. When the α_i were allowed to vary, the values for α_1 given are for temperatures 1.6°, 9.6°, 20°, 25°, 30°, and 40°C.

other ways. If there are two (*cis* and *trans* based) conformations of the wild-type protein, the microenvironment of Trp-140 is apparently similar for these conformations. The indole side chain of Trp-140 lies at the surface of the protein and, from x-ray diffraction studies, appears to be relatively mobile (22). Its nonexponential decay may be due to dipolar interactions with water and polar functional groups in its microenvironment.

While the disproof of our hypothesis is a negative result, we think that this finding has significance. If we had demonstrated a correlation between the existence of *cis*⟶*trans* proline isomers and the nonexponential decay of the trp residue of nuclease, then proline isomerism would have been indicated as a possible general, and perhaps major, cause of the nonexponential decay of trp residues in proteins.

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