Fluorescence Lifetime Distribution of Folded and Unfolded Proteins

E. GRATTON, N. SILVA, G. MEI, N. ROSATO, I. SAVINI, AND A. FINAZZI-AGRO

Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois at Urbana-Champaign, 1110 W. Green St., Urbana, Illinois 61801 (E. G., A. S.); Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università' di Roma "Tor Vergata," Via O. Raimondo 8, 00173 Rome, Italy (G. M., N. R., I. S., A. F.-A.)

Abstract

Time-resolved fluorescence of single tryptophan proteins have demonstrated the complexity of protein dynamic and protein structure. In particular, for some single tryptophan proteins, their fluorescence decay is best described by a distribution of fluorescence lifetimes rather than one or two lifetimes. Such results have provided further confirmation that the protein system is one which fluctuates between a hierarchy of many conformational substates. With this scenario as a theoretical framework, the correlations between protein dynamic and structure are investigated by studying the time-resolved fluorescence and anisotropy decay of holo and apo human superoxide dismutase (HSOD) at different denaturant concentrations. As a function of guanidine hydrochloride (GdHCl), the width of the fluorescence lifetime distribution of HSOD displays a maximum which is not coincident with the fully denatured form of HSOD at 6.5M GdHCl. Furthermore, the width of the fluorescence lifetime distribution for the fully denatured forms of holo and apo HSOD is greater than that of the native forms.

Introduction

One of the goals of internal protein dynamics studies is understanding the role of protein motions in determining protein function. From the physical point of view, proteins are mesoscopic systems that display unusual properties and provide an unique experimental system. In the protein dynamics literature, these two parallel approaches, i.e., functional versus physical, have received complementary and equal attention. For example, theoretical studies of internal protein motions center on the physical and chemical basis for the proper description and understanding of internal protein motions, whereas most of the experimental studies focus on the relationship of some specific detectable motion with some functional properties of the macromolecule. One of the most salient properties of protein dynamics is the enormous time scale over which dynamics can be detected. In one of the earlier reviews of protein dynamics [1], it was pointed out that protein time events can occur from picoseconds to several thousand seconds. There was also an attempt to identify the events occurring in a given time scale with some functional property of the protein. During the past decade, a large amount of experimental data has accumulated to provide a better insight.
into this peculiar characteristic of protein systems. Two important aspects were partially clarified. One relates to the identification of the hierarchical nature of protein dynamics that parallels the well-known hierarchy of structures in the protein [2, 3]. The second point relates to the observation that a single well-identifiable process (e.g., CO rebinding after photolysis in heme proteins [4]) can also extend over an enormous time scale, from nanoseconds to days. These experiments also showed that slight modifications of the native protein structure can produce large changes in the protein dynamics. One of the aims of current research in protein dynamics is the elucidation of the mechanism by which, using subtle conformational changes, a protein can regulate its biological function. Another important related area of research is the physical understanding of protein hierarchical dynamics: How the different structural and dynamical hierarchies are interrelated and how they can influence and regulate each other.

Because of the different aspects in which protein dynamics can be manifest, there is no unique or proper method of study. However, all the alternative and complementary methods have some limits. For example, theoretical methods based on molecular dynamics provide a description at the atomic level of protein motions, which is, of course, one of the ultimate goals of protein dynamics. However, present theoretical methods are restricted to a time range of less than a nanosecond. In this respect, they are able to explore only a small portion of the dynamics. X-ray scattering methods also provide structural information at the molecular level. Dynamic information using X-ray diffraction is obtained by the temperature factor, which provides an average value only and which lacks time information per se. Dynamic X-ray scattering has an extremely high potential, but the method is in its infancy. There are a number of other methods capable of providing information at the atomic level on specific protein motions, and, of course, fluorescence is one of them. From the beginning of protein dynamics, fluorescence had a very special role. During the 1950s, G. Weber demonstrated that tryptophan residues in proteins can have a relatively high local mobility [5, 6]. In the 1970s, Lakowicz and Weber showed that molecular oxygen can penetrate and diffuse in globular proteins with a diffusion rate that is only a factor of 5 to 10 less than the diffusion of molecular oxygen in water [7, 8]. Although fluorescence is not capable of providing dynamic information on all protein atoms, nevertheless, it has the sensitivity and characteristic time scale adequate to study some of the internal protein motions. It is important to discuss the kind of dynamic information that fluorescence can provide on protein dynamics and how to properly analyze fluorescence decay data to obtain the rate and amplitude of protein motions.

1. Using intrinsic protein fluorescence (typically tryptophan), we can test one particular protein residue, its environment, and some hydrodynamic properties of the whole molecule.

2. The fluorescence lifetime of tryptophan in proteins is never longer than 10 ns. Dynamic events longer than 10 ns must be studied using specialized methods, such as fluorescence steady-state or lifetime kinetics, using the phosphorescence properties of tryptophan or using some extrinsic long-lived probes.
3. The value of the lifetime per se, which is in the nanosecond range, is not direct evidence of protein dynamics in that time scale since processes internal to the tryptophan moiety are responsible for the fluorescence decay. However, the variation of the lifetime value due to some external agent can be evidence of competing rate processes in the nanosecond or shorter time scale.

4. The decay of the emission anisotropy of the tryptophan residue is generally direct evidence for protein motions. Nonmotional fluorescence depolarization can occur in conjunction with energy transfer phenomena or other excited-state processes, but these processes play a marginal role in polarization experiments in proteins. The decay of the emission anisotropy in proteins is due to at least two different kind of motions: the motion of the whole protein and the motion of the tryptophan with respect to the protein. By changing temperature or viscosity, one motion can be amplified at the expense of the other to facilitate its study.

One of the premises of our approach to protein dynamics is that proteins can exist in a large number of conformational substates, one slightly different from the other and rapidly interconverting at room temperature. The question is, How this kind of structure and dynamics will manifest itself in the fluorescence measurement?

1. Starting with spectral properties (steady-state excitation and emission spectra), it appears that the excitation spectrum is only partially sensitive to the structure of the protein, in contrast to the emission spectrum that is largely affected by the protein's structure. For example, the emission maximum of tryptophan in proteins ranges from 308 to 360 nm, for the extreme cases. These large spectral shifts have been extensively used to monitor conformational changes. Steady-state fluorescence kinetics is also often used to follow the time course of conformational changes induced by several agents. However, emission spectral properties are not necessarily correlated with the dynamics of the protein. Temperature studies of the behavior of the spectral moments have been performed with the purpose of obtaining dynamical information, but with little success.

2. The fluorescence lifetime is also extremely sensitive to the nature of the protein surroundings. Note that this sensitivity is not correlated with the spectral sensitivity. For example, a protein with very blue emission can have very long lifetimes (apoazurin emits with a maximum at 312 and has a lifetime of about 5 ns). The tryptophan fluorescence lifetime in proteins ranges from 10–20 ps to about 10 ns. This thousandfold variation is generally due to specific quenching mechanisms that are critically dependent on the particular location and orientation of neighboring protein residues. Therefore, it appears that the lifetime of the tryptophan excited-state will reflect subtle changes in protein local configuration. If the decay is properly analyzed, it will provide information on the multitude of local tryptophan environments. If interconversion between different local environments in an individual protein molecule occurs during the lifetime of the excited-state, these dynamics will be reflected in the fluorescence decay properties. There is a large number of fluorescence lifetime experiments that support this observation, showing that except for some unusual cases the fluorescence decay is not single exponential [9]. The common point of view used to ex-
plain this singular phenomenon is that proteins have two or three well-defined conformations, each corresponding to the component observed in the fluorescence decay. However, those protein components have seldom been seen other than in the fluorescence experiments.

3. Recently, we proposed [10–12] an alternative explanation for these multiple components observed in the fluorescence decay based on a different approach to data analysis, which is compatible with our view of protein dynamics. We observed that data corresponding to closely spaced double or triple exponential decays can be equally well represented by a continuous distribution of lifetime components. Although this observation can at first appear trivial, it corresponds to a specific view of protein structure and dynamics. As a natural consequence of this approach and on the basis of a physical model of protein structure [2], we made the prediction that the width of the lifetime distribution should increase as the protein temperature decreases. This prediction has been verified in a number of protein systems. Using the conventional approach in which at most two or three protein conformations exist in equilibrium, it is difficult to explain why a decrease in temperature should always give a larger separation between lifetime values that will mimic the broadening of the lifetime distribution. Furthermore, we have demonstrated that for some single tryptophan proteins the number of discrete components necessary to describe the fluorescence decay must increase at low temperature. Clearly, these results contradict the assumption that there are only two or three protein conformations at most. The distribution approach to data analysis provides a useful framework for the interpretation of the fluorescence decay in terms of protein conformational substates and interconversion dynamics. Recently, we have developed specific models to interpret the changes of the lifetime distribution as a function of temperature and other relevant thermodynamic variables [13].

The distributional analysis of the fluorescence decay is particularly suited to study systems in which a multitude of states, both microscopic and macroscopic, coexist. One biophysical problem, which has been in the literature for some time, is the characterization of the so-called denatured state. This state is by definition not well defined and supposedly corresponds to a collection of different protein configurations. From the fluorescence point of view, the denatured state always gives a relatively red-shifted spectrum with an emission maximum in the 350 nm region [14]. Given the uniformity of the emission spectral properties among all proteins, it is interesting to determine whether this apparent homogeneity corresponds to a tryptophan residue essentially exposed to the solvent and with motional and lifetime characteristics similar to NATA in solution or if it is due to an average of many protein conformations such that all protein systems look alike. NATA fluorescence decay is single exponential, and the molecule rotates very fast (rotational correlation times of about 60 ps in water). The rotational rate of tryptophan in the denatured protein is also relatively fast as documented by the large amount of fluorescence polarization data on denatured proteins that show that the denaturation processes is always accompanied by a decrease of polarization.

We have previously studied the temperature denaturation of apomyoglobin [15]. The general pattern is that as the temperature increases the width of the
lifetime distribution decreases while in the native state, in accordance with our previous studies that show that a temperature increase causes a narrowing of the distribution. However, when the denaturation region is reached, there is a substantial increase in the distribution width that reaches a maximum at the denaturation midpoint. These results indicate that for apomyoglobin there is an increase of the number of different protein conformations in the unfolded state. We have studied the unfolding induced by GdHCl and urea of HSOD, a dimeric protein composed of two identical subunits, each containing one tryptophyl residue exposed to the solvent [16]. It is known that bovine SOD in the presence of 8.0 M urea [17] and of SDS [18] does not dissociate into subunits. The great conformational stability of the enzyme seems to be a common feature of all SODs, which have large structural homology [19].

Materials and Methods

HSOD was purified from human erythrocytes [20]. Metal-depleted HSOD was prepared by dialyzing the protein against 1 mM EDTA in 0.05 M sodium acetate buffer, pH 3.5. The samples used for fluorescence experiments were dissolved in 0.1 M potassium phosphate buffer, pH 7.6. For denaturation experiments, each sample was prepared by diluting 0.3 mL of protein stock solution to 2 mL with GdHCl or urea-containing buffer. In all fluorescence experiments, the absorbance at 295 nm was in the range 0.04–0.10. Fluorescence measurements were carried out at 20°C after 20 or 36 h incubation in the presence of denaturant at 4°C. Protein unfolding was followed by changes of the steady-state intrinsic fluorescence and fluorescence polarization at various concentrations of GdHCl and urea using a single photon-counting [ISS, Inc., Champaign, IL, Model GREG PC] or a Jobin-Yvon Model 3D spectrofluorometer. All data have been corrected for changes of the refractive index of the solution as the amount of denaturant was increased [21]. Frequency domain methods were employed for the measurement of intrinsic fluorescence decay and the fluorescence depolarization [22]. The frequency range used was from 10 to 240 MHz. The light source was a high-repetition mode-locked Nd-YAG laser, and the harmonic content of the laser was used as previously described [23]. Excitation was at 295 nm (through a polarizer oriented at the “magic angle”) while emission was observed through a WG335 cutoff filter to remove scattered light. Data analysis was performed by minimizing the reduced chi-square with a routine based on Marquardt algorithm using the Globals Unlimited software [24].

Results

a. Steady-state Measurements

Fluorescence emission spectra of native and metal-depleted HSOD are structureless, centered at 344 nm, and similar to the spectrum of N-acetyltryptophanamide (NATA) [25]. The relative fluorescence intensity of holo and apo protein at 344 nm is reported in Figure 1(a) as a function of GdHCl or urea concentration. The unfolded apo protein showed a difference in intensity in 3 M GdHCl with respect to 7 M urea, possibly due to a quenching effect of gua-
Figure 1. Dependence of relative fluorescence intensity (a) and steady-state anisotropy (b) on denaturant concentration for holo-HSOD (filled symbols) and apo-HSOD (open symbols) in the case of GdHCl (circles) and urea (squares).

A similar quenching effect by urea is also found for NATA. Figure 1(b) shows the effect of the two denaturants on the steady-state fluorescence anisotropy of holo and apo HSOD. These results show that holo and apo protein forms are unfolded by GdHCl at 6.0 M and 3.0 M, respectively, indicating that the metal ions have a stabilizing effect on the protein. Urea is much less effective, as only the metal-depleted protein is denaturated at 7–8 M urea.

b. Fluorescence Intensity Decay

Fluorescence decay data were fitted using different models, namely, one and two exponential functions or a continuous Lorentzian-shaped distribution of lifetimes [10, 11]. The unimodal distributed Lorentzian lifetime model gives the better fit. As a comparison, one discrete lifetime component is needed to fit the decay of NATA. Also, the protein samples denaturated with GdHCl and urea
can be best fitted by a distribution of lifetimes. Figure 2(a) shows the variation of the distribution center and width as a function of the denaturant concentration. In the presence of GdHCl, the width increases continuously up to a maximum that occurs more or less at the denaturant midpoint as detected by steady-state anisotropy and fluorescence intensity experiments. Then it decreases, leveling off at higher concentrations of GdHCl.

c. Anisotropy Decay

The anisotropy decay of HSOD has been measured at different denaturant concentrations. Two rotational correlation times were always required to fit the data both for native and GdHCl or urea-treated samples [Fig. 3(a) and Table I]. The native sample shows a long rotational component of 15.9 ns and a short component of 0.09 ns. The slow rotation is due to the tumbling of the whole
Figure 3. (a) Rotational correlation times of holo-HSOD measured as a function of GdHCl concentration. (b) Steady-state fluorescence anisotropy of holo-HSOD (filled circles) and calculated steady-state fluorescence anisotropy values (open circles), [using the values reported in (a)], as a function of GdHCl concentration.

| Sample                  | One rotation | Two rotations |
|-------------------------|--------------|---------------|
|                         | $\chi^2$     | $\Phi$ | $r_0$ | $\chi^2$ | $\Phi_1$ | $\Phi_2$ | $r_1$ | $r_2$ |
| holo HSOD               | 3.9          | 16.61 | 0.23 | 2.0       | 15.90 | 0.09 | 0.22 | 0.06 |
| apo HSOD                | 2.8          | 11.90 | 0.22 | 2.2       | 13.77 | 0.16 | 0.21 | 0.07 |
| holo + 6.5 M GdHCl      | 7.9          | 1.22  | 0.20 | 5.3       | 1.26  | 0.15 | 0.15 | 0.13 |
| holo + 8.6 M urea       | 5.3          | 10.09 | 0.21 | 3.7       | 11.44 | 0.09 | 0.20 | 0.08 |
| apo + 3.0 M GdHCl       | 10.2         | 1.57  | 0.15 | 3.5       | 4.10  | 0.22 | 0.07 | 0.21 |
| apo + 6.0 M urea        | 24.3         | 1.92  | 0.15 | 4.4       | 4.05  | 0.22 | 0.08 | 0.20 |

$\Phi$, $\Phi_1$, $\Phi_2$: rotational correlation times in nanoseconds ($\delta\Phi \sim \pm 0.03$ ns). $r_0$, $r_1$, $r_2$: zero-time anisotropies ($\delta r \sim \pm 0.01$). $\chi^2$ = reduced chi-square.
molecule. The short component is in the range expected for local, fast rotation of the indolyl side chain [26]. If we use the time-resolved data to calculate the steady-state fluorescence anisotropy of holo-HSOD as a function of GdHCl concentration, we obtain a good agreement with the observed steady-state anisotropy data [Fig. 3(b)].

Discussion

The model of a protein as a complex physical system fluctuating among a large number of similar conformational substates has been already proposed [4]. In particular, both local and global small movements have been discussed in terms of interconverting processes among substates [4]. The time scale of interconversion depends on the protein and is affected by the same factors that influence the stability of the macromolecular structure (i.e., temperature, pH, denaturants). We propose to use the width of the Lorentzian lifetime distribution to qualitatively describe protein conformational substates and fluctuations of the protein. With this hypothesis in mind, we come to the following conclusions:

First, consider the ratio between the widths of apo and holo HSOD in the absence of denaturants. This ratio is about 2.5, suggesting that the heterogeneity of the fluorophore microenvironment is greater in the metal-depleted molecule, as expected on the basis of structural considerations [27-29].

Second, Figure 2(b) shows that the unfolded states are always characterized by broader distributions of lifetimes. This finding suggests that the fluorophore experiences a larger number of environments in the unfolded states because of the loss of a defined 3-dimensional structure.

| GdHCl (M) | One Lorentzian fit* | Two fixed Lorentzians fit* |
|-----------|---------------------|---------------------------|
|           | $\chi^2$          | $C$  | $W$  | $\chi^2$ | $F_1$ |
| 0.0       | 1.3                | 2.20 | 0.25 | 1.3       | 1.00  |
| 1.0       | 1.6                | 1.99 | 0.26 | 1.8       | 1.00  |
| 2.0       | 2.6                | 2.07 | 0.44 | 5.5       | 0.92  |
| 3.0       | 1.4                | 2.21 | 0.59 | 10.8      | 0.89  |
| 4.0       | 2.5                | 2.49 | 0.82 | 17.9      | 0.71  |
| 4.5       | 1.9                | 2.84 | 0.90 | 8.8       | 0.40  |
| 5.0       | 1.6                | 3.13 | 0.81 | 2.6       | 0.17  |
| 6.0       | 2.4                | 3.28 | 0.67 | 2.4       | 0.04  |
| 6.5       | 1.6                | 3.35 | 0.68 | 1.6       | 0.00  |

*Phase and modulation data are fitted with a single Lorentzian-shaped continuous distribution of lifetimes. $C =$ center of Lorentzian in nanoseconds, $\delta C = 0.02$ ns; $W =$ width of Lorentzian in nanoseconds, $\delta W = 0.03$ ns.

*Phase and modulation data are fitted with the sum of the Lorentzian relative to the native protein ($L_1$: $C_1 = 2.2$ ns, $W_1 = 0.25$ ns) plus that relative to the unfolded one ($L_2$: $C_2 = 3.35$, $W_2 = 0.68$ ns). $F_1 =$ fraction of fluorescence relative to $L_1$; $\chi^2 =$ reduced chi-square.
Besides the initial and final state, the shape of the transition curves for the width of the distribution as a function of denaturant concentration must be discussed. The simplest model that can be considered is a two-state unfolding mechanism. In this case, one assumes that only the folded and the unfolded conformations of the protein are present in the solution. Following this model, the decay data at various denaturant concentration should be fitted with the sum of two distributions, namely, that relative to the native protein and that relative to the completely unfolded protein, with a relative fraction that is a function of the denaturant concentration. The results of this fit using the two-state model for holo HSOD at different concentrations of GdHCl are shown in Table II together with the single-distribution fit. A single distribution is by far better to describe the decay as compared to two distributions corresponding to the native and to the unfolded state. The complex behavior of the distribution width as a function of denaturant concentration may be explained (1) by assuming that the heterogeneity of the tryptophan environment is greatest at the denaturation midpoint. At high denaturant concentration, the indolyl side chain senses a smaller number of different environments, though still larger than in the native state. This observation is compatible with a multipath denaturation process including a "molten globule" [30, 31]. (2) A dynamic explanation is also tenable. Assume that the rate of interconversion among a discrete number of different states is essentially constant but that the fluorescence lifetime increases as the denaturation proceeds. During the (larger) lifetime span in the denatured state, the protein will explore a larger number of conformations resulting in a reduction of the distribution width. The rate of internal rotation is essentially constant during the whole denaturation process [see Fig. 3(a)], suggesting that there is not a large change in the local mobility. However, more quantitative experiments are needed to check these hypotheses and are, in fact, in progress in our laboratory. In conclusion, we believe that our analysis in terms of distribution of lifetime values provides new information on the large number of protein substates for the unfolded form.

Acknowledgments

The experiments and data analyses described in this manuscript were performed at the Laboratory for Fluorescence Dynamics (LFD) at the University of Illinois at Urbana-Champaign (UIUC). The LFD, E. G. and N. S. are supported by the Division of Research Resources of the National Institutes of Health (PHS-P41-5-RR03155) and by UIUC. N. R., G. M., I. S. and A. F.-A. are supported by the Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Universita' di Roma "Tor Vergata."

Bibliography

[1] G. Careri, P. Fasella, and E. Gratton. Crit. Rev. Biochem. 3, 141 (1975).
[2] H. Frauenfelder and E. Gratton, in Methods in Enzymology, Biomembranes, Protons and Water: Structure and Translocation, Vol. 127, Academic Press; Harcourt Brace Jovanovich, New York, pp. 207–216 (1985).
[3] A. Ansari, S. Berendzen, S. F. Bowne, M. Frauenfelder, I. E. Iben, T. B. Sanke, E. Shymsunder, and R. Young, Proc. Natl. Acad. Sci. U.S.A. 82, 5000 (1985).
FLUORESCENCE LIFETIME OF FOLDED AND UNFOLDED PROTEINS

[4] R. H. Austin, K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. Gunsalus, Biochemistry 14, 5355 (1975).
[5] G. Weber and F. W. J. Teale, Biochem. J. 65, 476 (1957).
[6] G. Weber, Biochem. J. 75, 345 (1960).
[7] J. R. Lakowicz and G. Weber, Biochemistry 12, 4154 (1973).
[8] J. R. Lakowicz and G. Weber, Biochemistry 12, 4171 (1973).
[9] J. M. Beechem and L. Brand, Annu. Rev. Biochem. 54, 43 (1985).
[10] R. Alcala, E. Gratton, and F. Prendergast, Biophys. J. 51, 587 (1987).
[11] R. Alcala, E. Gratton, and F. Prendergast, Biophys. J. 51, 597 (1987).
[12] R. Alcala, E. Gratton, and F. Prendergast, Biophys. J. 51, 925 (1987).
[13] N. Silva and E. Gratton, Biophys. J. 57, 80a (1990).
[14] T. E. Creighton, Prog. Biophys. Mol. Biol. 33, 231 (1978).
[15] E. Bismuto, E. Gratton, and G. Irace, Biochemistry 27, 2132 (1988).
[16] N. Rosato, E. Gratton, G. Mei, and A. Finazzi-Agro’, Biophys. J. 58, 817 (1990).
[17] D. P. Malinowski and I. Fridovich, Biochemistry 18:5055 (1979).
[18] H. J. Forman and I. Fridovich, J. Biol. Chem. 248, 2645 (1973).
[19] E. D. Getzoff, J. A. Tainer, M. M. Stempien, G. I. Bell, and R. A. Hallewell, Proteins 5, 322 (1989).
[20] W. H. Bannister, D. G. Dagleish, J. V. Bannister, and E. J. Wood, Int. J. Biochem. 3, 560 (1972).
[21] J. B. Birks, Photophysics of Aromatic Molecules (Wiley-Interscience, New York, 1970), pp. 97–100.
[22] E. Gratton and M. Limkeman, Biophys. J. 44, 315 (1983).
[23] E. Gratton, D. M. Jameson, and R. Hall, Annu. Rev. Biophys. Bioeng. 13, 105 (1984).
[24] J. M. Beechem and E. Gratton, Proc. S.P.I.E. 909, 70 (1988).
[25] N. Rosato, G. Mei, E. Gratton, J. V. Bannister, W. H. Bannister, and A. Finazzi Agro’, Biophys. Chem. 36, 41 (1990).
[26] I. Munro, I. Pecht, and L. Stryer, Proc. Natl. Acad. Sci. U.S.A. 76, 56 (1979).
[27] H. E. Parge, E. D. Getzoff, C. S. Scandella, R. A. Hallewell, and J. A. Tainer, J. Biol. Chem. 261, 16215 (1986).
[28] C. G. Biliaderis, R. J. Weselake, A. Petkau, and A. D. Friesen, Biochem. J. 248, 981 (1987).
[29] J. A. Tainer, E. D. Getzoff, K. M. Beem, J. S. Richardson, and D. C. Richardson, J. Mol. Biol. 160, 181 (1982).
[30] D. A. Dolgikh, R. I. Gilmanshin, E. V. Bychkova, G. V. Semisotnov, S. Yu. Venyaminov, and O. B. Ptitsyn, FEBS Lett. 136, 87 (1981).
[31] K. Kuwajima, Proteins 6, 87 (1989).

Received September 3, 1990
Accepted for publication November 28, 1990