Lifetime and Quenching of Tryptophan Fluorescence in Whiting Parvalbumin†

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ABSTRACT: The fluorescence lifetime of the single tryptophan in whiting parvalbumin has been measured by time-correlated single-photon counting. In the presence of saturating calcium, >2 mol/mol of protein, the decay of fluorescence is accurately single exponential with a lifetime of 4.6 ns (0.1 M KCl, 20 mM borate, 1 mM dithiothreitol, 20 °C, pH 9). Upon complete removal of calcium from parvalbumin with ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid the emission decay becomes biphasic, and a second more rapid decay process with a lifetime of 1.3 ns comprising ~18% of the fluorescence emission at 350 nm is observed. The fluorescence emission of the calcium-saturated form is not measurably quenched by iodide. In contrast, upon complete removal of calcium, the fluorescence is completely quenchable as shown by extrapolation of the data to infinite iodide concentration. These results indicate that there is a large increase in the accessibility of the tryptophan residue in the protein to solvent upon removal of calcium. Stern–Volmer plots of the quenching data are nonlinear and indicate that there is more than one quenchable conformation of the calcium-free protein. The lifetime and quenching results are consistent with the presence of significant concentrations of only two stoichiometric species, apoparvalbumin and parvalbumin–Ca_2, at partial occupancy of the calcium binding sites.

Parvalbumins are small, soluble acidic proteins which bind two calcium ions per molecule of protein with submicromolar affinity. They occur in high concentration in fish and amphibian skeletal muscle and in somewhat lower concentrations in the muscles of higher vertebrates. The structure of carp parvalbumin with 2 mol of calcium bound per mol of protein has been determined to atomic resolution (Nockolds et al., 1972; Moews & Kretsinger, 1975). The single tryptophan at position 102 in the amino acid sequence of whiting parvalbumin provides a useful intrinsic probe for structural changes in the molecule. By analogy with the carp parvalbumin which has a phenylalanine residue at the homologous position, the tryptophan is expected to be in a very hydrophobic environment in the interior of the calcium-saturated protein. There is, however, relatively little information concerning the structural changes that must undoubtedly occur upon removal of calcium from the parvalbumins. Large changes in the absorption and fluorescence emission spectra are attendant upon calcium removal, which suggests that the tryptophan is in a much more polar environment in the calcium-free protein (Permyakov et al., 1980; Closset & Gerday, 1975; White, 1988). In the preceding paper (White, 1988) it has been shown, in contrast with earlier results (Permyakov et al., 1980), that the mechanism of calcium binding to whiting parvalbumin is highly cooperative with a Hill coefficient equal to 1.8. Permyakov et al. find that $K_I \gg K_{II}$ in eq 1, whereas White finds that $K_{II} \gg K_I$.[1]

$$PA_0 + 2Ca \overset{K_I}{\rightleftharpoons} PA\text{-}Ca_1 + Ca \overset{K_{II}}{\rightleftharpoons} PA\text{-}Ca_2 \quad (1)$$

High sensitivity and fast response time make fluorescence a method of choice for investigating the structural and dynamic properties of proteins. In the present paper we have used fluorescence lifetime and quenching measurements to obtain information about the structural changes and mechanism of calcium binding to whiting parvalbumin.

Materials and Methods

Whiting parvalbumin was prepared as previously described (Rao et al., 1969). EGTA and dithiothreitol were obtained from Sigma Chemical Co. Special grades of potassium chloride, Suprapur grade from EM laboratories, <0.1 ppm calcium, and potassium iodide, Puratronic grade from Alfa Chemical Co., 2 ppm calcium, were used to reduce contamination by calcium. All other chemicals of reagent grade were analyzed for calcium content by atomic absorption spectroscopy. Plastic labware, washed with 1 mM EGTA–10 mM sodium carbonate and thoroughly rinsed with double glass-distilled water, was used exclusively for experiments in which knowledge of caclium–protein stoichiometry was required. All solutions were made in distilled–deionized water containing less than 0.5 μM calcium. Stock calcium chloride solutions were prepared by neutralization of weighed amounts of calcium carbonate with hydrochloric acid. A molar extinction coefficient of 7400 M^{-1} cm^{-1} at 278 nm (Closset & Gerday, 1975) was used to determine the concentration of whiting parvalbumin.

To avoid complications due to the instability of the apoprotein solutions even in 1 mM DTT (White, 1988), all measurements in this study were performed by starting from PA-Ca_2, pH 9, solutions containing 1 mM DTT. To obtain protein solutions at different degree of calcium saturation, calcium was subtracted in situ by addition of known amounts of EGTA, which at pH 9 removes this ion in a stoichiometric fashion. White (1988) and Permyakov et al. (1980) have both shown that calcium binding to whiting parvalbumin is inde-

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[1] Abbreviations: NATA, *N*-acetyltryptophanamide; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PA_0, apoparvalbumin; PA-Ca_2, parvalbumin with two calcium atoms bound; PA-Ca_1, parvalbumin with one atom of calcium bound. PA-Ca_1 should not be confused with half-saturated parvalbumin which refers to the fraction of sites with calcium bound and may contain a mixture of PA_0, PA-Ca_1, and PA-Ca_2.

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Table I: Dependence of the Fluorescence Lifetime upon Calcium Binding to Whiting Parvalbumin[a]

	single exponential[b]		double exponential[c]				
	τ (ns)	RMS	a_1	τ_1 (ns)	a_2	τ_2 (ns)	RMS
PA-Ca_2	4.580	0.0037	0.11	4.574	0.42	4.583	0.0037
PA-Ca_2[d]	4.651	0.0040	0.56	4.652	0.03	4.642	0.0040
PA/Ca = 1	3.96	0.0106	0.18	1.37	0.45	4.53	0.0043
PA_0	3.40	0.0141	0.29	1.28	0.40	4.26	0.0039
NATA[e]	2.967	0.0028	0.60	2.967	0.03	2.954	0.0028

[a] Experimental conditions: 1 M KCl, 0.1 mM DTT, 20 mM borate, pH 9, 20 °C, and 0.04 mM whiting parvalbumin. PA-Ca_2, PA/Ca = 1, and PA_0 refer respectively to full calcium bound protein ($CaCl_2$, 0.1 mM), half-calcium-saturated protein (EGTA, 0.04 mM), and apoprotein (EGTA, 0.18 mM). Optical densities were <0.1 at the excitation wavelength (296 nm). Emission was measured at 350 nm. [b] Decay parameters were obtained by fitting the data to the equation $I(t) = I_0e^{-t/\tau}$. [c] Decay parameters were obtained by fitting the data to the equation $I(t) = a_1e^{-t/\tau_1} + a_2e^{-t/\tau_2}$. [d] PA_0 was converted to PA-Ca_2 by the addition of $CaCl_2$ to obtain the final free calcium concentration of 0.1 mM. [e] NATA decay parameters are shown as an example of a single-exponential fluorescence decay.

pendent of pH in the range 7–9.

Steady-state fluorescence measurements were made with a Spex Fluorolog, photon-counting fluorometer equipped with double monochromators for both excitation and emission. Quenching experiments were done with the addition of 1 M potassium iodide to parvalbumin solutions in buffer containing 1 M potassium chloride to maintain constant ionic strength. Appropriate corrections were made to the emission intensity for dilution. Relative fluorescence quantum yields were measured by the areas under the corrected emission spectra; the excitation was set at 290 nm (1.0-nm band-pass), which is an isosbestic point (White, 1988).

Fluorescence lifetime measurements were made by the time-correlated single photon counting method (Ware, 1971). The excitation source was a thyratron gated, nitrogen spark gap lamp running at 10 kHz. The 296-nm line was selected by a 0.25-m Jarrell Ash monochromator with a 1.0-nm band-pass and passed through a Lyot depolarizer (Karl Lambrecht Corp.). Emission was selected by interference filters at 325 ± 5, 350 ± 5, or 365 ± 5 nm and focused onto a 56 DUVP Philips photomultiplier with the dynodes wired for single-photon detection (Lewis et al., 1973). An RCA 1P28 wired for fast response monitored the excitation source to provide the start pulse. Standard Ortec NIM modules, Model 454 timing-filter-amplifier, Model 473A constant fraction discriminators, and Model 457 time-to-amplitude converter, and an Inotech Model 5200 multichannel analyzer were used to collected the data. The sample and reference solutions were alternated every 30 s in the excitation beam. The excitation intensity was adjusted to limit the count rate to ~100 Hz, 1% of the excitation rate. Peak counts were typically 10K–20K. For a few measurements excitation was provided by a Spectra Physics cavity-dumped mode-locked synchronously pumped Rhodamine 6G dye laser. The laser output was frequency doubled to 295 nm by a KDP crystal. The pulse width was ~10 ps and the repetition rate 1 MHz. Due to the high collection rate, ~10 kHz, data collection was made in inverted configuration (Haugen et al., 1978). Emission was passed through a polarizer set at 54.7° to the vertical to eliminate polarization artifacts.

In order to obtain the decay parameters, the impulse function due to the "source" was calculated from the emission profile of a reference compound (Wahl et al., 1974). The decay curves of the sample emission and the lamp were then analyzed by an iterative nonlinear least-squares method (Grinvald & Steinberg, 1974). Single- and double-exponential fits of each data set were compared to determine if a single-exponenential fit adequately explained the data or if double-exponential fitting was required. In this method it is essential to use the "correct" value of the reference lifetime to obtain accurate results. Deoxygenated *p*-terphenyl in cyclohexane was used with a lifetime of 1.025 ns, which was determined by two methods of internal calibration (Castelli, 1985). This procedure enabled us to obtain the decay parameter of the reference compound with a high degree of confidence so that the parvalbumin data could be reliably analyzed in terms of two exponentials.

Results

Fluorescence Lifetime Measurements. Representative results of the data analysis from the fluorescence profiles at 350 nm for the calcium-saturated, the half-calcium-saturated, and the calcium-free whiting parvalbumin are shown in Table I. It appears that the fluorescence decay of the fully bound protein (excess calcium) is accurately single exponential with a lifetime of 4.6 ns. In fact for each set of data τ_1 and τ_2, the two lifetimes of the double-exponential fit are both the same, within less than 1%, as τ_0, the lifetime of the single exponential fit. The validity of this criterion has been proven previously (Castelli, 1985). In Table I it is also shown that *N*-acetyltryptophanamide fluorescence, which is known to decay by a single exponential (Ross et al., 1981; Szabo & Rayner, 1980), fulfills this criterion. Weighted residuals between calculated and experimental curves are randomly distributed for both systems with reduced χ^2 values of 1.5 for PA-Ca_2 (Figure 1) and 1.3 for NATA. However, we have also observed that, if the calcium-saturated protein was stored on ice or frozen in solution for a long period of time (~6 months), the decay curves could not be accurately fit by single-exponential decay functions. We attribute this behavior to partial denaturation of the protein. Lyophilized protein stored at −20 °C was stable by this criterion.

Data analysis reported in Table I and Figure 1 shows that two components are required to fit the emission decay of whiting apoparvalbumin (excess EGTA). The fractions of the emission intensity associated with the two components are $a_1\tau_1/(a_1\tau_1 + a_2\tau_2)$ and $a_2\tau_2/(a_1\tau_1 + a_2\tau_2)$, respectively. The calculated values at different emission wavelengths are reported in Table II: 82% of the emission intensity at 350 nm decays with a lifetime similar to that of PA-Ca_2, 4.3 ns; the faster component with a lifetime of ~1.3 ns accounts for the remaining 18% of the emission. Upon the addition of excess calcium to the apoprotein, which had been obtained in the prescence of DTT, the single-exponential emission decay was restored. The changes in the fluorescence decay are thus attributable to the reversible binding of calcium to the protein. A two-component fit yields essentially the same lifetimes for parvalbumin at half-saturating calcium concentration as for the apoprotein, but the contribution of the faster component is reduced to 11%. The fluorescence decay parameters measured at 365 nm are similar to those at 350 nm except for the higher contributions of the fast component in the emission from

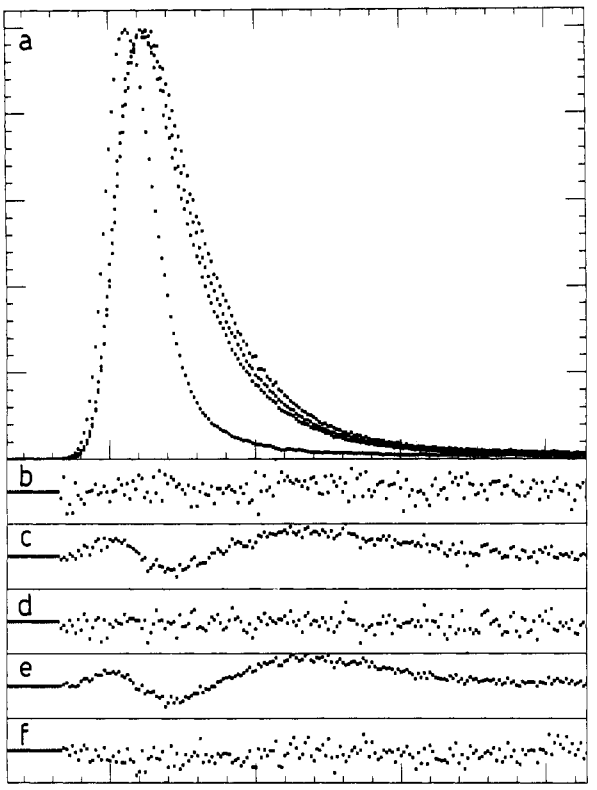

FIGURE 1: Whiting parvalbumin fluorescence decay analysis as a function of calcium saturation. Experimental conditions are reported in Table I. (a) Emission intensities (number of counts) vs time (channel number). Decay length increases in the following order: PTP (reference compound); apoparvalbumin (PA_0); half-calcium-saturated parvalbumin; full calcium saturated parvalbumin ($PA\text{-}Ca_2$). The peak count number is ~20000 for all the curves, the total number of channels is 200, and the channel width is 0.262 ns. (b–f) Weighted scaled residual plots for (b) $PA\text{-}Ca_2$ decay single-exponential fit with $\chi^2 = 1.5$; (c and e) half-calcium-saturated parvalbumin and PA_0 decay single-exponental fits with $\chi^2 = 26$ and 42, respectively; (d and f) half-calcium-saturated parvalbumin and PA_0 decay double-exponential fits with $\chi^2 = 2.0$ and 1.9, respectively. The decay parameters used to calculate the residuals are those reported in Table I. All residual plots are over the same time range of the emission intensity profiles.

Table II: Fractional Fluorescence Contribution of the Short-Lifetime Component (f_{exp}) in Apoprotein and Half-Calcium-Saturated Protein[a]

emission wavelength (nm)	$f_{exp}(PA_0)$[b]	f_{exp}(half-saturated protein)[b]	$f_{calcd}(PA\text{-}Ca_2/PA_0 = 1)$[c]
325	0.14	0.06	
350	0.18	0.11	0.10
365	0.26	0.16	0.15

[a] Experimental conditions are the same as described in Table I except for the emission wavelengths. Values are the average of at least three measurements. The values of 325 nm are affected by scattered light (see text). [b] $f_{exp} = a_1\tau_1/(a_1\tau_1 + a_2\tau_2)$. [c] Predicted values of f for an equimolar solution of $PA\text{-}Ca_2$ and PA_0: $f_{calcd} = f_{exp}(PA_0) \times I(PA_0)/[I(PA_0) + I(PA\text{-}Ca_2)]$, where $I(PA_0)$ and $I(PA\text{-}Ca_2)$ are the steady-state emission intensities at the indicated wavelength for calcium-free and calcium-saturated solutions of the same protein concentration. These quantities were measured under the same conditions as the emission decays (excitation 296 nm; emission, interference filter 350 ● 5 or 365 ± 5 nm). The values for $I(PA_0)$ and $I(PA\text{-}Ca_2)$ in arbitrary units are respectively 910 and 755 at 350 nm and 1335 and 965 at 365 nm.

the apoform and the half-calcium-saturated protein. Since the 325-nm interference filter did not totally block the exciting radiation, lifetime measurements at this emission wavelength were affected by scattered light. As a result, a very fast component ($\tau = 0.03$ ns, the low-limit value set in our fitting program) appeared in the analysis of $PA\text{-}Ca_2$ emission decay. However for the other systems, since two components were already present, the effect of the scattered light was to increase the contribution of the fast component. Analysis for three components has not been attempted. Thus the correct values for the fast-component contributions at 325 nm must be somewhat smaller than the values reported in Table II. In any case it is clear that this contribution increases with wavelength in this range.

The data reported in Tables I and II show that both the lifetime values and the emission contributions of the two components for the half-calcium-saturated protein are equal to the values expected for an equimolar mixture of PA_0 and $PA\text{-}Ca_2$.

The lifetime results suggest that any fluctuations in the microenvironment either are fast on the nanosecond time scale or do not affect the fluorescence lifetime of the tryptophan residue in calcium-saturated parvalbumin. In the apoprotein, however, an energy barrier prevents rapid interconversion between two classes of conformers whose emission spectra are red shifted with respect to that of $PA\text{-}Ca_2$. The larger contribution of the fast-decaying component of the fluorescence emission of the apoprotein at longer wavelengths indicates that the tryptophan residue is in a more polar environment in this conformation. The above interpretation about the double-exponential decay for the apoprotein is supported by the fluorescence-quenching studies described below. For a review on tryptophan emission decay, see for instance Beechem and Brand (1985).

Fluorescence Quenching by Potassium Iodide. In the presence of excess calcium the tryptophan fluorescence of freshly prepared whiting parvalbumin is not measurably quenched (<2%) by iodide at concentrations up to 1 M at any wavelength. In the presence of excess calcium significant quenching (5–20%) was only observed for parvalbumin that had been stored frozen in solution for long periods of time (>6 months). In this case the observed quenching was larger at longer wavelengths and was essentially completed at low iodide concentrations as would be expected if small amounts of quenchable impurities were present. The appearance of a quenchable component is correlated with nonexponential fluorescence emission decay, further evidence that slow denaturation or conformational changes occur in "old" protein.

Quenching of the tryptophan fluorescence emission of whiting parvalbumin is easily measured for the apoprotein. In view of the lifetime results which indicate that the emission contributions change with wavelength, quenching was measured at 325 and 365 nm. If iodide affects the parvalbumin fluorescence in the two conformations in different degrees, the quenching plots at these wavelengths must differ substantially. Stern–Volmer plots of the data are shown in Figure 2. Quenching at both wavelengths shows negative deviations from linearity, which indicates that more than one component is being quenched (Eftink & Ghiron, 1981). Furthermore, substantially more quenching is observed at 365 nm than at 325 nm. This implies that the emission contribution of the component which is more easily quenched must increase at longer wavelengths.

We have analyzed quantitatively the PA_0 quenching data in terms of two quenching constants, K_1^{sv} and K_2^{sv}, and two fractional emission contributions, f and $1 - f$, according to the Stern–Volmer analysis for two quenched species shown in eq 2. More complex equations have been derived to separate

$$F/F_0 = f/(1 + K_1^{sv}[I^-]) + (1 - f)/(1 + K_2^{sv}[I^-]) \quad (2)$$

dynamic and static terms (Eftink & Ghiron, 1981; Acuna et

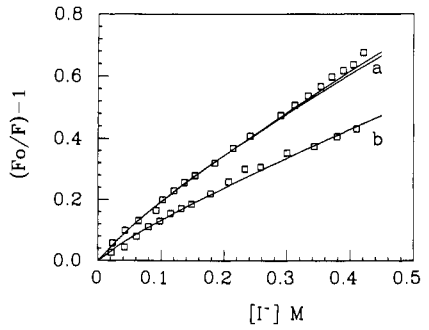

FIGURE 2: Quenching of whiting parvalbumin fluorescence by iodide. Stern–Volmer plots at (a) 365 and (b) 325 nm. The solid curves represent the best fits obtained with the values of the quenching parameters reported in Table III. At 365 nm the top curve is obtained by fitting the data with three variable parameters and the bottom curve with two variable parameters (f = constant). Initial experimental conditions were 0.02 mM apoprotein (0.1 mM excess EGTA) 1 M KCl, 0.1 mM DTT, 20 mM borate, pH 9, and 20 °C. Sequential aliquots of 1 M potassium iodide, 20 mM borate, pH 9, and 0.1 mM sodium thiosulfate solution were added to give the indicated concentrations of potassium iodide. Appropriate corrections were made to the emission intensity for dilution.

Table III: Fractional Emission Contributions and Stern–Volmer Quenching Constants in Apoprotein[a]

emission wavelength (nm)	f	K_1^{sv} (M^{-1})	$1-f$	K_2^{sv} (M^{-1})
365[b]	0.20	8.0	0.80	1.00
365[c]	0.26	6.7	0.74	0.85
325[b]	0.11	8.6	0.89	0.80

[a] Parameter values are obtained by fitting the experimental data (Figure 2) to a Stern–Volmer equation for the quenching of two emission components (see text). [b] All three parameters allowed to vary. [c] f fixed at the value obtained from the lifetime measurements.

al., 1982). However, the data obtained here are adequately explained by dynamic quenching. Moreover, it is generally accepted that iodide quenching is primarily collisional at low concentrations of quencher. The data at 365 nm were fit by a nonlinear least-squares method in which the parameters K_1^{sv}, K_2^{sv}, and f were allowed to vary. Alternatively, the number of parameters was reduced to two by using as a constant the value of f which was determined by the lifetime measurements made at the same excitation and emission wavelengths. Although a slightly better fit is obtained if all three parameters are allowed to vary, the results obtained by the two procedures are very similar (Table III). In Figure 2 calculated quenching curves for the two sets of parameters are shown together with the experimental values. Within the experimental errors both sets of parameters reproduce the experimental data. Thus there is a good quantitative agreement between lifetime and quenching results, and this supports the analysis of the data in terms of two components.

Fitting the data at 325 nm yielded similar values for the two quenching constants (see Table III), but as expected, the emission contribution of the component which is more easily quenched is much smaller. Thus, the quenching and lifetime data are mutually consistent with the existence of two conformers of PA_0. The component with the shoter lifetime is quenched more than the component with the longer lifetime. These results predict a shift toward the blue with increasing quenching. A small, but definite, blue shift of 2–3 nm in the emission from PA_0 occurs upon increasing iodide from 0 to 0.37 M (data not shown).

A plot of $F_0/(F_0 - F)$ vs $[I^-]^{-1}$ (Lehrer, 1971) indicates that all of the emission of apoparvalbumin is quenchable (Figure

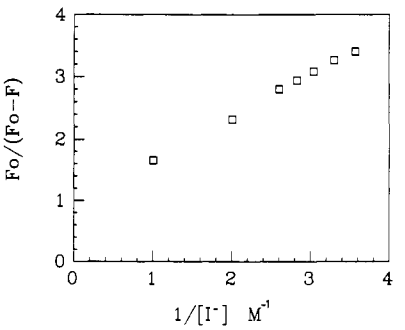

FIGURE 3: Quenching of whiting apoparvalbumin fluorescence by iodide. Lehrer plot at 365 nm. Extrapolation of the data at infinite iodide concentration indicates that all fluorescence is quenchable. In these measurements the initial concentration of potassium iodide was 1 M. The quencher concentration was then reduced by the addition of buffer containing potassium chloride, 1 M. All the other conditions were the same as in Figure 2.

3). Iodide quenching was measured also for half-calcium-saturated protein as a function of iodide concentration. However, quantitative analysis of the data was hampered by the fact that potassium iodide was calcium contaminated, and therefore the ratio of Ca/protein could not be kept constant. Corrections for the effect proved to be uncertain, and therefore we have not attempted to obtain precise values for the quenching parameters. Alternatively, fluorescence quenching may be measured at constant iodide concentration, varying the fraction of bound calcium. The spectra obtained upon titrating the calcium from $PA\text{-}Ca_2$ in 1 M potassium iodide are shown in Figure 4a. Two moles of EGTA per mole of parvalbumin in excess of that required to bind the free calcium in the potassium iodide, ~5 μM, is required to complete the spectral change. Similar results were previously observed in potassium chloride (White, 1988). However, the changes in the spectra observed upon titrating the removal of calcium from parvalbumin are larger in the presence of iodide, due to the selective quenching of PA_0. The fluorescence emission intensity at 350 and 365 nm varies linearly with respect to the fluorescence emission at 325 nm as is shown in Figure 4b. This means that the emission intensity at a given wavelength can be predicted exactly at any fractional calcium saturation from the values of the fluorescence intensities of $PA\text{-}Ca_2$ and PA_0 in absence of quencher and from the extent to which PA_0 emission is quenched. Thus it appears that at any fractional calcium saturation only two stoichiometric species, $PA\text{-}Ca_2$ and PA_0, are present in significant concentrations. Relative fluorescence quantum yields for calcium-saturated, half-calcium-saturated, and calcium-free protein were also measured. A decrease of 45% was observed from $PA\text{-}Ca_2$ to PA_0 with the yield of the half-calcium-saturated protein being intermediate. The results we have reported here about lifetime and quenching from Whiting parvalbumin are quite different from those previously reported by Permyakov et al. (1980, 1985) and Bushueva et al. (1980). The results of this group of authors may be explained with the presence of different, but always substantial, amounts of impurities (denaturated protein). In fact, in the earliest paper (Bushueva et al., 1980) quenching of $PA\text{-}Ca_2$ emission was reported linear up to the highest iodide concentration measured (0.3 M). However, in the latest paper (Permyakov et al., 1985) only ~25% of $PA\text{-}Ca_2$ fluorescence is reported to be quenchable at infinite iodide concentration. This last result is more comparable with the results we have obtained with "old" protein. In agreement with our results with old protein, Permyakov et al. (1985) have also found that two components were necessary to fit the decay of $PA\text{-}Ca_2$.

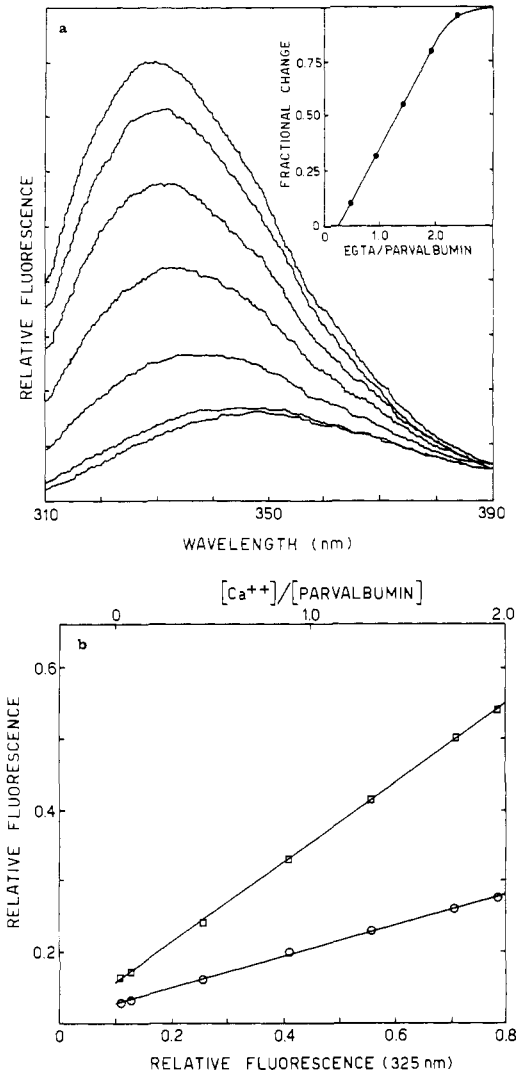

FIGURE 4: Dependence of the emission spectra of whiting parvalbumin upon calcium occupancy in 1 M potassium iodide. Experimental conditions: 1 M potassium iodide, 20 mM borate, 0.1 mM dithiothreitol, 0.02 mM whiting parvalbumin, pH 9, and 20 °C, in a total volume of 2 mL. (a) The spectra were recorded after adding sequential 10μL aliquots of 2 mM EGTA to make the final EGTA concentration, from top to bottom, 0, 10, 20, 30, 40, 50, or 60 μM. The excitation wavelength is 290 nm, and emission and excitation bandwidths are 5 nm. The fractional change of the fluorescence measured at 325 nm is shown vs the molar ratio of EGTA to parvalbumin in the inset. (b) The emission intensity at 350 (□) and 365 (○) nm is plotted vs the emission intensity at 325 nm.

DISCUSSION

Effect of Calcium Binding upon the Environment of Tryptophan-102. The fluorescence quenching and lifetime measurements are consistent with tryptophan-102 in whiting PA-Ca_2 being buried in a rigid structure that cannot be penetrated by iodide in the solvent. If multiple conformational substates exists, they must have a very similar environment with respect to tryptophan-102. Single-exponential decays of tryptophan fluorescence are uncommon even among proteins containing only a single tryptophan. Apoazurin is the only other known protein in which the decay of tryptophan fluorescence is single exponential (Szabo et al., 1983). This unusual behavior of PA-Ca_2 may be explained by structural considerations. The crystal structure of the calcium-bound carp parvalbumin shows that the two calcium binding regions consist of 29-residue domains with a characteristic helix–loop–helix conformation which has been termed EF hand [Moews and Kretsinger (1975) and references cited therein]. The two EF hands are paired by helix–helix and loop–loop interactions and are related by an approximate 2-fold axis. These conformations (EF hands and pairs of EF hands) are very stable and have been thought to be characteristic of calcium-regulated proteins [Kretsinger (1980) and references cited therein]. The crystal structures of ICaBP, a vitamin D dependent calcium binding protein from bovine intestine (Szebenyi et al., 1981), and of troponin C (Sundaralingam et al., 1985) have shown that this point of view is basically correct. In carp parvalbumin, residue 102, a phenylalanine, is part of the F helix and furthermore has a close contact with residue 97, an isoleucine, which forms the EF calcium binding loop (Moews & Kretsinger, 1975). In these conditions, residue 102 is in a well-defined, rigid structure and therefore in only one conformational state. Due to the stability of the EF-hand conformations, a similar situation must prevail even in solution for the calcium-bound form of the protein. NMR data have shown that metal-bound parvalbumins maintain in solution a highly ordered structure: in particular the structures of the carp parvalbumin EF site in solution and in crystal form are very similar (Lee & Sykes, 1983). Thus in whiting parvalbumin, PA-Ca_2, the tryptophan-102 residue must also be in a well-defined conformational state, and a single-exponential decay of the fluorescence obtains. It should be recognized that our results are also consistent with a very narrow distribution of lifetimes around a mean lifetime, which would correspond to a distribution of very similar conformational substates. Since the ionic energy of the calcium bonds is a large part of the stabilization energy of the EF hands, these conformations can be thought of as structural domains induced or, at least, stabilized by the calcium ions which determine the uniqueness or apparent uniqueness of the tryptophan conformational state and the single-exponential fluorescence decay. Upon removal of the calcium, because of the electrostatic repulsion between the carboxylate groups, conformational changes are to be expected, and the more common situation of more conformational states prevails with multi-exponential decay. Despite the complexity of the physical system, the lifetime and quenching data for the apoprotein can be explained in terms of two conformations or, more likely, two distributions of conformations. James et al. (1985) have shown that these two physical models may yield the same results. Thus we may speak of two main conformations which are characterized by different fluorescence spectra, quantum yields, lifetimes, and quenching constants. It is possible to obtain the relative amounts of the two main conformations. It is known that $1/\tau = k_r + k_{nr}$ where k_r is the radiative rate constant extended to all the radiative processes which originate the entire emission band and k_{nr} is the unimolecular rate constant for all the nonradiative processes. A specific radiative rate constant k_r^λ would then refer to the radiative process which originates the emission at a specific wavelength. The preexponential terms a_1 and a_2 found in the analysis of the emission decays are proportional to the products of the specific radiative rate constants at the wavelength of measure times the molar fractions of the two components, provided that the molar absorption coefficients are the same. Thus, for species which have the same emission spectra and the same radiative rate constants, the ratios between the preexponential terms, measured at any wavelength, yield directly the relative molar contributions. In the case of apoparvalbumin the emission spectra of the two conformers are different, and the ratio a_1/a_2 is a function of the emission wavelength. However, considering that the tryptophan residue is in both conformations exposed

to solvent and that the emission spectra differ only by a small shift, we may assume that the radiative rate constants are the same; since the maximum region does not present sharp peaks and it is fairly wide, the ratio between the specific radiative rate constants at 350 nm, which is in the maximum region (see Figure 4a), can be considered to be equal to the ratio between the radiative rate constants. Then the ratio $a_1/a_2 = 0.73$, as determined by the decay analysis at 350 nm, yields the relative molar contribution of the fast component with respect to the slow component. Since the tryptophan radiative rate constant is expected to decrease with increasing solvent exposition (Privat et al., 1979), the relative molar contribution of the conformer with the shorter fluorescence lifetime and the more solvent-exposed tryptophan could be slightly higher than the above value.

It is of some importance to consider the relationship between the changes in lifetime and quantum yield upon calcium removal. From the above considerations it should be clear that the weighted lifetime, defined as $\bar{\tau} = (a_1\tau_1 + a_2\tau_2)/(a_1 + a_2)$, is really a weighted average in terms of molar contributions only when the emission spectra and the radiative rate constants of the two components are the same. For PA_0 the weighted lifetime also is a function of the emission wavelength; however, by arguments similar to the ones used before to estimate the molar fractional contributions, the values determined at 350 nm can be compared with the quantum yields. Contrary to the results of Permyakov et al. (1985), who reported a mean lifetime increase in contrast with a quantum yield decrease, we find that upon removal of the two calcium ions the weighted fluorescence lifetime decreases by 35% and the quantum yield decreases by 45%. This small difference can be easily explained in terms of a decrease in the radiative rate constant. In fact, the quantum yield Φ is equal to k_r/τ, and in this case, the spectral shift is large (20 nm), and the radiative rate constant of the tryptophan residue is expected to decrease significantly in a polar environment (both conformations of PA_0) with respect to a nonpolar environment ($PA\text{-}Ca_2$) (Privat et al., 1979).

In particular, it appears that relative to the slow-decaying component the behavior of tryptophan emission in whiting parvalbumin upon calcium removal is quite similar to that of indole emission upon transfer from a nonpolar to a polar environment. The increase in the nonradiative rate constant is somewhat compensated by a decrease in the radiative rate constant and the lifetime remains nearly constant (Privat et al., 1979). Thus, it would seem that in this component the lifetime is mainly determined by the polarity of the environment rather than by quenching from specific residues. The bimolecular iodide quenching rate constant $k_q = K^{sv}/\tau$ for the slow component of apoparvalbumin, 2×10^8 M^{-1} s^{-1}, is rather small and indicates that the access of iodide to the tryptophan is restricted.

The shorter lifetime and the more red shifted emission spectrum of the fast component indicate larger structural changes. The tryptophan is more exposed to external quenching in this conformation and is also quenched by neighboring residues. The iodide quenching rate constant, $(4\text{–}5) \times 10^9$ M^{-1} s^{-1}, is very similar to the value measured for the fluorescence quenching of *N*-acetyltryptophanamide (Lehrer, 1971) and indicates that the tryptophan, in this conformation of the protein, is quenched as if it were completely exposed to solvent. These results are consistent with a model of two conformations (or major classes of conformations) for the calcium-free protein: one in which some organized structure remains, particularly around the tryptophan, and a second in which there is unrestricted penetration by solvent and iodide. However, also in the former case large changes must occur with respect to the core of the native conformation since the tryptophan is now largely exposed to solvent and can be reached by the large iodide ions. Williams et al. (1986) have also shown by ^1H NMR and CD data that the apo form of rat α-parvalbumin exists in solution in only two conformations. In this case, the predominant conformation at 25 °C is highly ordered and very similar to the conformation of the full calcium bound protein, while the other conformation is essentially unfolded.

Mechanism of Calcium Binding. Unequivocal evidence for a mechanism of positive cooperativity for calcium binding to whiting parvalbumin is shown in the preceding paper (White, 1988). Most significantly, equilibrium data at calcium concentrations lower than the dissociation constants yield a linear Hill plot and a Hill coefficient equal 1.8. The value of K_I, calculated from the on and off rate constants, is much smaller than the square root of the product K_I K_{II} obtained from equilibrium data.

In the present paper, we have verified that also for lifetime and quenching measurements the results are consistent with the mechanism of positive cooperativity. Actually, this mechanism most simply explains all our results. In fact, all data at intermediate degrees of calcium saturation are consistent with the presence of significant concentrations of only two stoichiometric species, PA_0 and $PA\text{-}Ca_2$, as is predicted if K_{II} is much larger than K_I; i.e., calcium binding is cooperative. However, the interpretation of the results, in this case, is not unequivocal. In fact, the data, which show the existence of only two different species (see lifetime results), are also consistent with a mechanism of independent binding under the following restrictive conditions: (1) The calcium binding constants of the two sites, which are not structurally equivalent, are the same. (2) The observed changes in the tryptophan fluorescence properties with respect to PA_0 are exclusively due to the filling of one specific site. Under these conditions, the two species with one calcium bound will be present in equal concentrations at any degree of calcium saturation, one species behaving as PA_0 and the other one as $PA\text{-}Ca_2$.

On the other hand, a sequentially ordered mechanism, as proposed by Permyakov et al. (1980), seems unrealistic since it is very unlikely that the putative single species, $PA\text{-}Ca_1$, with only one specific site filled, would exhibit at different wavelengths fluorescence decays with the same lifetime values observed for PA_0 and $PA\text{-}Ca_2$ with the emission contributions being exactly those expected for an equimolar mixture of these two species. Analogous arguments can be made for the quenching measurements.

On the basis of the results shown in the present and the preceding paper (White, 1988), we conclude that the mechanism of calcium binding to whiting parvalbumin is cooperative. The difference between our results and those of Permyakov et al. must be due to different preparation and handling of the protein.

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Cross-Linked Amino Acids in the Protein Pair S13-S19 and Sequence Analysis of Protein S13 of *Bacillus stearothermophilus* Ribosomes

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ABSTRACT: The 30S ribosomal subunits from *Bacillus stearothermophilus* were cross-linked under native conditions with the bifunctional reagent diepoxybutane. The dominant protein-protein cross-link in the 30S ribosomal subunit between proteins S13 and S19 [Brockmöller, J., & Kamp, R. M. (1986) *Biol. Chem. Hoppe-Seyler 367*, 925-935] was isolated on a preparative scale. The presence of a single cross-link site between cysteine-83 of protein S13 and histidine-68 of protein S19 was established by microsequence analysis of isolated cross-linked peptides. This cross-link site was further confirmed by different analytical methods including fast atom bombardment mass spectrometry of the cross-linked peptide. The cross-linking site is located in the highly conserved C-terminal regions of proteins S13 and S19. In addition, the complete amino acid sequence of protein S13 from *B. stearothermophilus* is determined. Sequence comparison with the homologus *Escherichia coli* protein S13 revealed 58% identical amino acid residues.

Protein cross-linking has found wide application in the study of the protein topography of prokaryotic and eukaryotic ribosomes. The results from this method combined with those from other approaches have already given valuable information about the topography of ribosomes (Hardesty et al., 1986). For construction of more detailed structural models of the ribosomal particles, further cross-linking studies are necessary for the exact determination of the cross-linking sites at the amino acid level. At this time in the cross-linked *Escherichia coli* ribosomal protein pair S5-S8 (Allen et al., 1979) and within a cross-linked pair of the proteins L7/L12 (Massen et al., 1981) have the two cross-linked amino acids been identified.

In recent years considerable progress in crystallizing ribosomes, ribosomal subunits, and ribosomal proteins has been achieved. Such crystals are mainly obtained from *Bacillus stearothermophilus* and from halobacterial ribosomes (Yonath et al., 1986). These studies are complemented by direct amino acid sequence analysis of ribosomal proteins from these organisms. Since the amino acid sequences of all ribosomal

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