Excited states of tryptophan in cod parvalbumin

Identification of a short-lived emitting triplet state at room temperature

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ABSTRACT The fluorescence and phosphorescence spectra of model indole compounds and of cod parvalbumin III, a protein containing a single tryptophan and no tyrosine, were examined in the time scale ranging from subnanoseconds to milliseconds at 25°C in aqueous buffer. For both Ca- bound and Ca-free parvalbumin and for model indole compounds that contained a proton donor, a phosphorescent species emitting at 450 nm with a lifetime of ~20–40 ns could be identified. A longer-lived phosphorescence is also apparent; it has approximately the same absorption and emission spectrum as the short-lived triplet molecule. For Ca parvalbumin, the decay of the long-lived triplet tryptophan is roughly exponential with a lifetime of 4.7 ms at 25°C whereas for *N*-acetyltryptophanamide in aqueous buffer the decay lifetime was 30 μs. In contrast, the lifetime of the long-lived tryptophan species is much shorter in the Ca-free protein compared with Ca parvalbumin, and the decay shows complex nonexponential kinetics over the entire time range from 100 ns to 1 ms. It is concluded that the photochemistry of tryptophan must take into account the existence of two excited triplet species and that there are quenching moieties within the protein matrix that decrease the phosphorescence yield in a dynamic manner for the Ca-depleted parvalbumin. In contrast, for Ca parvalbumin, the tryptophan site is rigid on the time scale of milliseconds.

INTRODUCTION

The photochemistry of tryptophan (trp) is implicated in disease states such as cataract formation (1, 2) and tumorgenesis induced by ultraviolet light (3). Fluorescence and phosphorescence of trp are also commonly used to study protein structure and dynamics. To understand the observed photochemical and spectral features of trp, the nature of the excited state molecule should be known. For instance, the existence of photoreactions such as electron ejection, intramolecular proton, and electron transfer have been suggested on the basis of fluorescence decay and excited state reactions for isolated indole derivatives (4–9). The role of the protein in affecting the rates of such reactions, the radiative and nonradiative decay rates, and intersystem crossing rates is relatively unknown.

In this work we are using transient absorption and time-resolved fluorescence and phosphorescence emission measurements to examine metastable excited state species of trp. Our choice of protein is cod parvalbumin type III, a member of Ca and Mg binding proteins of low molecular weight (M_r ~ 11,500). Of the large family of parvalbumins, ~40 have known structure. Cod parvalbumin type III has the same structural features of the other parvalbumins, but its amino acid composition is unusual in that it contains a single trp and no tyrosine. Because of this, absorption in the 280–300 nm range is due only to trp, and unlike most proteins complications in kinetic analysis due to energy transfer from tyrosine to trp is not a problem. The photochemistry of parvalbumin is in itself of interest because parvalbumin undergoes a large conformational change when Ca is added, resulting in an ~20 nm blue shift of the trp fluorescence maximum, corresponding to the burial of the indole ring (10, 11). Ca-bound parvalbumin exhibits phosphorescence at room temperature with a lifetime of ~5 ms, whereas the Ca-depleted protein shows no evidence of this long-lived emission (12). By examining the transient excited state species we hope to determine whether this is due to lower quantum yield of formation of the triplet state or whether the phosphorescence of trp is quenched by reactions occurring after its formation. To this end we wanted to examine the spectral features of excited state trp in the early times of triplet formation from the excited singlet state. Our results indicate that there are two emitting triplet states at room temperature.

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Abbreviations used in this paper: NATA, *N*-acetyl-l-tryptophanamide; trp, tryptophan; W(1), 3-carboxy-1,2,3,4-tetrahydro-2-carboline.

MATERIALS AND METHODS

Materials

N-acetyltryptophanamide (NATA) was obtained from Sigma Chemical Co. (St. Louis, Missouri). W(1), 3-carboxy-1,2,3,4-tetrahydro-2-carboline, was a kind gift of Dr. M. D. Barkley and other chemicals were obtained from usual commercial sources.

Parvalbumin was prepared from frozen cod fillets locally obtained. The procedure used incorporated minor modifications of published methods (13, 14). Frozen cod fillets (125 g) were homogenized with 200 ml of 10 mM Tris buffer (pH 8.7), 2 mM EDTA, and 2% glycerol in a blender, stirred for 1 h at 4°C, and then centrifuged (10,000 rpm for 30 min). The supernatant was subjected to a 45% acetone (vol/vol) added dropwise and centrifuged for 75 min at 10,000 rpm. The supernatant was taken to 80% acetone (vol/vol), the mixture was centrifuged, the pellet was resuspended in 10 mM Tris buffer, at pH 7.6 in 1 mM $CaCl_2$, 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 mM piperazine at pH 5.7 overnight and then chromatographed (0.5 ml/min) on a cellulose column (2.5 × 43 cm) (model DEAE-52; Whatman Inc., Clifton, New Jersey) equilibrated with the same buffer. The column was washed for ~24 h until the

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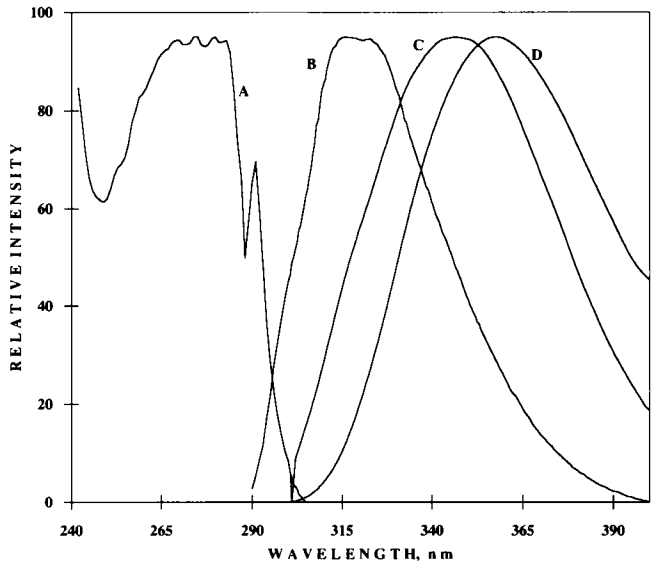

FIGURE 1 Absorption (*A*) and fluorescence emission (*B*) spectra of Ca(II)-bound parvalbumin in 0.01 M Tris and 0.10 M NaCl at pH 7.0. Fluorescence of Ca(II)-depleted parvalbumin (*C*) and NATA (*D*) in the same medium except for (*C*) where Ca(II) is removed by adding 5 mM EDTA. Excitation wavelength was 282 nm.

absorbance at 282 nm fell to ~0.03 and then was eluted with a NaCl gradient (500 ml, 0–0.1 M NaCl, 0.5 ml/min). The eluted protein was detected by absorbance at 282 nm. Peaks of the two parvalbumin species were separated, and the trp-containing species was dialyzed against water and lyophilized. The ultraviolet spectrum and fluorescence emission of the parvalbumin preparations used by us resemble that described in the literature (13, 14) (Fig. 1). The removal of Ca from parvalbumin by addition of 5 mM EDTA shifts the fluorescence emission maximum from 320 to 350 nm, broadens the spectrum, and produces a decrease in fluorescence yield. Further addition of EDTA (10 mM) had no further effect on the spectrum. In comparison, the emission of NATA is centered at 360 nm. A red shift in fluorescence of trp is generally considered to be an indication of increased exposure to solvent.

Instrumentation

Steady state absorption spectra were obtained with a spectrometer (model 200; Perkin-Elmer). Steady-state fluorescence and phosphorescence spectra were recorded on a Perkin-Elmer LS-5 luminescence spectrometer.

Transient absorption spectra of the excited state species were acquired using the instrument described in detail previously (15). The actinic light, obtained from a Q-switched Nd:YAG laser, was 8 ns fwhm in duration and with a repetition of 10 Hz. The exciting wavelength was 282 nm. The instrument includes a Triplemate flat-field spectrograph to resolve the spectrum and a dual-diode array system (model DIDA-512; Princeton Scientific Instruments, Inc., Monmouth Junction, New Jersey) to detect the signal. A continuous xenon lamp was used for probe light and the absorption in an area of the cuvette not illuminated by the actinic light was used as reference. Baseline corrections and conversion of transmittance to absorbance was carried out by the computer software. This apparatus allows us to obtain the difference absorption spectrum in a range of ~140 nm at variable times after the actinic pulse. The same instrument was used for transient emission studies, but the probe light was not used. The gate duration for data accumulation was 5 ns therefore the instrument is suitable for emission studies in the submicrosecond time range. For determining longer-lived emission, a PTI instrument (Princeton, New Jersey) with an integration time of 0.5 ms was used.

For measuring emission in the nanosecond time scale, a time-correlated single-photon counting instrument, previously described (16), was used. The exciting light was a Coherence Antares modelocked YAG laser (Palo Alto, CA), which synchronously pumped a cavity-dumped dye laser. A polarizer was placed at the magic angle on the emission side to avoid polarization artifacts in the decay. The data were analyzed using the Global Unlimited program (Univ. of Illinois at Urbana-Champaign) on the basis of the procedure of Knutson et al. (17).

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 micro stir 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 nM, respectively. The enzyme system reduced 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.

RESULTS

Protein fluorescence spectra and decay

Because the excited singlet state is the precursor to the triplet state, it is of interest to characterize the fluorescence decay of trp in the protein. The fluorescence decay curves of Ca(II)-bound and -depleted forms of parvalbumin were measured at different wavelengths. For both forms of the protein, attempts to fit data with a single exponential did not give satisfactory results as indicated by the large χ^2 values. Good fit of the data was obtained when data was analyzed with a sum of two exponential decays with lifetimes τ_1 (4.3 ns) and τ_2 (1.0 ns). The long-lived species contributed >90% of the total fluorescence intensity. This result is in agreement with that found previously (11, 18, 19). Calcium removal results in a decrease in the lifetimes although two components were still required to fit the data. The lifetime of τ_1 is 3.5 ns and τ_2 is 0.5 ns, with the long component contributing more of the total light intensity. Another short-lived component (lifetime ~ 300 ps that contributes <4% of the intensity) has been reported (19). The data were not examined for the existence of this component; suffice it to say, the general features of the fluorescence decay are consistent with those reported in the literature. The experiment was repeated using 275, 285, and 295 nm as excitation wavelengths, with no detectable change in results, indicating that there was no contamination from another fluorescing species or significant spectroscopic heterogeneity in the ground state molecule.

To further characterize the fluorescence emission, the decay of anisotropy was analyzed. The rotational time for the Ca-bound parvalbumin was 4.35 ns, a value within the range found previously (11). The results of the Ca-bound parvalbumin is consistent with rotation of the entire protein assuming that the protein has an ~16

Å radius and is approximately spherical. The rotational time for trp in Ca-depleted parvalbumin is 0.4 ns. Ferreira (11) also found the anisotropy to decrease with removal of Ca. The group of parvalbumin isoproteins to which parvalbumin III belongs is known to lose most of its tertiary structure upon removal of calcium (20). For Ca-depleted parvalbumin, the rotational time indicates motion independent of the entire protein and, along with the red shift in emission, the result would indicate that the trp is solvent exposed.

Transient absorption and emission spectra of Ca(II)-bound parvalbumin

In the experiments described above using the time-correlated single-photon counting instrument, we also monitored the emission at longer wavelengths where phosphorescence is likely to occur. The intensity of emission integrated for 5-ns intervals is shown for various times after the exciting flash in Fig. 2, where the log of intensity is plotted as a function of wavelength. The decay of intensity in the range 345–365 nm shows a time response that is essentially the same as the fluorescence measured at the fluorescence peak. However, in the range from 420 to 460 nm there is deviation from the exponential type of decay, and a new emitting species is apparent. Both Ca-bound parvalbumin and Ca-free parvalbumin showed evidence for this species (Fig. 2, *A* and *B*).

To determine the decay kinetics of this new emitting species, the emission of light was then examined over a longer time range using the diode array apparatus. In Fig. 3, the emission of light in the range of 10–60 ns after excitation is presented. The pulse laser was ~8 ns in duration and the measuring gate was 5 ns, therefore, the time of measurement relative to excitation has up to ~10 ns uncertainty relative to the lamp flash but the delay between successive measurements is accurate. At the earliest measurement, the fluorescence at 340 nm maximum is clearly seen. The decay at 340 nm is ~5 ns, consistent with the more precise fluorescence lifetime values determined by time-correlated single-photon counting. At longer wavelengths, another emitting species becomes apparent; its emission maximum is at 450 nm. By ~60 ns this species had decayed so that it could not be detected above the noise.

The short gate (integration) time of the instrument used to measure the emission precludes measurement of long-lived emission. To measure the long-lived emitting species, an instrument with a gate time of 0.5 ms was used. The spectrum observed integrating the light intensity from 0.4 to 9 ms after excitation by a xenon flash is shown in Fig. 4. The vibrational features of trp phosphorescence are clearly observed in the spectrum. The decay time of the long-lived emitting species was also measured. As seen by the inset, its lifetime was ~5 ms.

These data show that phosphorescence decay for trp in the protein has complex kinetics. The maximum of the emission spectrum of the short-lived triplet species, T_1,

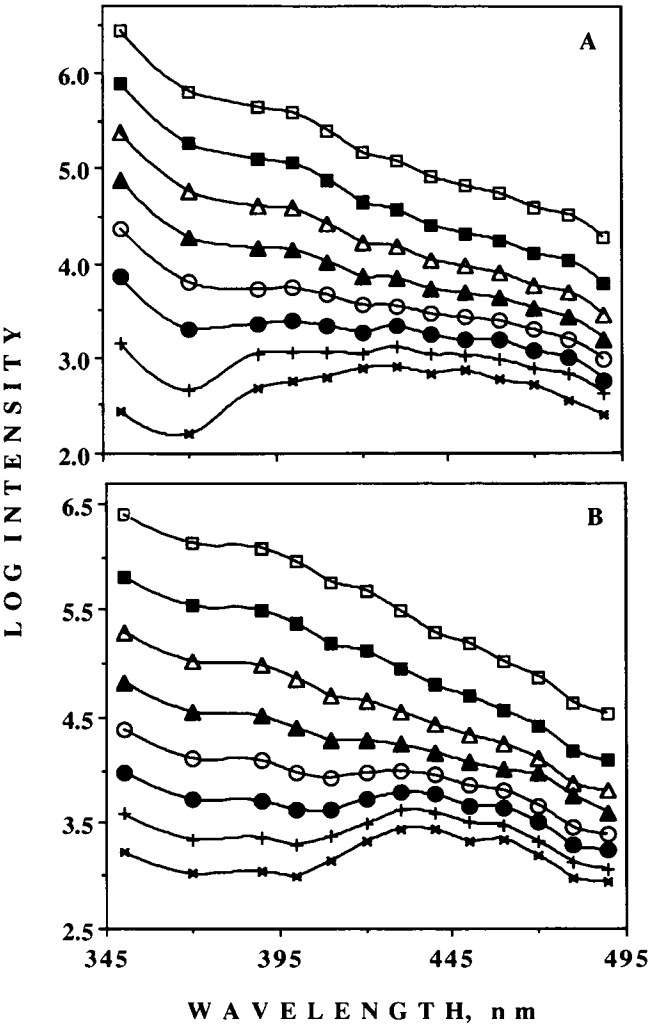

FIGURE 2 Log of emission intensities obtained at different wavelengths, using excitation at 295 nm, (*A*) Ca-bound parvalbumin and (*B*) Ca-depleted parvalbumin. Data is collected by integrating the fluorescence decay curve at the following times (in ns): (□) 1–5, (■) 6–10, (△) 11–15, (▲) 16–20, (○) 21–25, (●) 26–30, (+) 31–35, and (X) 36–40. Sample contained 1.4 mg/ml of parvalbumin in 0.01 M Tris and 0.1 M NaCl at pH 7.0. Ca-depleted parvalbumin also contained, in addition, 5 mM EDTA.

is at 450 nm, the same maximum as the long-lived phosphorescence. This suggests that both arise from a triplet state. Further verification is obtained from the transient absorption spectrum measured by Bent and Hayon (21), which showed an absorbing transient species for indole samples in the same time scale where we observed emission. For technical reasons we were unable to observe the transient absorption in these short times, but the transient absorption spectra of Ca-bound parvalbumin in oxygen-free buffer at pH 7.0 and 25°C is shown in Fig. 5 at delay times ranging from 30 ns to 10 ms after excitation with a laser pulse at 282 nm. At the earliest times the absorbance goes below the baseline in the spectral range ≤ 400 nm; this is due to the fluorescence of the sample. At times long enough to allow the fluorescence

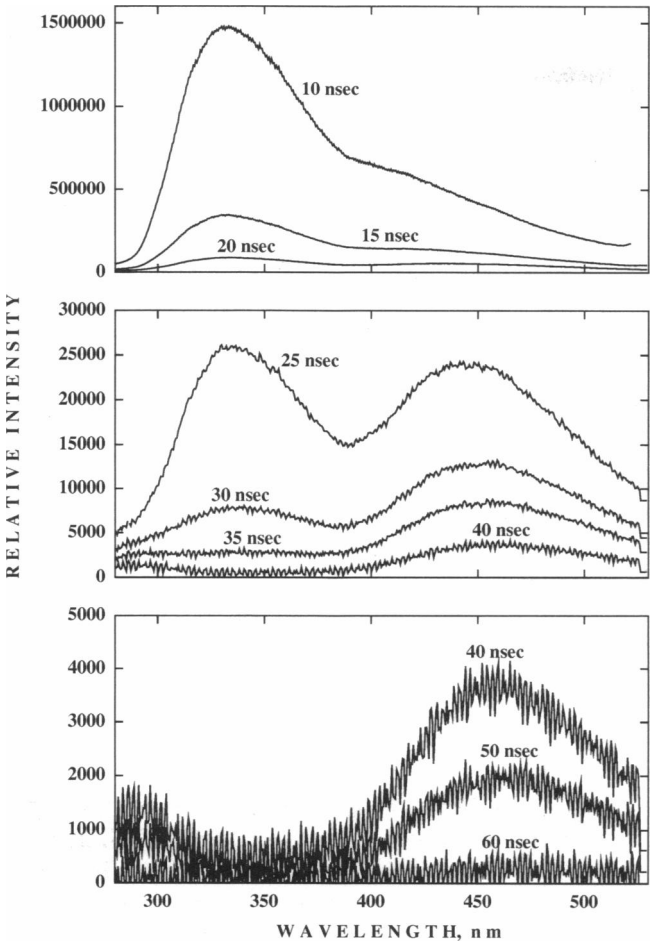

FIGURE 3 Luminescence spectra and decay of Ca(II)-bound parvalbumin on excitation at 282 nm. Delay times are indicated on the figure. Samples were prepared by dissolving 1.4 mg parvalbumin/ml of 0.01 M Tris, 0.3% glucose, and 0.1 M NaCl at pH 7.0. Deoxygenation was achieved using the enzyme system and other procedures as described in Methods.

to decay, absorbance in the region of 450 nm could be measured. In the time from 30 ns to 1 μs, there appeared to be a fast decaying species. Within the accuracy of the experimental data no shift in the band maxima is found for the measurements at different delay times. The absorption maximum is the same as reported previously for indole triplet in protein (22).

Transient absorption and emission spectra of Ca(II)-depleted parvalbumin

The emission of the Ca(II)-depleted parvalbumin at variable times after excitation is shown in Fig. 6. The fluorescence maximum centered at 360 nm is predominant at short times. The red shift in fluorescence relative to that observed for Ca(II)-bound parvalbumin is consistent with the red-shifted emission maximum obtained with a conventional fluorimeter (Fig. 1). The appearance of the triplet emission can be clearly seen at 450 nm. The triplet absorption spectra of Ca(II)-depleted parvalbumin was also studied in the time of 30 ns to 10 ms after the laser pulse. The emission and transient absorption spectra resembled those observed for the Ca-bound protein (Fig. 5).

Transient absorption and emission spectra of trp analogues

The emission of NATA, as an analog of trp, is shown in Fig. 7. Because NATA is red shifted relative to trp in the protein (see Fig. 1), the spectra of the two species are not as well separated as in the protein cases but it can be clearly seen that NATA also shows a triplet species with emission maximum at 450 nm. The emission maximum at 370 nm is attributed to the fluorescence. No changes in the spectral characteristics were observed when the excitation wavelength was changed from 282 to 266 nm (data not shown). The absorption spectra of NATA taken at variable times after excitation are shown in Fig. 8. In this case, there is an indication of the existence of an ionized form, as seen by the absorption around 570 nm. Note that absorption at these wavelengths was not seen in the case of protein (Fig. 5).

The emission spectra of W(1), a trp derivative where because of cyclization the position of the carboxyl and amino groups are fixed relative to the indole ring, were studied at pH 5.5 (Fig. 9 *A*) and 10.5 (Fig. 9 *B*). The fluorescence of both pH forms are observed with maximum at ~350 nm for low pH and ~370 nm for high pH. These maxima coincide with the published maximum for the two forms (23). At low pH, the short-lived (T_1) species is formed within the 10 ns and by 20 ns (shown in Fig. 9) the triplet emission predominates. In contrast, at high pH very little short-lived species is observed. The pK for ionization is 8.9 and, therefore, at pH 10.5 the percentage of ionized form is ~4%. The appearance of a small amount of emission of the short-lived species can largely, or indeed solely, be due to some zwitterion being present.

The transient absorption and emission of indole in aqueous buffer (0.01 M Tris, pH 7.0 and 0.1 M NaCl) and in cyclohexane was also examined. In these studies we did not see the short-lived transient triplet emission for indole in either solvent (data not shown). Control experiments were undertaken to see whether the short-lived species could arise from the buffer or cuvette. In the absence of protein or indole derivative no emission or transient absorption could be detected.

Summary of data

The kinetic information given in the spectra shown above are summarized in Fig. 10. In Fig. 10 *A*, the linear/log plot of emission is given, showing that the decay of the short-lived triplet species could be approximated by a first order process. The computer best fit gave decay times of 24.5, 23.4, and 19.5 ns for Ca-bound parvalbumin, Ca-depleted parvalbumin, and NATA, respec-

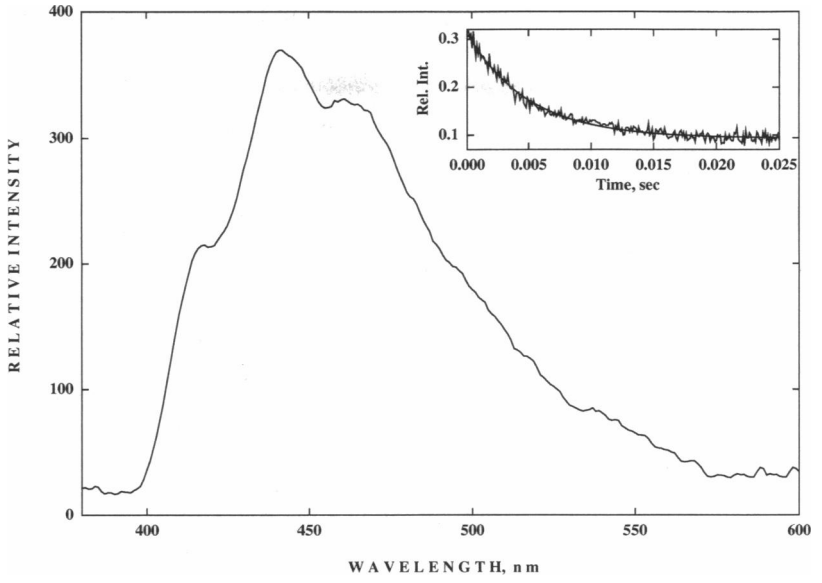

FIGURE 4 Phosphorescence spectrum of Ca(II)-bound parvalbumin using 1-ms integration time and 1-ms delay from xenon flash. The excitation wavelength was 282 nm. Conditions given in Fig. 3. (*Inset*) Intensity at 450 nm after excitation.

tively. For the low pH form of W(1), the lifetime was 48 ns. The decay lifetime for the high pH form was 36 ns but, as noted, the intensity is greatly reduced, therefore at the high pH the zwitterionic form may be contributing to the observed emission.

The long-lived species shows marked differences between the buried trp (in Ca parvalbumin) and the exposed trp (in Ca-depleted parvalbumin or in NATA) (Fig. 10 *B*). The decay of the long-lived phosphorescence (Fig. 4, *inset*) and the long-lived transient absorbing species for Ca-bound parvalbumin were the same, indicating that the transient absorption originates from the triplet state. The decay of Ca parvalbumin approximated an exponential at times > 1 μs, but nonsingle exponential behavior is evident in the time scale of 0.1–1 μs. The triplet decay of NATA also approximates an exponential; its lifetime was 30 μs, a value in the range of that reported for indole derivatives in water (21). In contrast to the exponential decay behavior of trp in Ca(II)-parvalbumin or NATA, the decay of the Ca-depleted parvalbumin shows nonsingle exponential behavior over the entire time range. The dotted curve simulates a three exponential decay function; this curve is not used to fit the data but to indicate that the data cannot be fit uniquely and at least a three exponential fit is needed to describe the decay behavior. The samples of Ca-bound and -depleted parvalbumins were matched to give the same optical density. It is therefore possible to compare the absorbance changes. At the shortest time measured, ~100 ns, the optical density of the Ca-depleted sample was ~30% reduced over the Ca parvalbumin. The behavior of Ca-depleted sample therefore contrasts greatly for both the case of the Ca parvalbumin and the free indole.

DISCUSSION

Parvalbumin, both Ca bound and Ca depleted, and the indole derivatives, W(1) and NATA, show a short-lived emitting species with maximum at 450 nm. Bent and Hayon (21) previously observed a short-lived transient species by absorption spectroscopy, which, based on the similarity to the longer-lived triplet species, they tentatively identified as a triplet state. Light emission from this species was not reported and our observation of emission positively identifies the species as an emitting triplet state molecule. Bent and Hayon (21) also reported that the short-lived species was only seen in indole derivatives where an amino group is present. Consistent with their observations, we too did not see evidence for short-lived triplet species from indole in either water or cyclohexane. The rigid indole derivative W(1) showed a longer lifetime than observed in the protein. At pH 10.3 there was little emission from W(1). The small emission can be accounted for by 4% zwitterionic species at this pH and the pH dependency of W(1) suggests that only the zwitterionic form shows the short-lived species.

The transient absorption spectrum of trp in both forms of the protein did not show evidence of an ionization product (Fig. 5) whereas NATA shows an increased absorption centered at 570 nm (Fig. 8). The free radical species has been identified as absorbing in this region (21, 24). The ionized species decayed more rapidly than the triplet state (compare the spectra taken at 50 ns and 1 μs in Fig. 8), showing that the triplet state is not the precursor of the ionization species. Picosecond laser studies suggest that photoionization occurs within picoseconds of the laser pulse and for indole or trp in aqueous solution ion-pair recombination does not occur on the

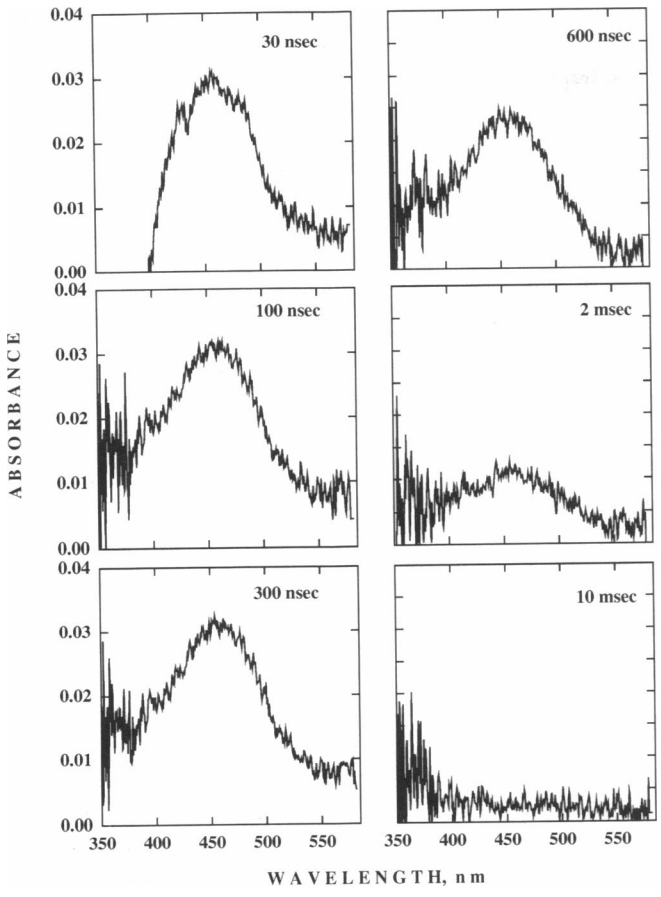

FIGURE 5 Transient absorption spectrum of tryptophan in Ca(II)-bound parvalbumin after excitation at 282 nm. The delay times are indicated. The gate time is 5 ns. Sample conditions given in Fig. 3.

time scale of fluorescence (25). The lack of appreciable ionization for trp in the protein may indicate that recombination occurs much faster in the protein so that it could not be detected by our measurement (i.e., <5 ns) or that ionization did not occur to an appreciable extent.

A major difference in the excited state properties of trp in the Ca-bound and -free protein can be seen in the decay behavior of the long-lived triplet state. The decay of the long-lived T_2 species in the Ca derivative shows approximate exponential decay behavior with a lifetime of ~5 ms. In contrast, the triplet decay of the Ca-free derivative was markedly nonsingle exponential over the entire time range studied (Fig. 10). The decay behavior contrasts with the decay of a solvent-exposed indole (i.e., NATA in aqueous buffer), and since the decay of trp in Ca-depleted parvalbumin is nonexponential over the entire time range, both ground state conformational heterogeneity and structural flexibility of the protein within the lifetime of the excited state can contribute to the quenching. The failure to observe long-lived phosphorescence from the Ca-depleted protein at room temperature must therefore in part be due to the quenching of the triplet state after its formation. In addition, it must be emphasized that the formation of T_2 is reduced for the exposed trp, as can be seen by the lower absorbance at early times (Fig. 10).

Ultimately, the photochemistry should be related to the environment of trp in the protein. The single trp of parvalbumin III from cod is at position 102 in the 108 amino acid primary sequence (19). Parvalbumin was shown by x-ray analysis to consist of six α-helices that are separated by loops (26). Between the C-D and E-F helices are loops containing acidic amino acids that each bind one Ca ion. The removal of Ca results in rather large conformational changes as seen from increase thermal instability (27). The exposure of trp to water in the Ca-depleted protein cannot entirely account for the decay behavior of its excited triplet state, however, since the decay in the protein contrasted with that of the model compound in aqueous solution in terms of the exponential decay behavior that was observed for the latter (Fig. 10). The decay behavior of the Ca-depleted parvalbumin suggested either that there are multiple structures of the protein or that the protein undergoes

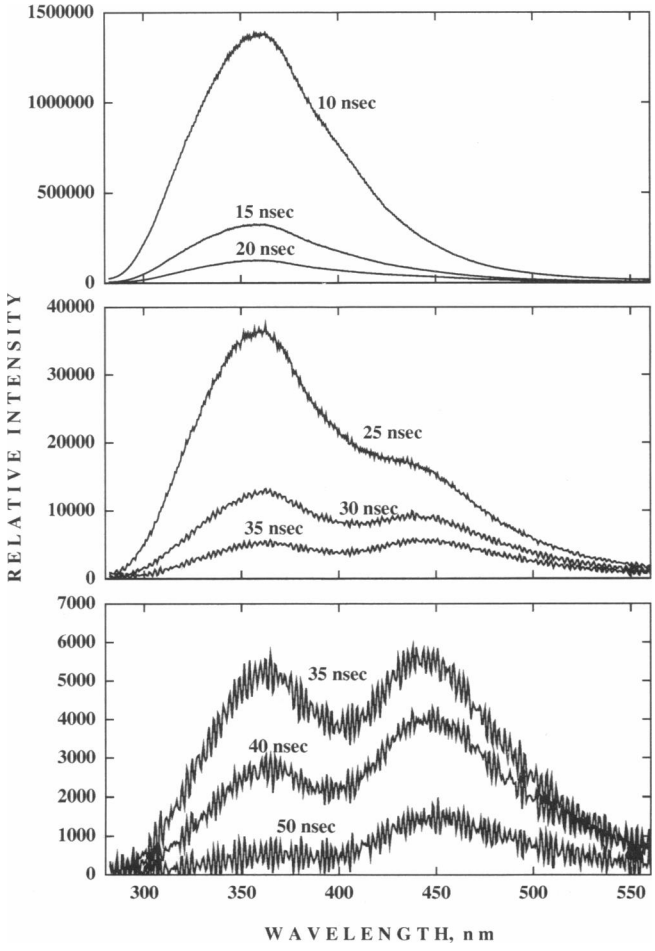

FIGURE 6 Luminescence spectra and decay of tryptophan in Ca-depleted parvalbumin on excitation at 282 nm. The delay times are indicated. Ca(II) is depleted by inclusion of 5 mM EDTA; other conditions are the same as in Fig. 3.

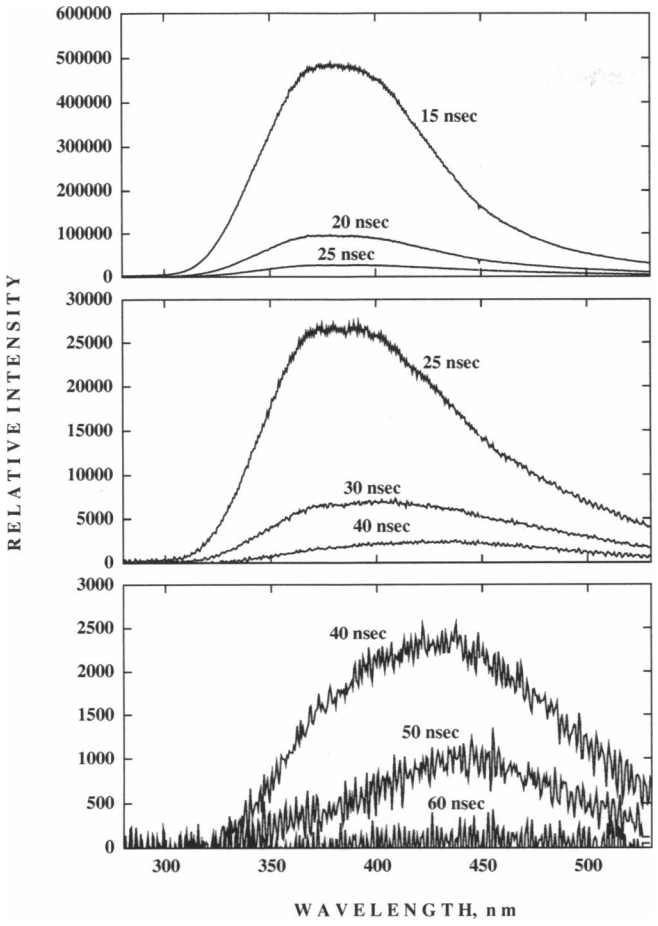

FIGURE 7 Luminescence spectra of NATA after excitation at 282 nm. The gate time is 5 ns. NATA was dissolved in 0.01 M Tris, 0.3% glucose, and 0.1 M NaCl at pH 7.0 to give an absorbance of 0.3 at 282 nm. The medium contains the deoxygenating enzyme system and was deoxygenated as described in Methods.

dynamic changes in the submicrosecond to millisecond time scale. This latter possibility is the more reasonable: it was noted for the fluorescence lifetime measurements that the same time-associated fluorescence emission spectra were obtained irrespective of excitation, indicating that by this criteria there are no significant spectral differences in ground state molecules.

The decay of Ca parvalbumin approximated an exponential in the time scale of 1 μs to 10 ms. Since trp phosphorescent lifetime at room temperature in a protein can approach 2 s (28) and it is ~6 s for the frozen protein (29), the implication is that in Ca parvalbumin the triplet state is quenched by something. In this case, if the quenching reaction arises from within the protein the quenching moiety must remain fixed relative to the trp on the time scale of the excited state; this argument is based on the supposition that segmental motion of the protein in the time that was examined would lead to nonexponential decay behavior. On the other hand, if the quenching process arises from something outside of the protein, then the quenching reaction is bimolecular, and in a nonviscous medium the diffusion of quencher would place the reaction in the fast diffusion limit. In this case, the position of the trp relative to the water surface would likely determine the quenching rate (30). To obtain an exponential decay this distance must also remain constant in the measured time scale. Therefore, in either case, the observation of exponential decay implies rigidity of the indole ring within the protein on the millisecond time scale.

Finally, although a detailed understanding of the chemistry and physics of excited state trp remains elusive, the demonstration of a short-lived emitting triplet species allows for the suggestion of additional experiments. Bent and Hayon (21) observed that the temperature dependence for the yield of T_1 and T_2 were different and they suggested that T_1 arises from a singlet state precursor that is different from T_2. The existence of two fluorescent states, ^1L_a and ^1L_a, for indole derivatives is well accepted. The lack of mirror symmetry, the lack of a clear 0,0 transition in the emission, and the red shift in fluorescence relative to the absorption (see Fig. 1) suggest that the singlet emission occurs from a state that is quite different from the ground state molecule. On the other hand, the structured phosphorescence emission (Fig. 4 and previous results by Saviotti and Galley [31] and Calhoun et al. [32]) suggests the geometry of the triplet excited state and the ground state resemble each other. In this regard, molecular orbital calculations suggest that the equilibrium geometry for the ^1L_b state is much the same as for the ground state molecule whereas the displacements for the ^1L_a state are much larger (33). We can, therefore, suppose that solvent rearrangement

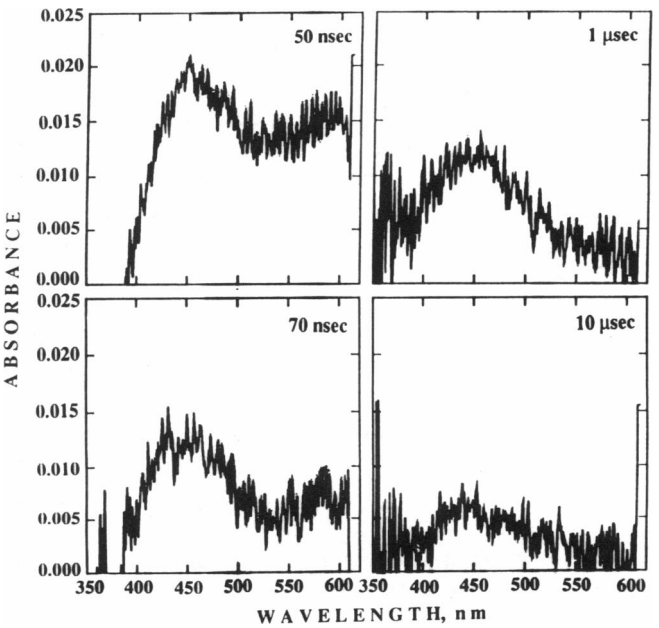

FIGURE 8 Transient absorption spectra of NATA. Conditions are same as Fig. 7. Times after excitation are indicated.

occurs around the excited singlet state and that there will then be relaxation of solvent around the triplet state molecule at early times after its formation by intersystem crossing. If there is equilibration between the two singlet states and both are precursors to the triplet state, the process of solvent relaxation could lead to nonexponential decay of phosphorescence. Further indication of this could be found by examining at higher spectral resolution the short-lived emitting species. Since the emission of phosphorescence for the long-lived species is somewhat vibrationally resolved at room temperature, one may expect that at the earliest times after intersystem crossing that the triplet emission would be unresolved but that at later times resolution would be seen. An additional effect to account for the decay of phosphorescence must be invoked to explain the observation that the fast decaying species is only seen in the case when an amino group can donate a proton. It seems possible, therefore, that charge interactions will lead to a decay of the triplet state. An inductive effect from the charged group is invoked to explain shifts in the fluorescence spectra of model indole compounds (34) and, in the paper by Bent and Hayon (21), it was suggested that the dipole of the excited state indole interacts with a proton donor to stabilize an intramolecular charge-transfer complex. On the other hand, for the singlet excited state there is demonstration for proton transfer from amino groups (8) and such a reaction might also lead to two emitting triplet states. Both temperature studies and the examination of isotope effects may help to distinguish the various possibilities.

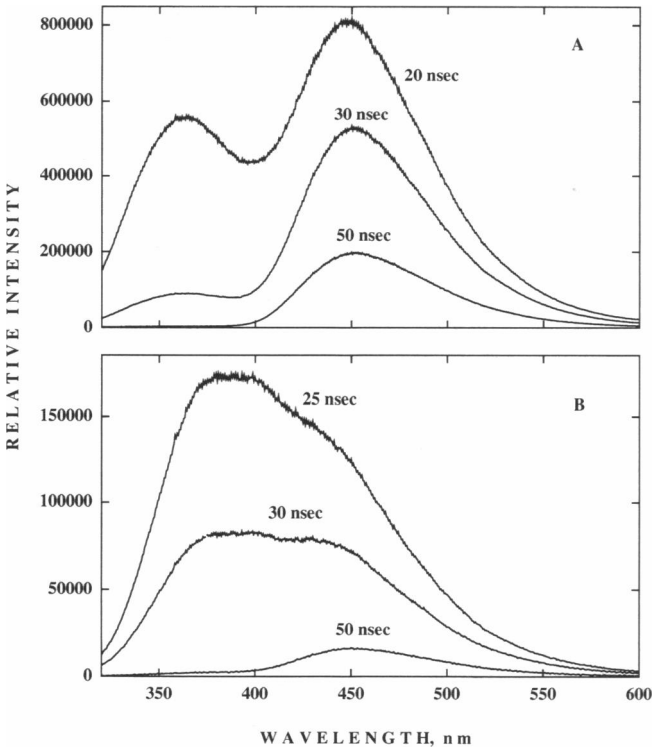

FIGURE 9 Luminescence spectra and decay of W(1), pH 5.5 (*A*) and pH 10.5 (*B*), after excitation at 282 nm. W(1) was dissolved in 0.01 M Tris, 0.3% glucose, and 0.1 M NaCl to give an absorbance of 0.28 at 282 nm. The sample also contained the oxygen removal system described in Methods. The measurement times are indicated.

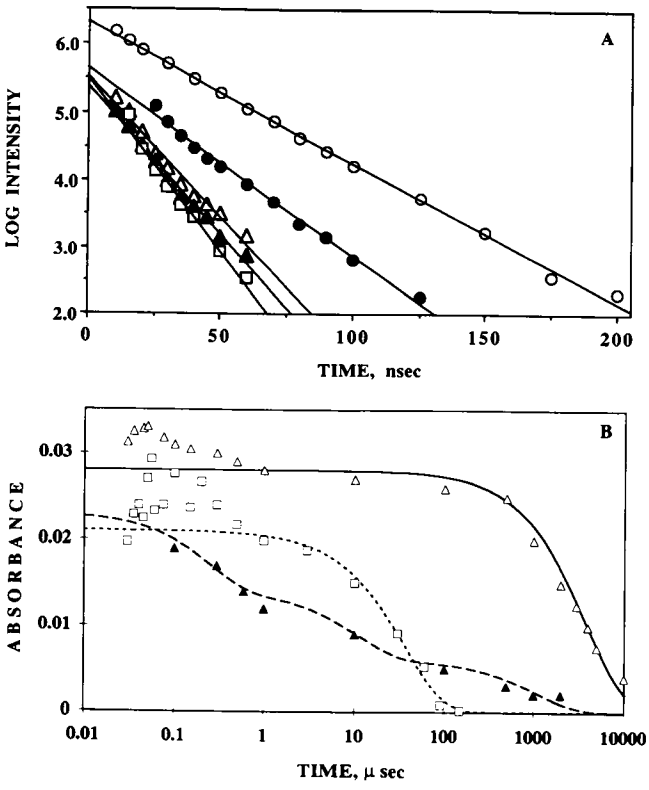

FIGURE 10 Decay kinetics of emission and absorption. (△) Ca-bound parvalbumin, 1.8 mg parvalbumin/ml. pH 7.0; (▲) Ca-depleted parvalbumin, same as for Ca-bound parvalbumin but with the addition of 5 mM EDTA. (□) NATA, pH 7.0; (○) W(1), pH 5.5; (●) W(1), pH 10.5. Buffer was 0.01 M Tris, 0.3% glucose, and 0.1 M NaCl. The concentrations of NATA and W(1) were adjusted to give ~0.2–0.3 absorbance at 282 nm. (*A*) Emission of short-lived species with the computed lifetimes of 25 (△), 23 (▲), 19 (□), 48 (○) and 36 ns (●). (*B*) Transient absorption (——) fit using an exponential 4.7 ms; (– – –) simulated decay curve with absorbance amplitudes of 0.009, 0.008, and 0.006 and lifetimes of 0.25 μs, 11 μs, and 1.1 ms, respectively; and (· · ·) fit with lifetime of 30 μs.

In conclusion, short-lived phosphorescence has been observed for trp in a protein. Second, the decay kinetics of trp in Ca-depleted protein shows nonexponential decay and the lack of observed long-lived phosphorescence is both due to lower formation of the T_2 state and increased quenching in a dynamic manner.

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