Dynamics of Parvalbumin Studied by Fluorescence Emission and Triplet Absorption Spectroscopy of Tryptophan†

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ABSTRACT: Fluorescence emission and triplet−triplet absorbance spectroscopy of the single tryptophan in cod parvalbumin were used to study the stability and dynamics of the protein as influenced by Ca^{2+} binding and interaction with a chaotropic agent. The concentrations for half-saturation for Ca binding were 3.6×10^{-9}, 3.3×10^{-4}, 7.1×10^{-3}, and 0.14 M in the presence of 0, 2, 3, and 4 M guanidine hydrochloride, respectively. As predicted for thermodynamic reversibility, the guanidine hydrochloride unfolding reaction depends upon Ca^{2+}, and the ΔG are as follows: 22.9, 29.3, 35.2, and 44.2 kJ/mol for no added Ca^{2+}, 1, 2, and 5 mM Ca^{2+}, respectively. The stability toward denaturation imparted by the binding of two Ca^{2+} is about −60 kJ/mol. For Ca^{2+}-bound parvalbumin in the presence of excess Ca^{2+}, the decay of the triplet state tryptophan is approximately exponential, and the lifetime decreases from 6.5 to 3.8 ms as the temperature increases from 10 to 40 °C. In contrast, the triplet decay of the calcium-free protein is nonexponential over the time range of microseconds to milliseconds, a result that may indicate that the Ca-free protein is molten-globule-like. At Ca^{2+} concentrations where the protein is partially saturated with Ca^{2+}, the lifetime of the longest decay component is less than that for the Ca-saturated protein; this finding suggests an exchange of Ca^{2+} and a conformational change during the triplet lifetime. From these data, a rate constant for the process that includes calcium-related protein conformational change can be surmised to range between 200 and 500 s^{-1}.

It is apparent from a number of experiments that the determinants of protein folding and stability can vary. Anfinsen, in his classical work on lysozyme, showed that the primary sequence of a protein can determine its secondary and tertiary structure (Anfinsen, 1973). For other proteins a pro form may aid in protein folding, which is followed by enzymatic cleavage to the active form (Bryan et al., 1992). But for many, the stability of the protein dramatically depends upon the presence of ligands or cofactors, and for some proteins these cofactors are required for folding (Jaenicke, 1987). In addition to thermodynamic considerations, cofactors play a role in the kinetics of protein folding. Protein folding occurs on a very wide range of time scales (Kuwajima, 1989; Doms et al., 1993; Matthews, 1993). For some proteins, folding occurs as the protein is synthesized on the ribosome (Helenius, 1993), whereas other folding processes can extend to hours (Schmid, 1993).

The effect of binding of the ligand calcium to the small globular protein parvalbumin (MW 12 000) is studied in this paper with respect to its stability and dynamics. Of the several types of parvalbumin, isoenzyme III from cod has been the most studied by spectroscopy because it contains a single Trp moiety. Ca^{2+} removal from parvalbumin type III by EDTA[1] induces large changes in the protein, as indicated by the Trp fluorescence maximum shifting from 324 to ~352 nm, the broadening of the emission spectrum, and a decrease in the intensity (Permyakov et al., 1980; White, 1988; Eftink & Wasylewski, 1989; Sudhakar et al., 1993a). Besides fluorescence, Trp also exhibits phosphorescence at room temperature, and the triplet lifetime for Trp in the Ca^{2+}-bound protein is about 5 ms at 20 °C (Vanderkooi et al., 1987). This time is comparable to the times for many kinds of protein conformational changes that often are not amenable to more direct methods such as rapid mixing experiments.

Several features of parvalbumin stability and dynamics will be examined using measurements of the excited singlet and triplet states of Trp. The stability imparted to the protein by the addition of Ca^{2+} will be determined by monitoring how Ca^{2+} protects against denaturation by guanidine hydrochloride (GdnHCl). The decay of the excited triplet state molecule will also be examined under conditions where there could be exchange between bound and free Ca^{2+}. The decay parameters will allow us to estimate the rates that characterize the reorganization of the protein when Ca^{2+} is released.

MATERIALS AND METHODS

Ethylenediaminetetraacetic acid (EDTA), *N*-acetyl-*l*-tryptophanamide (NATA), and GdnHCl were obtained from Sigma Chemical Co. (St. Louis, MO). All chemicals were

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; GdnHCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; NATA, *N*-acetyl-*l*-tryptophanamide.

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of a chemically pure grade. Parvalbumin was prepared from frozen cod (*Gadus callarius L*) fillets using a procedure (Sudhakar et al., 1993a) that is based upon other work (Haiech et al., 1979; Horrocks & Collier, 1981). The protein was dissolved in 10 mM tris(hydroxymethyl)aminomethane (Tris) and 0.1 M NaCl (pH 7.0). Added EDTA or $CaCl_2$ concentrations are indicated in the figure legends.

Instrumentation. Steady state fluorescence spectra were recorded with a Perkin-Elmer LS-5 luminescence spectrometer or a Perkin-Elmer 650 10S spectrometer.

The excited triplet state was monitored by its transient absorption spectrum acquired with a YAG laser, using the instrument as described in detail previously (Papp et al., 1990). Previous work has established that the decay of phosphorescence and the transient absorption of Trp in parvalbumin coincided, indicating that both are valid measures of the triplet state (Sudhakar et al., 1993a). The gate duration for data accumulation was 5 ns. A thermostated cell compartment was used to maintain the sample at 20 °C or the indicated temperature. The triplet decay was fit to an exponential:

$$I(t) = A_0 \exp(-t/\tau) \quad (1)$$

where A_0 is the initial intensity and τ is the lifetime. For many decay processes in proteins, a stretched exponential function is required to fit the data. This function has the following form (Williams & Watts, 1969):

$$I(t) = A_0 \exp(-t/\tau)^{\beta} \quad (2)$$

where β is an empirically derived value.

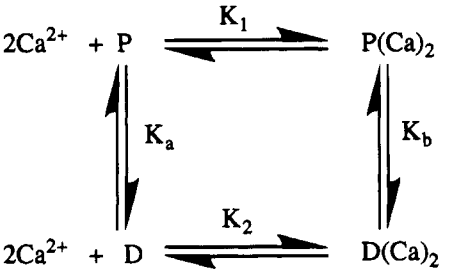
Oxygen Removal from Samples. For triplet state measurements, oxygen was removed from the sample. The buffer solution containing 0.3% glucose was initially degassed under an aspirator and then bubbled with argon. The protein was dissolved in the buffer and placed in a cuvette containing a glass-coated micro stir bar, and the air space was filled with argon. A small volume of solution containing glucose oxidase and catalase was then added to give final concentrations of 80 and 16 nM, respectively. The enzyme system reduces O_2 to H_2O_2 and then to H_2O. Throughout these operations, air was excluded by a constant flow of argon gas over the cuvette (Calhoun et al., 1988). The cuvette was closed with a glass stopper.

Ca^{2+} Binding and Denaturation Profiles. Values of f_N and f_D, the respective fractions of the protein present in the native and denatured states, were estimated from the fluorescence emission in two ways. In one, the intensity of fluorescence at 324 nm was used. The other involved analysis of the entire spectrum at different concentrations of GdnHCl. Each spectrum, $S(\lambda)$ was treated as a linear combination of two components. In the case of denaturation, the first, $S_N(\lambda)$ was an emission spectrum of parvalbumin without GdnHCl (the fully N state), while the second, $S_D(\lambda)$, was taken at high [GdnHCl], the fully denatured state. Assuming that

$$S(\lambda) = f_N S_N(\lambda) + f_D S_D(\lambda) \quad (3)$$

the spectral data were analyzed using the spreadsheet Microsoft Excel to find the best values for f_N and f_D. This procedure, which takes all wavelengths into account, minimizes scattering artifacts (Owen, 1992). The same procedure was used for the analysis of Ca^{2+} binding. The free Ca^{2+} was calculated using an equilibrium constant of 4.1×10^{10} M^{-1} for the Ca−EDTA complex at pH 7.0 (Martell & Smith, 1974).

Scheme 1



ANALYSIS

Addition of ions to proteins can stabilize the structure (Pace & Grimsley, 1988; Sugawara et al., 1991). In the description to follow, calcium binding will be treated as a single reaction with no intermediates, characterized by an equilibrium constant, K_1. The denaturant (GdnHCl) will interact with the two native conformations (form P, which is calcium-free, and $P(Ca)_2$, which is the calcium-bound state). It is assumed that only native and denatured forms exist. This assumption is based upon observations of globular proteins (Saito & Wado, 1983; Pace, 1986; Santoro & Bolen, 1988). The interactions of GdnHCl were also treated as equilibrium reactions, with the two equilibrium constants denoted as K_a and K_b. The stabilizing influence of calcium against denaturation will be seen in this analysis as a difference in the magnitudes of K_a and K_b. Finally, the binding of calcium to the denatured form D, producing form DCa, will be characterized by the equilibrium constant K_2.

The binding can be expressed in terms of a thermodynamic cycle, an approach also used to evaluate the stability of protein to denaturant as a function of pH (Bolen & Santoro, 1988) or the presence of inhibitor (Hirono & Kollman, 1990). The reaction cycle for Ca^{2+} binding is summarized in Scheme 1. Species $P(Ca)_2$ shows a spectral maximum at 324 nm. For this analysis we take all three other states to be relatively quenched forms with spectra that peak in the vicinity of 352 nm and that cannot readily be distinguished from each other. Experimental titrations as a function of [Ca^{2+}] or [GdnHCl] were analyzed by calculating the ratio of the fraction of the sample in the NCa state to the fraction in the three other states.

$$\frac{f_{324}}{1 - f_{324}} + \frac{[P(Ca)_2]}{[P] + [D] + [D(Ca)_2]} = \frac{K_1[Ca^{2+}]^2}{1 + K_a + K_b K_1 [Ca^{2+}]^2} \quad (4)$$

GdnHCl Titrations. Addition of GdnHCl to the protein affects the equilibrium system of Scheme 1 by increasing the equilibrium constants K_a and K_b. Following Pace (1986), we assume that the protein has a number of identical noninteracting sites for the binding of denaturant. By applying this to the denaturation reactions of both forms P and $P(Ca)_2$, the free energies of denaturation depend on the concentration

of denaturant according to (Pace, 1986)

$$\Delta G_a = \Delta G_a^B - \Delta n_a RT \ln(1 + K_G[\text{GdnHCl}]) \quad (5a)$$

$$\Delta G_b = \Delta G_b^B - \Delta n_b RT \ln(1 + K_G[\text{GdnHCl}]) \quad (5b)$$

In these equations, Δn_a and Δn_b refer to the increase in the number of denaturant sites between the native form (P or $P(Ca)_2$) and the respective denatured form (D or $D(Ca)_2$). The equilibrium constant K_G describes the binding of GdnHCl at each site. In the buffer-only limit of zero denaturant, the free energy takes on the limiting value ΔG_a^B or ΔG_b^B.

The equilibrium constants K_a and K_b are functions of denaturant concentration. Their values increase as increasing [GdnHCl] stabilizes the respective D and DCa_2 forms:

$$K_a([\text{GdnHCl}]) = e^{-\Delta G_a} = K_a(0)(1 + K_G[\text{GdnHCl}])^{\Delta n_a} \quad (6a)$$

$$K_b([\text{GdnHCl}]) = e^{-\Delta G_b} = K_b(0)(1 + K_G[\text{GdnHCl}])^{\Delta n_b} \quad (6b)$$

If one assumes that the number of denaturant sites revealed by GdnHCl binding is essentially the same in the calcium-free and calcium-bound states, then the addition of denaturant increases K_a and K_b by the same factor. We denote this factor as X for simplicity. The fraction of the sample, f_{324}, that gives rise to the 324 nm signal and the remaining sample ($1 - f_{324}$) are functions of X due to their dependence on K_a and K_b, so that eq 4 becomes

$$\frac{f_{324}(X)}{1 - f_{324}(X)} = \frac{K_1[Ca^{2+}]^2}{1 + XK_a(0) + XK_b(0)K_1[Ca^{2+}]^2} \quad (7)$$

where

$$X = (1 + K_G[\text{GdnHCl}])^{\Delta n} \quad (8)$$

In the region of experimental interest where significant denaturation has taken place, $XK_a(0) \gg 1$ and the ratio in eq 7 is simply proportional to X^{-1}. For the purposes of comparison with experiment, it can be written in logarithmic form:

$$\log\left\{\frac{f_{324}(X)}{1 - f_{324}(X)}\right\} = \log\left\{\frac{K_1[Ca^{2+}]}{K_a + K_bK_1[Ca^{2+}]}\right\} - \Delta n \log(1 + K_G[\text{GdnHCl}]) \quad (9)$$

As a result of the assumption that Δn is independent of Ca, the only calcium dependence in eq 7 is in the additive term. This predicts that plots of $f_{324}/(1 - f_{324})$ on a log scale vs [GdnHCl] will have a *shape* that is independent of $[Ca^{2+}]$, and *curves measured under conditions of different calcium concentrations will simply be shifted relative to each other.* If one makes the additional assumption that the equilibrium constant for GdnHCl binding is small ($K_G[\text{GdnHCl}] \ll 1$ for all attainable concentrations), then eq 7 becomes

$$\log\left\{\frac{f_{324}(X)}{1 - f_{324}(X)}\right\} = \log\left\{\frac{K_1[Ca^{2+}]^2}{K_a + K_bK_1[Ca^{2+}]^2}\right\} - K_G\Delta n[\text{GdnHCl}] \quad (10)$$

This equation predicts a series of parallel straight lines shifted by amounts that depend on $[Ca^{2+}]$.

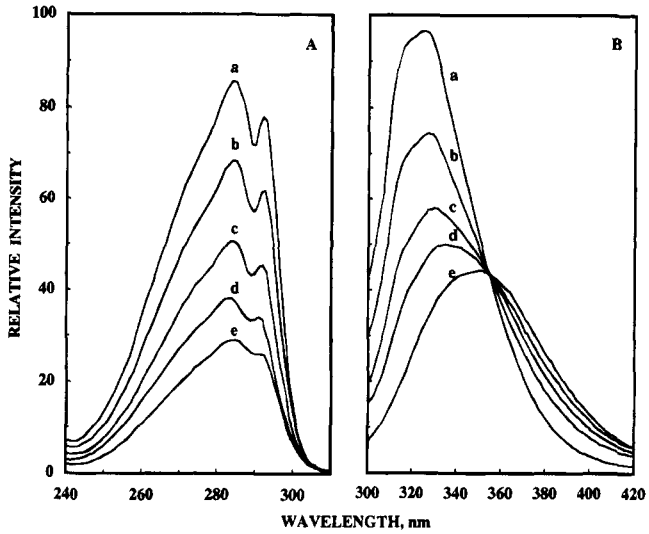


FIGURE 1: Fluorescence excitation (A) and emission spectra (B) of parvalbumin. The emission wavelength was 324 nm (A) and the excitation wavelength was 280 nm (B). Band pass was 2 nm. The sample contained 1 mg of parvalbumin/mL of 0.01 M Tris and 0.1 M NaCl at pH 7.0, and added EDTA concentrations are 0 mM (a), 0.2 mM (b), 0.4 mM (c), 0.8 mM (d), and 4 mM (e). Temperature: 20 °C.

RESULTS

Fluorescence Properties of Parvalbumin. The fluorescence excitation and emission spectra of parvalbumin in the presence of varying amounts of EDTA are shown in Figure 1A and 1B, respectively. The excitation spectrum shows vibrational resolution as calcium is removed (Figure 1A). The emission spectrum at saturating Ca levels shows some vibrational resolution, in that a shoulder can be seen at ~320 nm (Figure 1B, a). This type of emission is characteristic of indole in a largely hydrophobic environment (Konev, 1967). As noted earlier, upon the removal of Ca^{2+} there is a shift in the emission maximum from 324 to 352 nm and a decrease in the intensity of emission (Figure 1B, b–e).

The emission spectra were analyzed for the relative contributions of the Ca-bound and Ca-free forms. Within experimental error, the fluorescence emission spectrum of parvalbumin at intermediate Ca^{2+} concentrations could be described by a sum of contributions from the spectra for the fully saturated species and the Ca-free species, and when dilution factors were taken into account, an isoemissive point was observed. An example of the analysis of the spectra is shown in Figure 2. The observation that the emission spectrum has only two components shows that there is no significant amount of the singly Ca-bound species (or that the singly bound species has exactly the same spectral properties as either the unbound or fully saturated proteins).

Effect of Denaturant. The addition of GdnHCl to Ca–parvalbumin results in a shift in the fluorescence emission

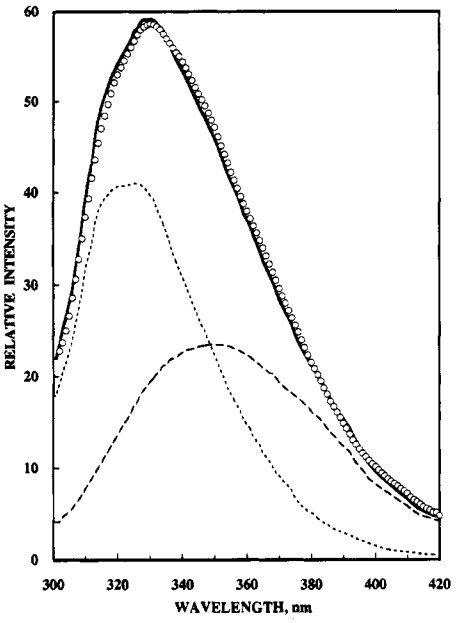


FIGURE 2: Resolution of the emission spectrum into two components: emission spectrum of parvalbumin in presence of 0.4 mM EDTA (O). Conditions are given in the legend to Figure 1. The solid line is the fit simulated by the two components, the Ca-bound form (---) and the Ca-free form (— —), using arbitrary values for f_N and f_D (eq 3).

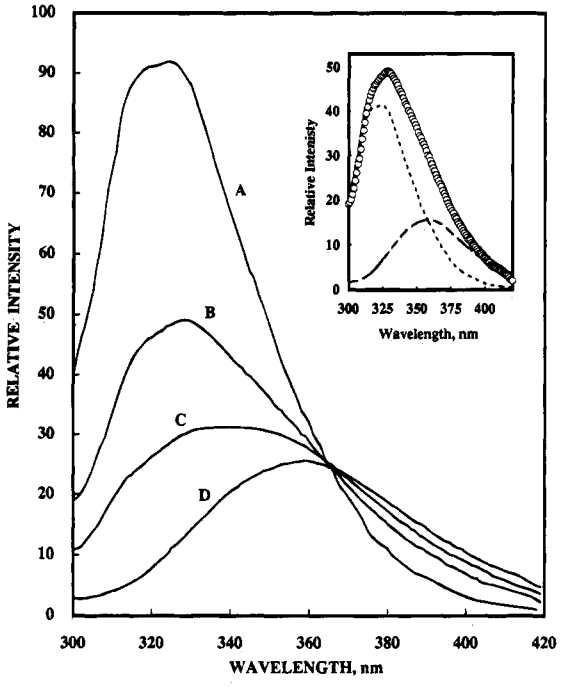


FIGURE 3: Fluorescence emission spectra as a function of GdnHCl. The excitation wavelength was 280 nm. The sample contained 1 mg/mL protein in 0.01 M Tris and 0.1 M NaCl at pH 7.0 and 0 (A), 2.4 (B), 2.7 (C), and 3.9 M (D) GdnHCl. Temperature: 20 °C. Inset: Fit of the emission spectrum (O) with two components, viz., the native (---) and denatured (— —) forms. The solid line is the fit simulated by the two components.

maximum from 324 to ~358 nm (Figure 3). As for the case of the shift with Ca^{2+} (Figure 2), the spectrum at intermediate values of GdnHCl showed a satisfactory fit to sums of spectra for the native and denatured forms (Figure 3, inset), confirming the assumption that the protein exists predominantly in only the native and unfolded forms. The effect of GdnHCl on the spectrum is reversible. This was tested by

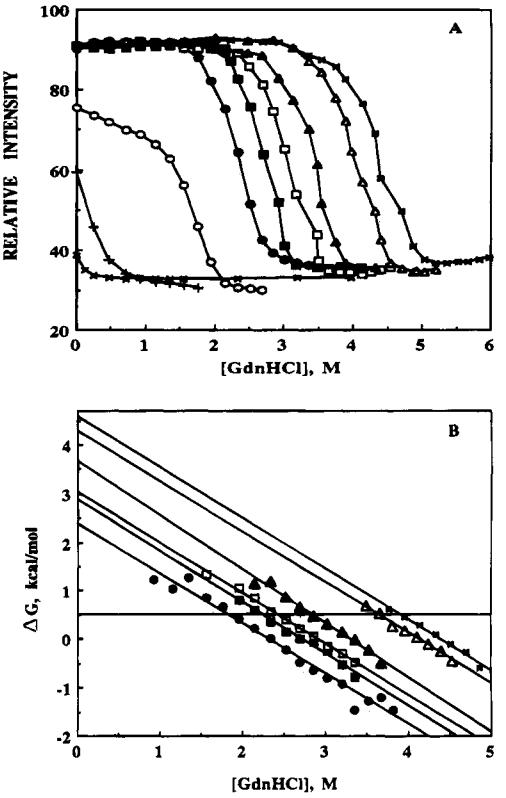


FIGURE 4: Denaturation profile of parvalbumin by GdnHCl at 20 °C. (A) Fluorescence intensity at 324 nm, using 280 nm as the excitation, as a function of denaturant at different concentrations of added Ca^{2+} or EDTA: native protein (●); 0.5 mM (■), 1 mM (□), 2 mM (▲), 3 mM (△), 5 mM (×) $CaCl_2$; 0.1 mM (○), 0.2 mM (+), and 3 mM (x) EDTA. (B) ΔG as a function of GdnHCl. The solid line is the least-squares curve fit.

diluting the high concentration of GdnHCl in the sample to a concentration where the GdnHCl had no effect on the protein: the spectrum reversed and it was identical with that of the native form.

The effect of GdnHCl on parvalbumin was examined as a function of $[Ca^{2+}]$. For Ca^{2+} concentrations ranging from 0.5 to 5 mM, the fluorescence spectrum in the absence of GdnHCl indicated that all protein initially had bound Ca (Figure 2); nevertheless, the higher Ca^{2+} concentrations protected against denaturation (Figure 4A). In the presence of 0.1 and 0.2 mM EDTA, where the fluorescence emission spectra indicate that 80% and 50% of the protein had bound Ca^{2+}, respectively, the addition of GdnHCl resulted in a shift in the emission maximum and a decrease in intensity, with the half-effects being seen at 1.7 and 0.3 M GdnHCl, respectively. The fluorescence intensity of the calcium-free protein (i.e., in the presence of 3 mM EDTA) is greatly reduced from that of the Ca-bound species, and further addition of GdnHCl decreases the fluorescence intensity by only about 20%, suggesting that the tryptophan in the Ca-free protein is already partially exposed to solvent.

Values for f_{324} and $(1 - f_{324})$ were calculated for each experimental [GdnHCl] and $[Ca^{2+}]$; ΔG was then calculated according to eq 5 and plotted in Figure 4B. The plots are approximately parallel, and the parallel lines are in agreement with eq 10. The values for the slopes, the midconcentration of GdnHCl, and the ΔG values are given in Table 1. These values are compared with the ΔG values of parvalbumin isotype II (Sudhakar et al., 1993b), which has a similar structure but has Phe at position 102 instead of Trp, and are

Table 1: ΔG Values for Parvalbumin Types II and III as a Function of $[Ca^{2+}]$

$[Ca^{2+}]$ (mM)	ΔG (kJ/mol)	[GdnHCl] (M) at zero G value	reference
none	22.9	2.30	*a*
0.5	27.6	2.65	*a*
1	29.3	2.85	*a*
2	35.2	3.35	*a*
3	41.2	4.10	*a*
5	44.2	4.35	*a*
none	23.7	2.65	*b*
2	36.1	3.90	*b*

[a] Data from this paper for parvalbumin type III. Conditions are as given for Figure 4. [b] Data for parvalbumin type II from Sudhakar et al. (1993b).

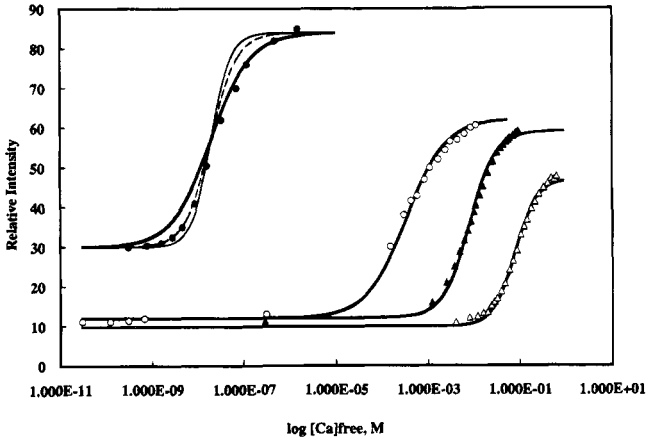


FIGURE 5: Ca^{2+} titration in the presence of no added GdnHCl (●), 2 M GdnHCl (○), 3 M GdnHCl (▲), and 4 M GdnHCl (△). The sample contained 1 mg/mL protein in 0.01 M Tris and 0.1 M NaCl at pH 7.0. The buffer also contained Ca and EDTA in different ratios, and free $[Ca^{2+}]$ was calculated with the help of the equilibrium constant for the Ca−EDTA complex. Temperature: 20 °C. Excitation wavelength was 280 nm; emission was at 324 nm. Solid lines are simulated binding curves with Ca at half-binding of 3.6×10^{-9}, 3.3×10^{-4}, 7.1×10^{-3}, and 0.14 M, respectively, assuming a simple binding isotherm without cooperativity. For Ca^{2+} titration of the protein with no addition of GdnHCl (●), the simulated curves show binding isotherms with $n = 2$ (thick solid line), $n = 1.5$ (dashed line), and $n = 1.2$ (thin solid line), respectively.

also given in Table 1. ΔG values for the two proteins are very close at corresponding Ca^{2+} concentrations.

Effect of Ca^{2+}. Thermodynamic considerations (Scheme 1) show that if Ca^{2+} decreases the effectiveness of denaturation by GdnHCl, then the converse is true: GdnHCl will decrease the binding affinity for Ca^{2+}. The experimental test for this is shown in Figure 5, where it can also be seen that the Ca^{2+} binding curves are parallel for the different concentrations of GdnHCl. The data did not fit perfectly to $n = 1$, and a higher power factor generally gave somewhat better results. The concentrations for half-saturation for Ca were 3.6×10^{-9}, 3.3×10^{-4}, 7.1×10^{-3}, and 0.14 M in the presence of 0, 2, 3, and 4 M GdnHCl, respectively.

Triplet Decay Kinetics as a Function of Calcium Binding. In the experiments described earlier, fluorescence was used to monitor Ca binding. A longer time scale is afforded by monitoring the excited triplet state of tryptophan.

The transient triplet absorption spectrum of the excited triplet state of tryptophan in Ca-bound parvalbumin in oxygen-free buffer at a 100 ns delay time after excitation at

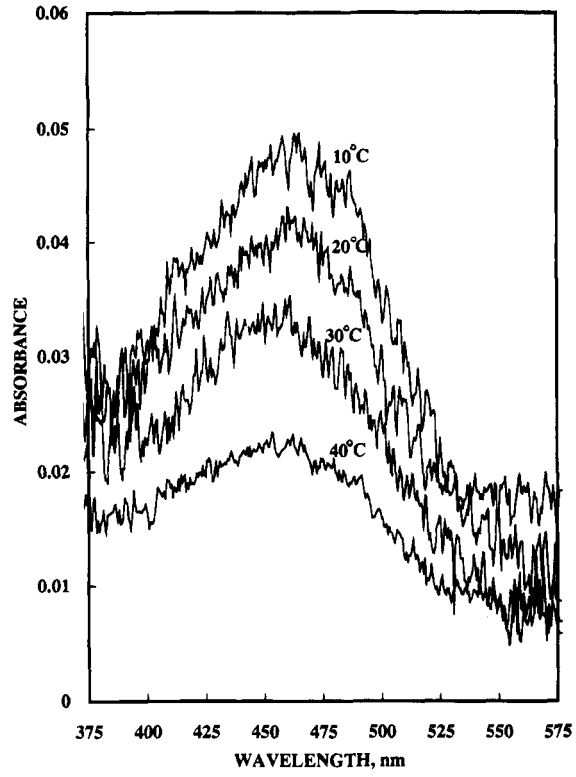


FIGURE 6: Triplet absorption spectra of parvalbumin at temperatures indicated in the figure after excitation at 282 nm. The delay time was 100 ns and the sampling time was 5 ns. The sample was prepared by dissolving 2 mg/mL protein into 0.01 M Tris, 0.3% glucose, and 0.1 M NaCl at pH 7.0. Deoxygenation was achieved using the enzyme system and other procedures as described in Materials and Methods.

282 nm is shown at temperatures ranging from 10 to 40 °C in Figure 6. An increase in temperature causes a decrease in the optical density measured 100 ns after excitation, without a change in the spectrum (within the noise and resolution levels). The absorption maxima were not detectably different for the Ca-bound and Ca-depleted proteins, as reported previously (Sudhakar et al., 1993a).

The decay kinetics of the Trp triplet in parvalbumin was determined at different Ca levels. The results (Figure 7) indicate that the decay is markedly dependent on $[Ca^{2+}]$. For Ca-bound parvalbumin, the decay is approximated by a single-exponential fit with a lifetime of 4.7 ms. [A short-lived component may be seen at times less than 1 μs, and we note that a short-lived emitting species has previously been detected (Sudhakar et al., 1993a).] For Ca-depleted parvalbumin, there is a relatively smaller yield of the triplet state, and a nonexponential type of decay profile is observed over the entire time scale, from <0.1 to >1000 μs.

For the partially Ca-saturated protein, the decay profile in the time range 1−100 μs is intermediate between the Ca-free and Ca-bound forms, suggesting that the decay is a simple sum of the Ca-bound and Ca-free species (Figure 7). But at times longer than ~100 μs, it is apparent that the decay of Ca-bound triplet state is *faster* than that of the Ca-bound form at saturation. The longest lived triplet component of the sample for the protein that is half-saturated with Ca^{2+} gave a lifetime of 1.9 ms; 3 ms was found for the longest component of the 75% Ca-bound form of the protein. For the 25% Ca-bound and the totally Ca-free forms of the protein, no long-lived exponentially decaying species could

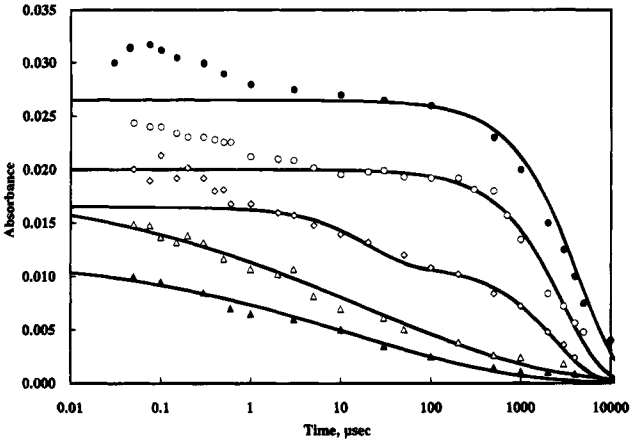


FIGURE 7: Triplet decay kinetics of parvalbumin at different Ca^{2+} levels. Native parvalbumin with Ca^{2+} bound at the following levels: 100% (●), 75% (○), 50% (◇), 25% (△), and 0% (▲). Conditions are the same as for Figure 6. The amount of Ca-bound form is determined with the help of Figure 2. Temperature: 20 °C. Solid lines are simulated decays. For 100% and 75%, the simulated exponential fits gave lifetimes of 4.7 and 3 ms, respectively. The decay for 50% saturation was fit to two exponentials with $\tau_1 = 15$ μs and $\tau_2 = 1.9$ ms. The 75% and 100% decays were fit with a stretched exponential function, i.e., $I(t) = A_0 \exp(-t/\tau)^{\beta}$, with lifetimes of 17 and 15 μs and β values of 0.15 and 0.2, respectively.

Table 2: Triplet Decay Lifetimes for Different Concentrations of Ca-Bound Protein

composition	triplet decay	β
100% Ca-bound protein	4.7 ms	
75% Ca-bound + 25% Ca-free protein	3.1 ms	*a*
50% Ca-bound + 50% Ca-free protein	1.9 ms	*b*
25% Ca-bound + 75% Ca-free protein	17 μs	0.22[c]
100% Ca-free protein	15 μs	0.25[d]

[a,b] Long-lived component of an exponential fit; 0.25[a] or 0.5[b] EDTA added. [c,d] Exponential fit to a stretched exponential (eq 2); 1[c] EDTA and 3[d] mM EDTA added. See Figure 7.

be seen. In these cases, a stretched exponential (eq 2) was used to fit the data. The fitting parameters are given in Table 2.

As a control, the effect of EDTA on the fluorescence emission and the triplet absorption of NATA was examined. No effect was seen at 10 mM EDTA, showing that EDTA does not directly quench the singlet or triplet state at this concentration.

Triplet Decay Kinetics in the Presence of Denaturant. The fluorescence emission spectrum of Ca-free parvalbumin resembles that for the protein in the presence of denaturant (compare Figures 1 and 3). In view that the Trp triplet of the Ca-free form shows such unusual nonexponential decay behavior (Figure 7), it was important to determine whether the denatured protein would show a similar profile. The triplet absorption spectrum of parvalbumin in the presence of GdnHCl shows an absorption maximum at ~450 nm, similar to native parvalbumin, but the decay of the triplet absorption (Figure 8) is approximated by a single exponential with a lifetime of 51 μs. This lifetime is comparable with the lifetime of 30 μs for NATA (Figure 8) or that of 14 μs reported for tryptophan in aqueous medium (Bent & Hayon, 1975).

Triplet Decay Kinetics as a Function of Temperature. The effect of changing temperature on the decay of the triplet

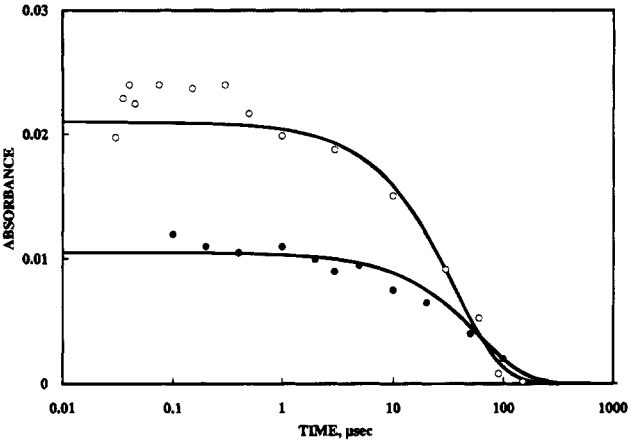


FIGURE 8: Triplet intensity as a function of time for NATA (○) and parvalbumin (●) denatured by 3.8 M GdnHCl. The concentration of NATA was adjusted to give an OD of ~0.2−0.3 at 282 nm, and 2 mg/mL protein was used. Other conditions are the same as for Figure 6. The solid line is the single-exponential simulated fit, with lifetimes of 30 μs for NATA and 51 μs for the protein.

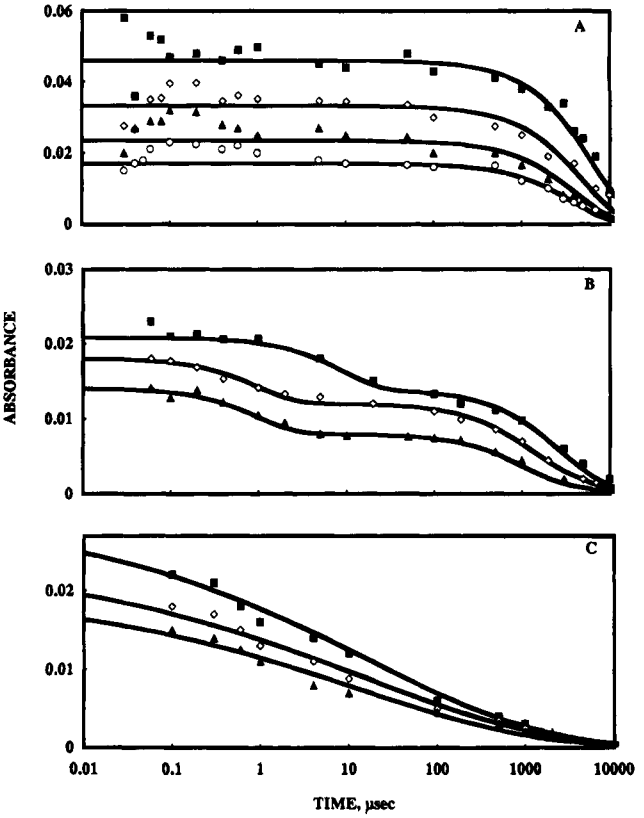


FIGURE 9: Triplet decay kinetics of parvalbumin at 10 (■), 20 (◇), 30 (▲) and 40 °C (○). (A) Ca^{2+}-bound parvalbumin; the solid line is a single-exponential fit using the lifetimes given in Table 2. (B) Half-saturated Ca^{2+}-bound parvalbumin. The solid line is a simulated two-exponential fit. (C) Ca-free parvalbumin. Conditions are as given for Figure 6.

state tryptophan was examined in the range 10−40 °C (Figure 9). A decrease in the triplet decay time as the temperature increases was seen. The lifetime for parvalbumin in the presence of Ca^{2+} was 6.0, 4.8, 4.2, and 3.8 ms at 10, 20, 30, and 40 °C, respectively. A second feature is that the total yield of the triplet state for the Ca-bound and Ca-free proteins decreases with temperature, a characteristic also evident in Figure 6.

DISCUSSION

Comparison of Trp Fluorescence and Phosphorescence. Both fluorescence and phosphorescence measurements give information about the exposure of Trp to solvent. A blue shift in the fluorescence spectrum correlates with the burial of Trp (Burstein et al., 1973), and in general, the phosphorescence lifetime is long (>milliseconds) at room temperature for buried Trp, but short (in the microseconds time regime) for Trp that is exposed to the aqueous phase (Strambini & Gonnelli, 1985; Vanderkooi et al., 1987; Papp & Vanderkooi, 1989). A secondary to tertiary transition in the protein that results in the burial of Trp will be sensed by an increase in phosphorescence lifetime and a blue shift in fluorescence. On the other hand, when the protein changes from an extended form to one with secondary structure, the indole ring is likely to remain exposed to the solvent, and the sensitivity of these techniques to this type of change may be less. In this regard, the information inferred from both luminescence measurements complements the results obtained by circular dichroism, the latter being sensitive to differences between the unfolded and secondary forms but relatively insensitive to the transition from secondary to tertiary structure.

The fluorescence decay of tryptophan in parvalbumin is in the 2−5 ns regime (Sudhakar et al., 1993a). On this time scale, one would not expect equilibration of Ca-free and Ca-bound forms or of the folded and unfolded forms. Indeed, the observation of isosbestic points in the fluorescence emission spectra as a function of Ca or denaturant (Figures 1B and 3) substantiates this. Therefore, fluorescence is a suitable probe for studying the equilibrium behavior of the protein. On the other hand, the decay of the triplet state is greater than microseconds. The long lifetime of phosphorescence suggests that it will be sensitive to the dynamic properties of the protein.

Protein Stabilization Induced by Ca^{2+}. Binding of Ca^{2+}, as indicated by fluorescence, stabilizes the protein to denaturant (Figure 4). A consequence of the thermodynamic cycle is that, even if the protein appears to be completely saturated with ligand, the stability of the protein can still depend upon the ligand concentration. The buffer-only (GdnHCl-independent) term in eq 9 is calcium-dependent for low calcium ion concentrations and becomes calcium-independent when

$$K_b K_1 [Ca^{2+}]^2 \gg K_a \qquad (11)$$

In the corresponding experiment (Figure 4), denaturant titration curves at different $[Ca^{2+}]$ values show decreasing separations as $[Ca^{2+}]$ rises above values of about 1 mM (Figure 4). This observation can be used with eq 11 to estimate the stabilizing influence of calcium upon denaturation. By taking 1 mM to be the calcium ion concentration that characterizes the transition from calcium-independent to calcium-dependent behavior, and by using the experimental value of 9×10^{16} M^{-2} for K_1, we obtain

$$K_a/K_b = (10^{-3}\ M)^2 K_1 = 10^{11} \qquad (12)$$

In free energy terms, a shift in an equilibrium constant by a factor of 10^{11} corresponds to an approximately −60 kJ/mol shift in the corresponding $\Delta G°$. This value can be construed to be the stability imparted by two Ca^{2+} ions and is similar in magnitude to values for other biological processes, for example, the hydrolysis of 2 mol of ATP.

Stabilization of protein folding by cations and anions has been studied previously (Pace & Grimsley, 1988; Sugawara et al., 1991), and questions concerning the binding thermodynamics of substrate or ligand can be considered as a subset of a larger problem concerning the stability and folding of proteins (Creighton & Kim, 1991). The technique of using denaturant to answer questions about binding could have general utility in the study of substances with very high binding affinities when it is difficult to measure the affinity because the concentration of the unbound form cannot be accurately measured. As denaturant is added, the binding affinity decreases and comes within a range that can be measured experimentally. When the concentration dependence of denaturation is known, then it is possible, by extrapolation, to determine the binding of ligand in the absence of chaotropic agent or at least to compare whether the binding affinities of the two forms of the protein are different (see Scheme 1).

Decay of the Trp Absorption in Parvalbumin. The major triplet component for the Ca-bound protein decays with a lifetime of ~5 ms at 20 °C; a short-lived component, which could not be analyzed with the data collected here, may be evident at times less than 1 μs [(Sudhakar et al. (1993a) and Figure 7]. When the temperature is raised, there is a shortening of the triplet lifetime (Figure 9). There is also a decrease in the population of the triplet state, measured at the earliest time, i.e., 60 ns, perhaps due to a decrease in triplet production from the singlet state.

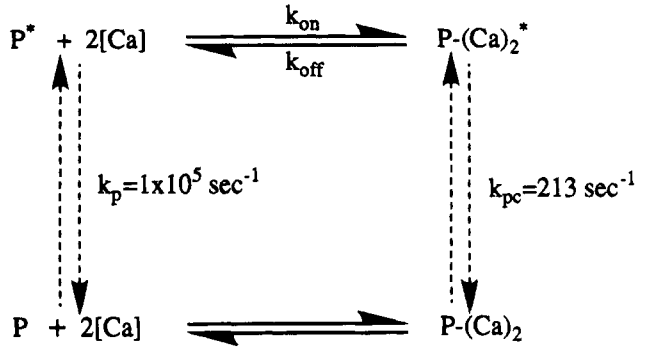
Focusing on the time regime >microseconds, we would like to emphasize three features of the triplet decay related to dynamic properties of the protein.

The first is the striking difference in the triplet decay between the parvalbumins when excess Ca is present and when Ca is depleted. The decay of the triplet state for the Ca-free proteins is nonexponential over the entire time regime from 0.1 μs to 10 ms, whereas the Ca-bound species showed exponential decay. Circular dichroism studies indicate that the Ca-free protein has about the same α-helix content as the Ca-bound protein (White, 1988; Hutnik et al., 1990), but the fluorescence spectral maximum shows that the Trp is largely exposed to water, and the nonexponential triplet decay suggests that there is a distribution of conformations. The calcium-free protein has the attributes of the molten-globule state, i.e., a protein with secondary structure (as indicated by CD) but without rigid tertiary structure (as seen by fluorescence and phosphorescence). Molten-globule structures are thought to resemble protein folding intermediates (Ikeguchi et al., 1986; Ptitsyn et al., 1990; Elove et al., 1992).

The second feature is the difference between Ca-free protein and GdnHCl-unfolded protein. Although the fluorescence spectra of Ca-free and GdnHCl-unfolded parvalbumins would indicate that the tryptophan is exposed to solution in both cases, the triplet decay reveals quite different properties (compare Figures 7 and 8). In the case of the GdnHCl-treated protein, the decay is exponential with a lifetime in the range of that shown by NATA in solution (Figure 8), suggesting that the indole remains water-exposed in the time regime of the excited state for the treated protein.

The third feature is seen in the decay profiles at intermediate Ca^{2+} concentrations (Figure 7). At times less than 10 μs, the decay appears to show contributions from both the

Scheme 2



bound and free forms, suggesting that there is interchange between the two forms on this time scale. At longer times, however, it is obvious that the decay of the intermediate form is not a simple sum of the decays of the component parts, as there is no evidence of the long-lived Ca-bound form. This indicates a second route for decay of the PCa* form, which we take to be the loss of calcium ion from parvalbumin during the time scale of the excited triplet state. We consider the kinetic scheme in Scheme 2, where k_p is an estimate for the rate of decay of the triplet state in the absence of Ca^{2+}. The value k_{pc} is the observed rate of decay for the Ca^{2+}-bound species. In the presence of excess calcium, the value for k_{on} can be expected to be much greater than that for k_{off}, and the equilibrium for the excited parvalbumin molecules will be shifted completely to the calcium-bound form. Under these conditions, the observed lifetime of 4.7 ms gives an accurate estimate for the reciprocal of the rate k_{pc}.

Between the two extremes of high and low [Ca], there will be conditions where the sample will not achieve equilibrium in the excited state. This condition has been examined for reactions in the singlet excited state (Birks, 1970; Loken et al., 1972). On the basis of the analyses of these workers, the following equations describe the decay of P* and P*$(Ca)_2$:

$$\frac{\partial}{\partial t}[P^*] = k_{off}[P^*\text{-}(Ca)_2] - X[P^*] \quad (13a)$$

$$\frac{\partial}{\partial t}[P^*\text{-}(Ca)_2] = k_{on}[Ca]^2[P^*] - Y[P^*\text{-}(Ca)_2] \quad (13b)$$

where

$$X = k_p + k_{on}[Ca]^2$$

$$Y = k_{pc} + k_{off}$$

When these are solved, it is found that *both* components P* and P*$(Ca)_2$ would exhibit *both* lifetimes (the same two lifetimes), and what will be observed is a biphasic decay with the following rates:

$$\text{rates} = \lambda_{1,2} = \frac{1}{2}[(X+Y) \pm \sqrt{(Y-X)^2 + 4k_{off}k_{on}[Ca]^2} \quad (14)$$

Since $k_p \gg k_{pc}$ (and since k_{off} is not large), a power series expansion of the square roots allows us to write

$$\lambda_{fast} = \lambda_2 \cong X + \frac{k_{off}k_{on}[Ca]^2}{X-Y} \quad (15a)$$

$$\lambda_{slow} = \lambda_1 \cong Y - \frac{k_{off}k_{on}[Ca]^2}{X-Y} \quad (15b)$$

In our system, we cannot solve these equations absolutely because the decay of the Ca-free protein was not exponential. However, using the value of k_p obtained for NATA and the values of the measured λ_{slow}, we can estimate k_{off}. The measured values of λ_{slow} were 500, 300, and 200 s^{-1} for 2×10^8, 6×10^{-8} and $\geq 10^{-6}$ M $[Ca^{2+}]$, respectively. Using three equations to solve for k_{pc}, k_{off}, and k_{on}, we determine their approximate values to be 200 s^{-1}, 460 s^{-1}, and 10^{20} M^{-2} s^{-2}.

The expressions given in eq 15 are still quite complex. We can better see the $[Ca^{2+}]$ dependence by noting that $k_{off} \ll k_p$. Then by substitution in the expression for X and Y, we obtain

$$\lambda_{fast} \cong k_p + k_{on}[Ca]^2 - \frac{k_{off}k_{on}[Ca]^2}{k_p + k_{on}[Ca]^2} \quad (16a)$$

$$\lambda_{slow} \cong k_{pc} + k_{off} - \frac{k_{off}k_{on}[Ca]^2}{k_p + k_{on}[Ca]^2}$$

$$= k_{pc} + k_{off}\frac{k_p}{k_p + k_{on}[Ca]^2} \quad (16b)$$

The equation for λ_{slow} is telling us that if we expect to see any effect of calcium-driven equilibrium shift upon the lifetime, it will require k_{off} to be comparable to k_{pc}. When we let the math take us from this intuitive observation to the point of using the two decay rates in Figure 7 (300 and 500 s^{-1} when expressed as rates) to calculate k_{on} and k_{off}, we obtain $k_{off} = 460$ (that is to say, k_{off} is somewhere in the range 200−500 s^{-1}). It should be noted that these rates render the release of Ca^{2+} from parvalbumin kinetically comparable to the time of muscle contraction. Furthermore, the k_{off} rates are temperature-dependent. The k_{off} values increase to ~800 s^{-1} in going from 10 and 20 to 40 °C.

The rate of Ca^{2+} release (which is presumably equal to our determined k_{off}) was measured by Breen et al. (1985) to be biphasic, with rates of 1 and 5 s^{-1} for Ca^{2+} dissociation from cod parvalbumin at pH 5.8 and 24.8 °C by stopped flow. These values are in the range found by Ogawa and Tankura (1986), but are greater than those found by Permyakov et al. (1987), who give dissociation rate constants in the ranges 0.03−0.8 and 0.18−5 s^{-1}. Ho and Hoshihara (1984) distinguished between two rate constants and found a range of 20−240 s^{-1} for the faster phase. While this range is close to the values we found, the values for k_{off}, based upon the triplet decay measurements, are still somewhat larger than those cited for Ca^{2+} release.

One consideration is that, in our case, the fastest process that affects the excited state molecule is being studied, whereas the processes that occur at times less than ~1 ms may not be resolved in stopped-flow experiments. Therefore, the two techniques could be measuring different processes.

We should also point out that it is possible that the structure of the protein is perturbed by the excited triplet state Trp, such that the release of Ca^{2+} is faster for the triplet state molecule. This possibility (which would be interesting because it would provide a method to produce a pulse of Ca^{2+} with excitation by light) seems unlikely since excited triplet molecules in general resemble the respective ground state molecule in terms of geometry and reactivity, as measured by excited and ground state pK's (Parker, 1968). Another possible difference between the experiments in the literature and our experiment is that the initial Ca^{2+} concentrations are different. In our case, Ca^{2+} release from a population of parvalbumin that is half-saturated was monitored, whereas the starting condition for the literature experiments was protein in the presence of excess Ca^{2+}. The observed rates may be influenced by singly bound Ca^{2+}, and the protein may also be stabilized by excess Ca^{2+}. It is also important to recognize that, because the triplet lifetime of the Ca-free parvalbumin is short, the measurement measures only k_{off} and not k_{on}, i.e., Ca^{2+} release and not Ca^{2+} binding as measured by the triplet decay.

Conclusions. The energetics of Ca^{2+} stabilization was determined to be −60 kJ/mol. Ca^{2+} also makes the protein more rigid, as indicated by the long phosphorescence lifetime for the Ca^{2+}-bound protein. Finally, the Ca^{2+} binding is dynamic on the time scale of the excited triplet state.

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