Journal of Fluorescence, Vol. 3, No. 2, 1993

Phenylalanine Fluorescence and Phosphorescence Used as a Probe of Conformation for Cod Parvalbumin

K. Sudhakar,[1] W. W. Wright,[1] S. A. Williams,[2] C. M. Phillips,[2] and J. M. Vanderkooi[1,3]

Received April 7, 1993; revised July 16, 1993

The fluorescence emission and triplet absorption properties of phenylalanine in cod fish parvalbumin type II, a protein that contains no Trp or Tyr, was examined in the time scale ranging from nanoseconds to microseconds at 25°C in aqueous buffer (pH 7.0). In the presence of Ca(II), the decay of fluorescence gave two lifetimes (5.9 and 53 ns) and the triplet lifetime was 425 μs. Upon removal of Ca, the fluorescence intensity decreased to values approaching that for free Phe, while the longest fluorescence decay component was 17 ns. At the same time, the decay of triplet showed complex nonexponential kinetics with decay rates faster than in the presence of Ca. Quenching and denaturation analyses suggest that the Phe's are present in a hydrophobic environment in the Ca-bound protein but that the Ca-free protein is relatively unstructured. It is concluded that Phe luminescence in proteins is sensitive to conformation and that the long lifetime of Phe excited states needs to be considered when studying its photochemistry in proteins.

KEY WORDS: Parvalbumin; phenylalanine; fluorescence; phosphorescence.

INTRODUCTION

Of all the amino acids in proteins, only the three aromatic amino acids, namely, phenylalanine, tyrosine, and tryptophan, exhibit fluorescence [1]. Whereas the fluorescence properties of Tyr and Trp are widely used to study proteins, the emission of Phe cannot be used to study proteins in most cases. Phe has a low molar absorptivity (19.6 $cm^2\ mol^{-1}$) in comparison to Tyr (~180 $cm^2\ mol^{-1}$) or Trp (~580 $cm^2\ mol^{-1}$) at their respective absorption maxima of 258, 275, and 280 nm. The quantum yield of Phe is low, and furthermore, its emission maximum at 282 nm overlaps the absorption of Tyr and Trp so that energy is effectively transferred to these moieties in proteins that contain them. These factors explain why in all proteins and peptides, except those few that contain Phe but no Trp or Tyr, the emission of Phe is not even seen [2].

For the above reasons, the fluorescence of Phe has not been scrutinized in the detail given to Tyr or Trp. In spite of this, its excited-state properties deserve some attention. In a few proteins and polypeptides where Phe is the only aromatic amino acid, its fluorescence can serve as a useful marker. In addition, Phe may be involved in the UV photochemistry of proteins, and with the increasing flux of UV irradiation due to the depletion of the ozone layer, its photoreactions may have increasing biological significance.

Proteins containing Phe, but devoid of both Tyr and Trp residues, occur rather seldom. One such protein is parvalbumin isozyme II from cod fish, which contains 10 Phe's but no Tyr's or Trp's [3]. It is a member of

[1] Johnson Research Foundation, Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104.
[2] Regional Laser and Biotechnology Laboratory, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104.
[3] To whom correspondence should be addressed.

1053-0509/93/0300-0057$07.00/0

the family of parvalbumins, and while the role of parvalbumins in vertebrate muscle is not fully understood, they seem to be indirectly involved in the relaxation process of fast-twitch glycolytic fibers through their ability to bind and release Ca(II) [4–7]. In this work, we studied the photophysical properties of Phe in parvalbumin II in Ca-bound and Ca-free forms of the protein.

MATERIALS AND METHODS

Materials

All chemicals were of chemically pure grade. Tris, EDTA, and guanidine hydrochloride (GHCl) were obtained from Sigma Chemical Co. (St. Louis, MO).

Parvalbumin was prepared from frozen cod fillets obtained from the local supermarket. The procedure used slight modifications of published methods [3,8]. Frozen cod fillets (125 g) were homogenized in a blender with 200 ml of 10 m*M* Tris buffer (pH 8.7), 2 m*M* EDTA, and 2% glycerol, stirred for 1 h at 4°C, and then centrifuged (16,300 *g* for 30 min). To the supernatant, acetone was added dropwise to give a 45% acetone (v/v) solution. It was then centrifuged for 75 min at 16,300 *g*. The supernatant was taken to 80% acetone (v/v), the mixture was centrifuged, and the pellet was resuspended in 10–15 ml of 10 m*M* Tris buffer at pH 7.6 and 1 m*M* $CaCl_2$, heated rapidly (~5 min) to 60°C, and then immediately cooled and centrifuged. Subsequent steps were at 4°C. The supernatant was dialyzed against 1.6 m*M* piperazine at pH 5.7 overnight and then chromatographed (0.5 ml/min) on a Whatman DEAE-52 cellulose column (2.5 × 43 cm) equilibrated with the same buffer. The column was washed for ~24 h with the piperazine buffer until the absorbance at 280 nm fell to ~0.03 and then eluted with a NaCl gradient (500 ml, 0–0.1 *M* NaCl, 0.5 ml/min). The eluted protein was detected by absorbance at 280 and 260 nm. As shown in Fig. 1, peaks of the two parvalbumin species were separated; the peak eluting at 92 m*M* NaCl exhibits an absorbance maximum at 280 nm and is identified as the Trp-containing species. The next peak, with minimal absorbance at 280 nm and a maximum at about 260 nm, is identified as the protein containing the Phe residues but no Trp or Tyr. It eluted at ~100 m*M* NaCl. Fractions between the arrows indicated in Fig. 1 were pooled, dialyzed against water, and lyophilized.

Analysis of Denaturation Profile

The procedure of Pace [9] was used to characterize the denaturation process of parvalbumin. Many globular proteins have been found to approach closely a two-state mechanism:

$$N \leftrightarrow D \tag{1}$$

in which only the native state, N, and the denatured state, D, are present at significant concentrations in the transition region. The values f_N and f_D, the respective fraction of the protein present in the native and denatured states at different concentrations of GHCl, are obtained in the transition region by extrapolation from the linear portions of the denaturation curve at low and high denaturant concentrations. ΔG is obtained from the following equation:

$$\Delta G = -2.303\, RT \log f_D/f_N \tag{2}$$

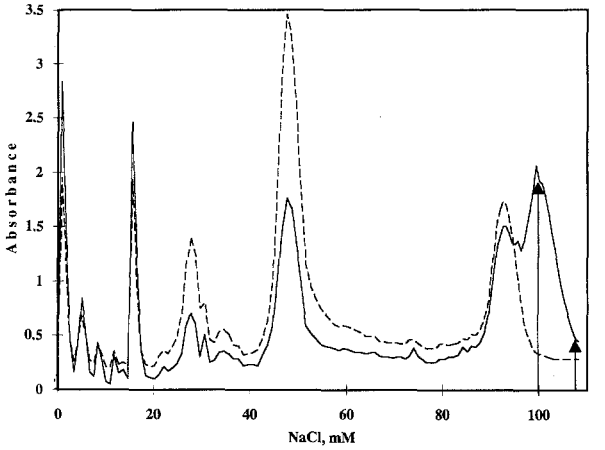

Fig. 1. Elution profile for DEAE cellulose as a function of salt gradient. The dashed line is the optical density at 280 nm; the solid line is the optical density at 260 nm. Fractions between the arrows were collected and used for this study.

Instrumentation

Steady-state absorption spectra were obtained with a Model 200 Perkin-Elmer spectrometer. Steady-state fluorescence spectra were recorded on a Perkin-Elmer LS-5 luminescence spectrometer.

Transient absorption spectra of the excited state species were acquired using the diode array instrument described in detail previously [10]. The actinic light, obtained from a Q-switched Nd:YAG laser, was 8 ns fwhm in duration and had a repetition rate of 10 Hz. The exciting wavelength was 266 nm. The instrument includes a Triplemate flat-field spectrograph to resolve the spectrum and a Princeton Instruments DIDA-512 dual-diode array system to detect the spectrum. A xenon arc

lamp (Hammamatsu, Middlesex, NJ) provided probe light for photoexcited and unexcited regions of the sample. Baseline corrections and conversion of transmittance to absorbance were carried out by the computer software. This apparatus allows us to obtain the UV/VIS difference absorption spectrum over a range of 300 nm at variable times after the actinic pulse. The same instrument was also used for transient emission studies. The gate duration for data accumulation was 5 ns, making the instrument suitable for emission studies in the submicrosecond time range and longer.

For measuring the decay of emission on the nanosecond time scale a time correlated single photon counting instrument, previously described [11], was used. The exciting light was a Coherent Antares modelocked YAG laser (Palo Alto, CA), which was quadrupled to give an excitation wavelength of 266 nm. Since the repetition rate of the pump source was 76 MHz, lifetimes in excess of 13 ns could not be measured with the modified instrumental configuration and the relative amplitudes of short and long components cannot be accurately determined.

The decay of the fluorescence and of the triplet transient species were analyzed by the standard RLBL LIFETIME program (Holtom. 1989), which accounts for the instrument response function or by Excel (Microsoft Corporation, Redmond, WA), which did not deconvolute the instrument response. An exponential function was used to describe the decay of fluorescence, f, where the lifetime, τ, is given by

$$f(t) = \sum_{i}^{N} A_i e^{-t/\tau_i} \qquad (3)$$

for i number of components ($i = 1.0$ for single exponential) and A is the amplitude.

Fluorescence Quenching

The quenchability of the Phe fluorescence was examined by monitoring fluorescence intensity, F, upon the addition of KI by this relationship [12]:

$$F_O/F = 1 + k\tau_O[KI] \qquad (4)$$

where F_O is the fluorescence intensity in the absence of quencher, τ_O is the fluorescence lifetime with no quencher, and k is the bimolecular quenching constant.

The solutions of KI were prepared immediately before use. A trace of sodium thiosulfate was added to the stock KI solutions to retard I_3^- formation.

Oxygen Removal from Samples

The buffer solution containing 0.3% glucose was initially degassed under an aspirator and then bubbled with argon. The protein was dissolved in the buffer and placed in a cuvette containing a glass-coated microstir bar, and the air space was filled with argon. A small volume of solution containing glucose oxidase and catalase was added to give a final concentration of 80 and 16 n*M*, respectively. This enzyme system catalyzed the reduction of O_2 to H_2O_2 and then to H_2O. The cuvette was then closed with a quartz stopper. Throughout these operations, air was excluded by a constant flow of argon gas over the cuvette.

Computer Graphics

The coordinates for parvalbumin was obtained from the Brookhaven Data base, entry 5CPV, based upon coordinates results of Kumar et al. [13] and plotted using a Silicon Graphics computer.

RESULTS

Absorption and Emission Properties

The UV absorption spectrum and fluorescence emission of the parvalbumin preparation used is shown in the Fig. 2. The fluorescence emission maximum is at 287 nm, which compares with 284 nm for Phe in water. The absorption maximum occurred at 259 nm; this wavelength did not detectably change between Phe in water and Phe in the protein environment (not shown).

The fluorescence lifetime of Phe in parvalbumin has been reported to be nonsingle exponential with a short lifetime of 5.4 ns and a longer lifetime estimated to be 62 ns, but which could not be accurately determined with the then available instrumentation [14]. For the Ca-bound protein studied here, a fluorescence species with a lifetime of 5.9 ns is seen using the time-correlated single photon counting apparatus (Fig. 3A). The data also had a contribution from a longer component of approximately 50 ns.

To determine the lifetime of the long component accurately, the fluorescence emission of Ca-bound parvalbumin was investigated using the transient absorption/emission diode array spectrometer, which has a time resolution >5 ns. The decay of fluorescence is shown in Fig. 3B; a fit to the curve gave a lifetime of 53 ns for the long-lived emitting component of protein in deoxygenated buffer. Because two instruments were used to

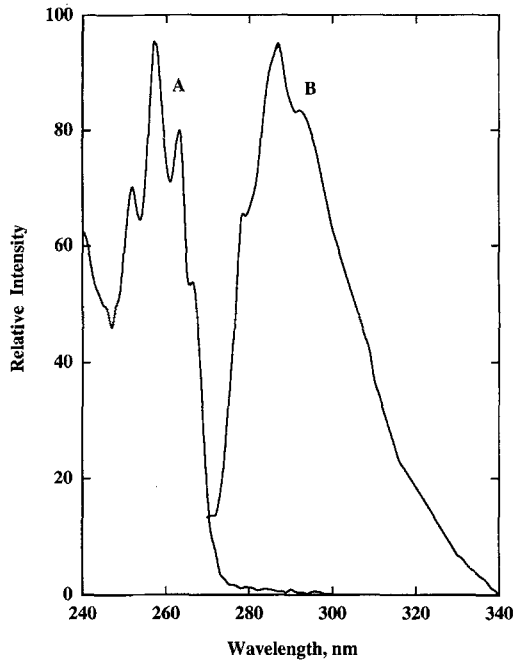

Fig. 2. Absorption (A) and fluorescence emission (B) spectra of parvalbumin. Medium contained 0.01 *M* Tris (pH 7.0) and 0.1 *M* NaCl. Excitation: 266 nm using a 3-nm effective bandpass. Temperature: 25°C.

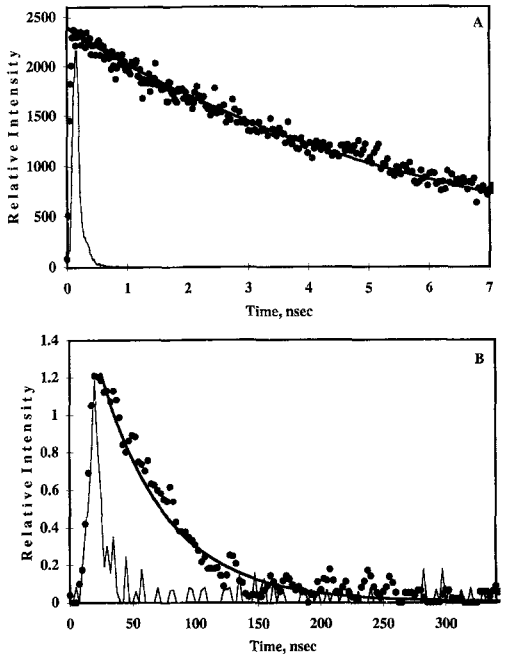

Fig. 3. Fluorescence decay using an emission wavelength of 295 nm and excitation at 266 nm. (A) Decay using the time-correlated single-photon counting instrument. The solid line indicates an exponential fit with a decay of 5.9 ns ($A = 1.31$) and 53 ns ($A = 0.002$). (B) Decay using the diode array instrument. The solid line shows an exponential fit with the lifetime of 53 ns.

determine the lifetimes, the relative amplitudes of the long and short components was not obtained.

The emission spectrum as a function of time was also recorded for the long-lived species. As shown in Fig. 4, the emission maximum remained at 287 nm, with no shifts in the emission spectra in the time scale of 15 to 100 ns. The emission in the range where phosphorescence is expected to occur, i.e., 325 to 375 nm, did not decay to zero in the time scale examined, indicating that a longer-lived species is also emitting. Because of the limited gate time of the instrument a clear spectrum of the phosphorescence could not be obtained.

At room temperature, the triplet state could be detected by its transient absorption using the diode array transient absorption spectrometer. The transient absorption spectrum observed 1 μs after excitation at 266 nm is shown in Fig. 5A. The spectrum was taken at different delay times, and while the absorbance decreased with increasing times, the spectrum showed no discernible shifts (not shown). The decay of the triplet in Ca-bound parvalbumin is shown in Fig. 5B; the lifetime was 425 μs.

The fluorescence and triplet absorption properties

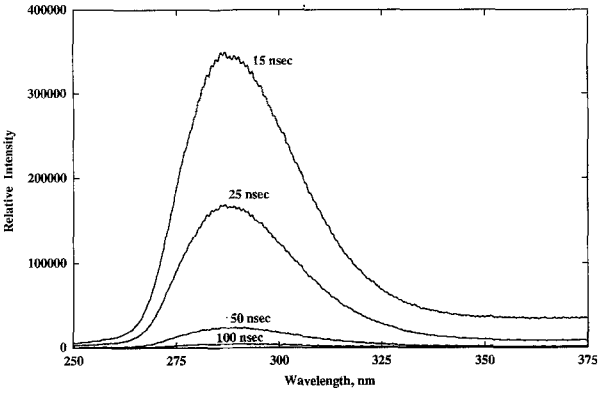

Fig. 4. Fluorescence emission spectra of Phe in cod parvalbumin, at the delay times indicated. Excitation wavelength, 266 nm; gate time, 5 ns. Samples contained 2 mg of protein/ml in 0.01 *M* Tris and 0.1 *M* NaCl at pH 7.0.

of Phe in the Ca-depleted parvalbumin and in free Phe were also examined. Addition of EDTA (2 m*M*) to the parvalbumin solution resulted in approximately 60% de-

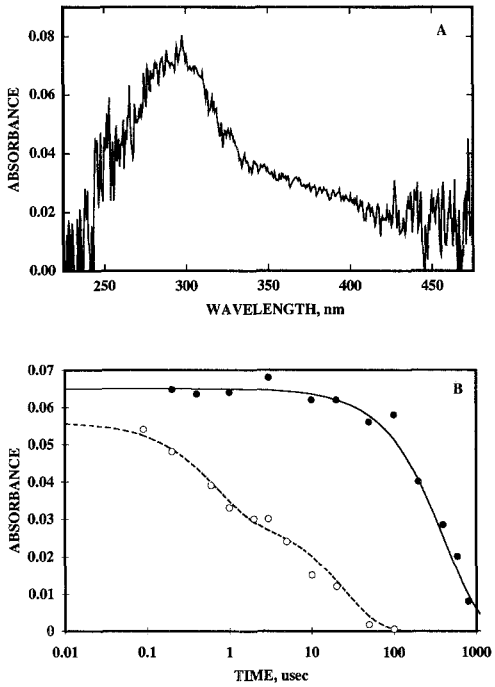

Fig. 5. (A) Transient absorption spectrum of Phe in cod parvalbumin after excitation at 266 nm. The delay time was 1 μs and the gate time 5 ns. (B) Decay kinetics of triplet; plotted absorption maximum of parvalbumin (•) and Ca(II)-depleted parvalbumin (○) at ~302 nm as a function of different delay times. The solid line is the simulated decay curve using the exponential 425 μs with the absorbance amplitude of 0.064 and the dashed line is the fit with absorbance amplitudes of 0.001, 0.025, and 0.03 and lifetimes of 0.09, 0.7, and 25 μs. Samples were prepared by dissolving 2 mg parvalbumin/ml of 0.01 *M* Tris, 0.3% glucose, and 0.1 *M* NaCl at pH 7.0. Deoxygenation was achieved using the enzyme system and other procedures described under Materials and Methods.

crease in fluorescence intensity and a decrease the lifetime of longer component from 53 to 17 ns. There was also a small blue shift (~2 nm) in the emission. The transient absorption spectrum of the excited triplet state species in the presence of EDTA was identical to that found for the Ca-bound parvalbumin, however, the decay of the triplet state was dramatically shortened. The absorbance as a function of delay time is also given in Fig. 5B for the Ca-depleted protein. The decay is nonexponential.

With regard to free Phe in aqueous solution, the transient triplet state lifetime is even further reduced. We found a decay time of 5 μs (data not shown), which compares well with the literature value for the triplet state lifetime of 3 μs [15].

Sensitivity of Phe Fluorescence to Protein Conformation

The decrease in fluorescence intensity and lifetime as well as the decrease in lifetime of the triplet state that was observed when Ca was removed from parvalbumin suggests that Ca removal results in exposure of Phe to the solvent. As a further indication of the exposure of Phe to solvent in the Ca-bound and Ca-free protein, quenching experiments were undertaken using KI. The data are shown in Fig. 6. The quenching constants can be determined from these data, using the fluorescence lifetimes. We used a lifetime of 5.9 ns for Phe in solution and 17 ns for Phe in the Ca-depleted protein and a lifetime of 50 ns for the Ca-bound parvalbumin, the respective Stern–Volmer quenching constants of 63.6, 19.3, and 4.5 $M^{-1}ns^{-1}$ for Phe in solution, for Ca(II)-depleted and Ca-bound parvalbumin. These values are approximately in view that the relative amplitudes of short- and long-emitting species could not be determined with our instruments. Therefore, no attempt was made to distinguish collisional and static effects [16].

The decrease in fluorescence intensity and increased susceptibility to quenching by I^- upon removal of Ca suggests that the Phe becomes exposed to the solvent. If so, addition of a denaturant to unfold the protein should also result in a decrease in fluorescence intensity. The addition of GHCl results in a decrease in fluorescence intensity (Fig. 7A) and a small (~2- to 3-

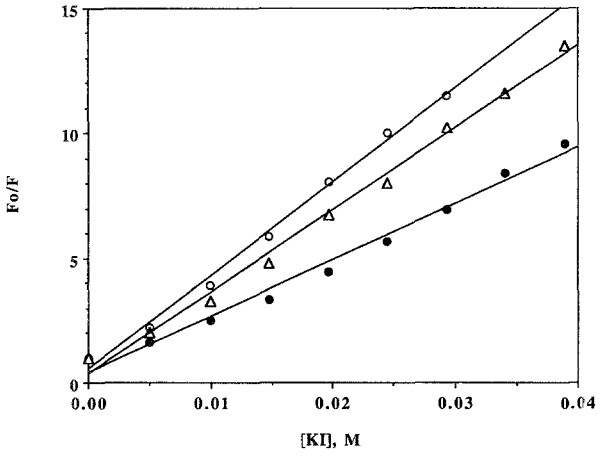

Fig. 6. Stern–Volmer plot for quenching of parvalbumin (•), Ca-free parvalbumin (Δ), and Phe (○) by KI. Parvalbumin, 1.4 mg/ml, or Phe, 10 μ*M*, was dissolved in 0.01 *M* Tris, pH 7.0, and 0.1 *M* NaCl. Ca(II) was removed by adding 5 m*M* EDTA. Quenching was measured by the decrease in steady-state emission intensity, using 266 nm for excitation and 287 nm for emission.

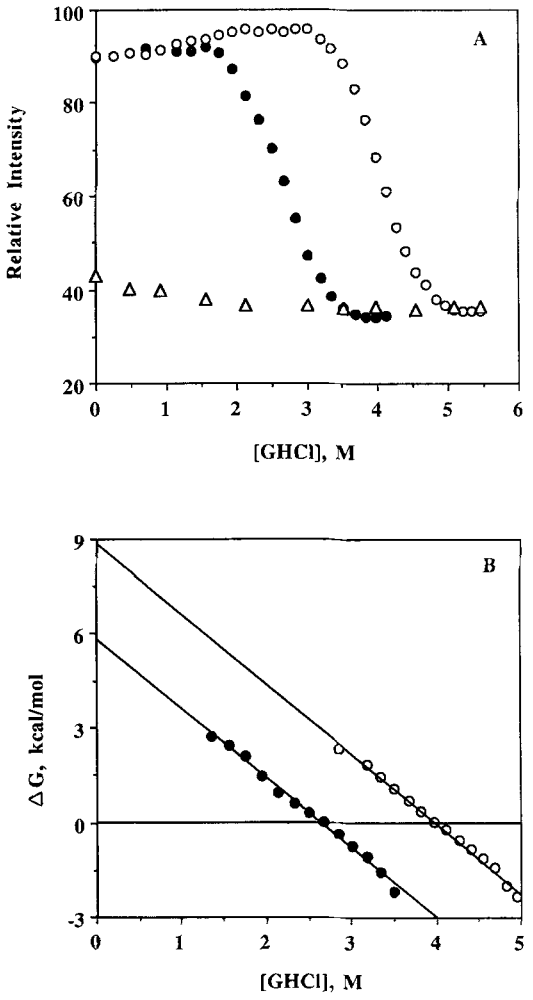

Fig. 7. (A) Effect of GHCl on the fluorescence intensity of parvalbumin (•), Ca(II)-added parvalbumin (○), and Ca(II)-depleted parvalbumin (Δ). Parvalbumin (1.4 mg/ml) was dissolved in 0.01 *M* Tris, pH 7.0, and 0.1 *M* NaCl. Ca(II) was depleted by adding 5 m*M* EDTA. Temperature: 25°C; excitation, 266 nm; emission, 287 nm. (B) ΔG as a function of GHCl molarity; the solid line represents the least-squares curve fit.

nm) blue shift in the emission spectrum (not shown). The concentration for the half-effect depends upon the Ca(II) concentration. With no Ca addition, the half-effect is at 2.65 *M* GHCl. Upon adding 2 m*M* $CaCl_2$ to this solution there is a shift in the concentration for the half-effect to 3.9 *M* GHCl. The conformational stability of the protein is 5.82 kcal/mol K (24.33 kJ/mol K) with no addition and 8.85 kcal/mol K (36.2 kJ/mol K) at the higher Ca(II) concentration (Fig. 7B). The results clearly indicate that Ca(II) protects against denaturation of parvalbumin by GHCl.

The effect of GHCl on calcium-free parvalbumin was also examined. As seen in Fig. 7A and noted above, the intensity of the calcium-free protein is greatly reduced from that of the Ca-bound species. Further addition of GHCl did not significantly affect the fluorescence intensity. This suggests that in the Ca-free species, the Phe is already exposed to solvent. As a control experiment, the effect of GHCl on the fluorescence intensity of free Phe was also studied; like calcium-free parvalbumin, there was little change in the intensity caused by the addition of GHCl.

The intensity of fluorescence for Phe is temperature dependent both in water and in the Ca-bound protein. The temperature profile is shown in Fig. 8, showing essentially no difference for Phe in the two situations.

DISCUSSION

Parvalbumins are members of a well-studied family of Ca(II) and Mg(II) binding proteins found originally in skeletal muscles but now recognized to be distributed among other tissues [17]. The crystal structures of several Ca-parvalbumins have been determined and found to contain six helical regions. Two high-affinity Ca(II) binding sites connect the two pairs of helices to form a compact protein structure. A representation of the structure of parvalbumin from carp in the presence of Ca is indicated in Fig. 9. (Most amino acids in this protein are conserved relative to the protein we studied, except that position 102 in the carp protein is Trp rather than Phe). In the absence of Ca(II), no parvalbumins have

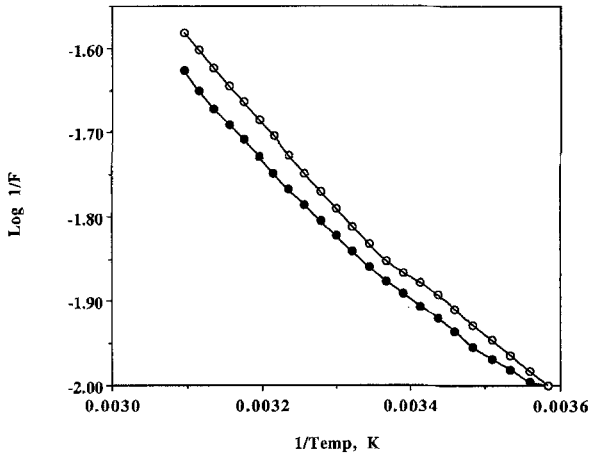

Fig. 8. Dependence upon temperature of the fluorescence intensities of 10 μ*M* Phe (○) and Phe in 2 mg of protein/ml (•) at pH 7.0. Excitation, 266 nm; emission, 287 nm.

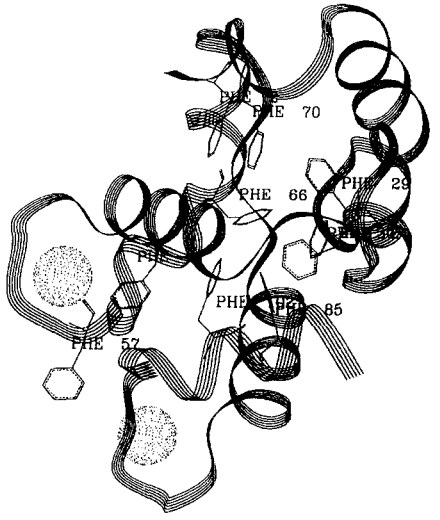

Fig. 9. Structure of carp parvalbumin showing Phe. The shaded area represents the Ca atoms.

been crystallized, and on the basis of a variety of physical studies, it has been proposed that the protein exists in a relatively less structured form [18].

In the present work, we use the differences between the Ca-bound and the Ca-free cod parvalbumin to characterize the excited singlet and triplet states of phe. The fluorescence intensity and lifetime are very dependent on the protein conformation. The lifetime of Phe in aqueous solution is 5–6 ns, whereas in the Ca-bound protein the lifetime of the long-emitting component is 53 ns. In comparison, the lifetime of Phe in solution is 6.8 ns [19], whereas the lifetime of benzene is 29 ns in cyclohexane and toluene shows a lifetime of 34 ns in cyclohexane at 20°C [20]. The long lifetime of the Phe in the protein could be an indication of Phe in the hydrocarbon-like environment of the protein interior and also could be a consequence of rigidity of the protein interior. Indeed, the crystallographic structure shows that all Phe's, except Phe57, are buried (Fig. 9).

The observation that denaturation results in a decrease in fluorescence intensity (Fig. 7) is consistent with the view that the quantum yield of buried Phe is higher than for aqueous exposed moieties. This is further substantiated by the increased quenchability by KI. It then follows that the decrease in fluorescence lifetime and intensity upon removal of Ca(II) results in the exposure of Phe to the solvent.

The triplet-state lifetime for Phe in Ca-bound parvalbumin is much longer than for Phe in aqueous solution. The transient absorption changes gave a triplet lifetime of ~425 μs for the Ca-bound protein. This is about 100 times longer than for Phe in solution (3 μs reported for the chromophore in aqueous solution [15] and ~5 μs observed by us). The protein in the absence of Mg(II) and Ca(II) showed a decrease in the phosphorescence lifetime, and the decay was not single exponential (Fig. 5B). In this regard, the Phe triplet state resembled the decay of Trp in Trp-containing parvalbumin [21]. The decay of Phe can be more directly related to the conformation of the protein, however, because Phe, unlike Trp, shows very little spectral shift and no evidence of unusual excited-state activity. The lifetime at room temperature for Phe in the protein and in solvent is, nevertheless, very much reduced from the phosphorescence lifetime at 77 K, which is 5.5 s [2].

The fluorescence spectrum is relatively insensitive to environment, with a small blue shift in going from a hydrocarbon to an aqueous phase for Phe and observed in the protein upon removal of divalent cations or under denaturation conditions. The triplet absorption spectrum showed no environmental responses, but in this case the instrumental band pass is wide (about 10 nm) and so small changes would not be detected (Fig. 5A). The dependence of fluorescence intensity was sensitive to changes in temperature and the Phe in solution and in the protein showed the same temperature dependence in the range where the protein remains native (Fig. 8). Leroy *et al.* [19] suggest that the temperature dependence of Phe indicates that internal conversion contributes to the decay from the singlet state.

Alterations in the structure of proteins related to parvalbumin as a function of Ca binding have been indicated by monitoring the fluorescence of the other aromatic amino acids. For the Trp containing parvalbumin (type III) anisotropy and spectral positions indicate that Ca increases the rigidity and burial of Trp [22,23]. In the case of calmodulin, a Ca binding protein with four Ca binding sites and that contains two Tyr's, the anisotropy becomes higher when Ca is bound [24,25]. Since all the fluorescent markers change in response to Ca binding, this suggests a rather global conformational response with increased rigid structures for the Ca-bound proteins. In the case of Phe in the parvalbumin studied here, it is distributed over the whole molecule (Fig. 8) and so its luminescence will be a composite of all events occurring in the protein. We note that upon removal of Ca, the Phe fluorescence lifetime and phosphorescence did not go to the values found in aqueous solution, suggesting that some residual structure remains in the Ca-free protein.

In conclusion, the lifetimes of both the excited-singlet and the triplet-state Phe molecules are remarkably long in parvalbumin. The lifetime of fluorescence for

Phe in the calcium-containing protein is greater than 50 ns and the triplet lifetime is greater than 400 μs. The removal of Ca (by the addition of a chelator) or a change in the protein structure (by the addition of denaturant which unfolds the protein) results in a decrease in both lifetimes to approximately the value found in aqueous solution. Since lifetime is a contributing factor to reactivity, the long lifetime of the Phe excited states in proteins means that they are more likely to participate in photoreactions. Additionally, the sensitivity of lifetime and intensity to environment means that Phe fluorescence can be used as a conformational marker in those proteins and peptides which contain Phe exclusively of the aromatic amino acids.

ACKNOWLEDGMENTS

This work was supported by NIH GM34448 and NIH RR01348. The authors thank Amy Vanderkooi for help in some experiments.

REFERENCES

1. F. W. J. Teale and G. Weber (1957) *Biochem. J.* **65**, 476–482.
2. J. W. Longworth (1971) in R. F. Steiner and I. Weinryb (Eds.), *Excited States of Proteins and Nucleic Acids*, Plenum Press, New York, pp. 319–484.
3. W. D. Horrocks, Jr., and W. E. Collier (1981) *J. Am. Chem. Soc.* **103**, 2856–2862.
4. M. R. Celio and C. W. Heizmann (1982) *Nature* **297**, 504–506.
5. C. Gerday and J.-M. Gills (1976) *J. Physiol.* **258**, 96–97P.
6. C. Gerday (1982) *Mol. Physiol.* **2**, 63–87.
7. G. Baron, J. Demaille, and E. Dutruge (1975) *FEBS Lett.* **100**, 156–160.
8. J. Haiech, J. DeRancourt, J. F. Pechere, and J. G. DeMaille (1979) *Biochimie* **61**, 583–587.
9. C. N. Pace (1986) *Methods Enzymol.* **131L**, 266–280.
10. S. Papp, J. M. Vanderkooi, C. S. Owen, G. R. Holtom, and C. M. Phillips (1990) *Biophys. J.* **58**, 177–186.
11. G. R. Holtom, H. P. Trommsdorff, and R. M. Hochstrasser (1986) *Chem. Phys. Lett.* **131**, 44–50.
12. O. Stern and M. Volmer (1919) *Phys. Z.* **20**, 183–188.
13. V. D. Kumar, L. Lee, and B. F. P. Edwards (1990) *Biochemistry* **29**, 1404–1412.
14. E. A. Permyakov, E. A. Burstein, V. I. Emelyanenko, Y. M. Alexandrov, K. V. Glagolev, V. N. Makhov, T. N. Syreishchikova, and M. N. Yakimenko (1983) *Biofizika* **28**, 393–397.
15. D. V. Bent and E. Hayon (1975) *J. Am. Chem. Soc.* **97**, 2606–2612.
16. M. R. Eftink and C. A. Ghiron (1976) *J. Phys. Chem.* **80**, 486–493.
17. T. C. Williams, D. C. Corson, K. Oikawa, W. D. McCubbin, C. M. Kay, and B. D. Sykes (1986) *Biochemistry* **25**, 1835–1846.
18. M. Jackson, P. I. Haris, and D. Chapman (1991) *Biochemistry* **30**, 9681–9686.
19. E. Leroy, H. Lami, and G. Laustrait (1971) *Photochem. Photobiol.* **14**, 411–421.
20. I. B. Berlman (1965) in *Handbook of Fluorescence Spectra of Aromatic Molecules*, Academic Press New York pp. 42–47.
21. K. Sudhakar, C. M. Phillips, S. A. Williams, and J. M. Vanderkooi (1993) *Biophys. J.* **64**, 1503–1511.
22. C. M. L. Hutnik, J. P. MacManus, and A. G. Szabo (1990) *Biochemistry* **29**, 7318–7328.
23. S. T. Ferreira (1989) *Biochemistry* **28**, 10066–10072.
24. I. Gryczynski, R. F. Steiner, and J. R. Lakowicz (1991) *Biophys. Chem.* **39**, 69–78.
25. K. Torok, A. N. Lane, S. R. Martin, J.-M. Janot, and P. M. Bayley (1992) *Biochemistry* **31**, 3452–3462.