Comparison of Metal Ion-induced Conformational Changes in Parvalbumin and Oncomodulin as Probed by the Intrinsic Fluorescence of Tryptophan 102*

Cindy M. L. Hutnik‡§, John P. MacManus‡, Denis Banville¶, and Arthur G. Szabo‡‖

From the ‡Division of Biological Sciences, National Research Council, Ottawa, Ontario K1A OR6, Canada and the ¶Biotechnology Research Institute, National Research Council, Montreal, Quebec H4P 2R2, Canada

The calcium-induced conformational changes of the 108-amino acid residue proteins, cod III parvalbumin and oncomodulin, were compared using tryptophan as a sensitive spectroscopic probe. As native oncomodulin is devoid of tryptophan, site-specific mutagenesis was performed to create a mutant protein in which tryptophan was placed in the identical position (residue 102) as the single tryptophan residue in cod III parvalbumin. The results showed that in the region probed by tryptophan-102, cod III parvalbumin experienced significantly greater changes in conformation upon decalcification compared to the oncomodulin mutant, F102W. Addition of 1 eq of Ca^{2+} produced greater than 90% of the total fluorescence response in F102W, while in cod III parvalbumin, only 74% of the total was observed. Cod III parvalbumin displayed a negligible response upon Mg^{2+} addition. In contrast, F102W did respond to Mg^{2+}, but the response was considerably less when compared to Ca^{2+} addition. Time-resolved fluorescence showed that the tryptophan in both proteins existed in at least two conformational states in the presence of Ca^{2+} and at least three conformational states in its absence. Comparison with quantum yield measurements indicated that the local electronic environment of the tryptophan was significantly different in the two proteins. Collectively, these results demonstrate that both cod III parvalbumin and oncomodulin undergo Ca^{2+}-specific conformational changes. However, oncomodulin is distinct from cod III parvalbumin in terms of the electronic environment of the hydrophobic core, the magnitude of the Ca^{2+}-induced conformational changes, and the number of calcium ions required to modulate the major conformational changes.

Oncomodulin is a low molecular weight ($M_r \approx 11700$) calcium-binding protein that was first detected in the soluble extracts of Morris rat hepatomas (MacManus, 1979). It is an oncodevelopmental protein which has been found to occur in approximately 85% of a variety of human and rodent tumors, as well as in extraembryonic structures during normal prenatal development (MacManus et al., 1985). The primary

structure has shown that oncomodulin belongs to the large superfamily of calcium-binding proteins which includes troponin C, calmodulin, parvalbumin, vitamin D-dependent calcium-binding protein, S-100, and calbindin 9K (MacManus et al., 1983a and 1983b). Specifically, all of these proteins possess what are known as "EF-hand calcium-binding sites." The name "EF-hand" is derived from the calcium-binding structure in parvalbumin that is formed by two of its six helices (the E-helix and the F-helix) and the peptide loop connecting these two α-helices (Strynadka and James, 1989).

Functionally, oncomodulin remains an enigma. Early reports suggested that oncomodulin possessed certain calmodulin-like regulatory properties. For example, oncomodulin was shown to mimic calmodulin by stimulating DNA synthesis in Ca^{2+}-deprived cells (Boynton et al., 1982). In addition, oncomodulin was shown to be able to activate certain calmodulin-dependent enzymes such as cyclic nucleotide phosphodiesterase (MacManus, 1981; Mutus et al., 1985b), nuclear protamine phosphokinase (MacManus and Whitfield, 1983), and calcineurin (Mutus et al., 1988). Recently, it has been reported that oncomodulin was not able to stimulate bovine heart cAMP phosphodiesterase (Clayshulte et al., 1990) contradicting the earlier finding of Mutus et al. (1988). This discrepancy cannot be explained at the present time, but clearly a modulatory role for oncomodulin requires additional verification. This, however, is beyond the scope of the present report. Although the physiological target of oncomodulin remains unknown, glutathione reductase has been considered a possibility owing to the inhibitory effect of oncomodulin on this enzyme (Palmer et al., 1989).

Although oncomodulin may, in some limited way, resemble calmodulin functionally, it has been found to more closely resemble other members of the family in terms of structure, namely the β-parvalbumins. On comparison of the amino acid sequences of rat oncomodulin and rat muscle parvalbumin, 55/108 residues were identical, with an additional 33/108 residues resulting from single base pair substitutions (MacManus et al., 1983b). Both proteins possess six stretches of α-helices, lettered A through F. The proteins are analogous in that the first two helices (A and B) flank a nonfunctional binding loop, whereas the latter four helices flank two functional Ca^{2+}-binding loops known as the CD- and EF-binding sites. Despite extensive homology, the two proteins have been shown to be immunologically dissimilar (MacManus and Whitfield, 1983). In addition, unlike both calmodulin and oncomodulin, parvalbumin has never been shown to possess any calcium-dependent modulator properties (Heizmann, 1984; Demaille, 1982; Wnuk et al., 1982).

Although it is not known what confers this ability, it has been suggested that Ca^{2+}-specific conformational changes

may play a role. For example, Ca^{2+}-binding to calmodulin is known to induce conformational changes that bring a hydrophobic area to the protein surface thereby facilitating protein-protein interactions (Klee and Vanaman, 1982; Tanaka and Hidaka, 1980). Ca^{2+} binding to oncomodulin has also been shown to induce specific conformational changes (MacManus *et al.*, 1984). The parvalbumins have also been shown to undergo conformational changes upon the binding of Ca^{2+}, which in some cases have been found to be Ca^{2+}-specific (Hutnik *et al.*, 1990; Moeschler *et al.*, 1980; Birdsall *et al.*, 1979). Thus, a higher affinity for Ca^{2+} than for Mg^{2+} may not be as important physiologically as the structural alterations to the protein produced upon Ca^{2+} binding.

The purpose of this study was to compare Ca^{2+}-induced conformational changes at an identical site in oncomodulin and parvalbumin. This was accomplished by comparing the intrinsic fluorescence of tryptophan, which occurs naturally in position 102 of cod III parvalbumin (Hutnik *et al.*, 1990) and in a site-specific mutant of oncomodulin in which the phenylalanine in position 102 has been replaced with tryptophan. X-ray crystallography of carp parvalbumin (Kumar, Lee, and Edwards, Brookhaven Protein Data Bank) and oncomodulin (Ahmed *et al.*, 1989) has revealed that position 102 is equidistant (≈ 11 Å) from both metal-binding sites and lies within the hydrophobic protein interior. By examining the effect of Ca^{2+} binding upon the fluorescence properties of the sensitive, identically located Trp probe in the two proteins, differences between the two proteins emanating from the metal-binding sites may be revealed.

EXPERIMENTAL PROCEDURES

Materials—Sodium cacodylate and DL-dithiothreitol were purchased from Sigma. Potassium chloride was purchased from Fisher Scientific Co., Ottawa, Ontario. Trichloroacetic acid was obtained from Anachemia, Montreal, Quebec. N-Acetyltryptophanamide was purchased from Aldrich Chemical Co. Calcium chloride and magnesium chloride were purchased from Thiokol/Ventron, Danvers, MA. All buffers were prepared using reverse osmosis quality water purified by the Milli-Q Water System, Millipore Canada Ltd., Mississauga, Ontario.

Methods—Cod III parvalbumin was purified from frozen fish fillets and judged to be homogeneous as previously described (Hutnik *et al.*, 1990). Oncomodulin mutagenesis, plasmid characterization by DNA sequencing, expression, and purification were performed according to the procedures outlined in detail elsewhere (MacManus *et al.*, 1989). In addition, a tryptic peptide map of the recombinant oncomodulin with the putative F102W mutation was obtained by high performance liquid chromatography. Three peptides were found to have absorbance at 280 nm. As the elution times of two of the peptides coincided with the established elution times of the two tyrosine-containing peptides in native oncomodulin, the additional peptide detected in the F102W mutant was further analyzed for its amino acid content. This peptide was shown by amino acid analysis (of both hydrochloric and methanesulfonic acid hydrolysates) to have the required composition of residues 97-108 (MacManus *et al.*, 1983a) with the phenylalanine missing, and one tryptophan added.

The calcium-loaded (holo) proteins were decalcified by precipitation with trichloroacetic acid (Haiech *et al.*, 1981). This method was found to be satisfactory in achieving greater than 96% decalcification as well as permitting reversible calcium reconstitution (Hutnik *et al.*, 1990). Calcium content following apo (metal-free) protein preparation was measured by inductively coupled plasma atomic absorption spectrometry by S. Willie, Division of Chemistry, National Research Council.

The method of time-correlated single photon counting was used in the fluorescence decay experiments which, in combination with the steady state fluorescence data, permitted the construction of decay-associated spectra (DAS).[1] Details of the time-resolved methods and

data analysis are identical with those described elsewhere (Hutnik and Szabo, 1989; Willis and Szabo, 1989). In addition, some of the data were collected on a multichannel analyzer which was a Nucleus Personal Computer Analyzer card installed in an IBM-compatible AT clone. Each data set was transferred to a Digital VAX 11/750 for data analysis. The steady state fluorescence measurements were performed on an SLM 8000C spectrofluorometer equipped with a Neslab Endocal refrigerated circulating bath for temperature control. All fluorescence emission spectra were measured with 4 nm slits and were corrected as described previously (Hutnik *et al.*, 1990). Fluorescence quantum yields (ϕ_f) were measured at 20 °C using N-acetyltryptophanamide in aqueous buffer, pH 7, as a quantum yield standard ($\phi_f = 0.14$, Szabo and Rayner, 1980).

The titration data were obtained with the aid of a macro program for the SLM 8000C spectrofluorometer. Aliquots (2 μl) of a 1 mM $CaCl_2$ solution (in 10 mM cacodylate, 150 mM KCl, pH 7) were added with continuous stirring to 1.5 ml of apoprotein (in the same buffer). The protein concentrations were 20-40 μM. Typically, 60 additions resulted in plateau values being recorded in the titration curves. Absorption spectra were recorded on a Varian DMS 200 UV-VIS spectrophotometer, as well as on a Cary 219 spectrophotometer. All spectroscopy was done with protein samples dissolved in 10 mM cacodylate buffer containing 150 mM KCl, pH 7.0. Initially, 1 mM dithiothreitol was added to the cod III parvalbumin solutions. However, as no discernible differences were apparent in its absence, it was subsequently omitted.

RESULTS AND DISCUSSION

Comparison of Composition and Structure—The recent solution of the x-ray crystallographic structure of oncomodulin (Ahmed *et al.*, 1989) has revealed a striking similarity between oncomodulin and carp pI 4.25 parvalbumin (Kumar, Lee, and Edwards, Brookhaven Protein Data Bank). Fig. 1 shows a superimposition of the peptide backbones and the location of the native phenylalanine residue in position 102 relative to the two Ca^{2+}-binding sites. While some differences in the backbone can be seen near the amino terminus, the main chains are remarkably similar.

The complete primary sequence of cod III parvalbumin has not been definitely determined. One attempt at sequencing an anomalously long 113-residue protein has been published (Elsayed and Bennich, 1975). Recently the carboxyl-terminal 20-amino acid segment was sequenced, and the single Trp residue was definitely located at position 102 (Hutnik *et al.*, 1990). Determination of the amino acid compositions of the
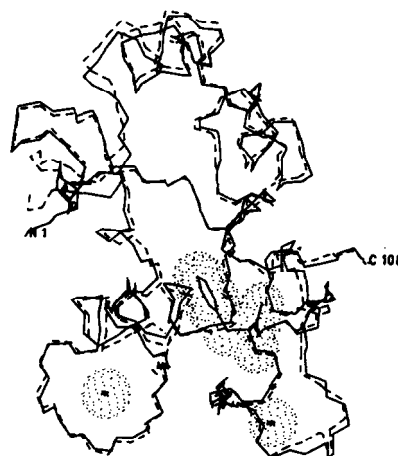

FIG. 1. **Superimposition of the peptide backbones of oncomodulin (*solid line*) and carp pI 4.25 parvalbumin (*dashed line*).** Phenylalanine in position 102 has been included with the atomic surfaces of the constituent atoms highlighted. The ionic spheres of the two calcium ions in the binding sites are also highlighted, with the CD-site located on the *bottom left side* of the figure and the EF-site located on the *bottom right*. The amino terminus and carboxyl terminus are marked on the *left* and *right sides* of the figure, respectively.

---

[1] The abbreviations used are: DAS, decay-associated spectra; PV, parvalbumin; F102W, the oncomodulin mutant protein in which the native Phe-102 has been replaced with Trp; SVR, serial variance ratio; ϕ_f, quantum yield of fluorescence.

remaining tryptic fragments and comparison to the published sequences of the anomalous cod III parvalbumin (Elsayed and Bennich, 1975) and whiting parvalbumin (Joassin and Gerday, 1977) allowed the estimation of a "best guess" amino acid sequence of cod III parvalbumin. In Fig. 2, a comparison of the known sequence of the oncomodulin mutant F102W and the estimated cod III parvalbumin (PV) is shown. Examination of the 20-amino acid carboxyl-terminal segments reveals that although the net charge on the segment is the same (−4), F102W has a greater number of both charged (8 in F102W *versus* 6 in PV) and polar residues (5 in F102W *versus* 2 in PV). Examination of the entire sequences shows that F102W possesses a larger number of acidic residues (24 in F102W *versus* 19 in PV).

Spectral Comparison of Holo(Ca²⁺-containing)proteins—The shape of the absorption spectra of the holoproteins were very similar (Fig. 3A) indicating that the aromatic amino acid residues resided in comparable molecular environments. These spectra are consistent with the amino acid compositions of the proteins. Cod III PV has 4 more phenylalanine (Phe) residues and 1 less tyrosine (Tyr) compared to F102W. As a result, in the spectrum of cod III PV, the vibronic structure associated with Phe (in the region 260–280 nm) is more evident, as well as the ^1L_b electronic transition of Trp,

which appears as a sharp subsidiary peak in the spectrum of the holoproteins. The location of the sharp subsidiary peak of the two proteins is different, with the PV peak at 289 nm occurring 4 nm to the blue of the F102W peak, the latter occurring at 293 nm. It has been shown that the local electrostatic potential of Trp is an important determinant of both the magnitude and position of the absorption transitions (Ilich *et al.*, 1988). The differences in the wavelength positions of these transitions in F102W and PV are the first indication that the proximity of point charges with respect to the indole rings may be different. An alternative possibility which explains the red shift of the ^1L_b absorption transition in F102W is the involvement of the indole N-H in hydrogen bond formation with a nearby protein moiety (Catalan *et al.*, 1986). However, the first possibility is the preferred explanation in lieu of the fluorescence measurements which are presented below.

When the proteins were excited at 295 nm, the corrected steady state emission spectra originating from the single Trp residue (Fig. 4, *Spectra 1* and *2*) were comparable in shape, as well as in the wavelength position of the maximum fluorescence intensity (F102W λ_{max} = 319 nm and cod III PV λ_{max} = 320–325 nm). However, the fluorescence emission spectrum of PV was broader, possessing a 9 nm greater band width at half-height (48 nm in PV *versus* 39 nm in F102W). The slightly shorter wavelength position of the F102W fluorescence spectrum and the slightly longer wavelength position of the absorption spectrum (Fig. 3A) were consistent with the idea that the electronic polarizability of the Trp in F102W, as well as its dipolar environment, may be considerably different when compared to cod III PV. Electronic transitions of Trp in less polar media have been shown to shift the absorption spectrum to longer wavelengths (Demchenko, 1986). In contrast, in an environment of low polarity, fluorescence spectra generally occur at shorter wavelengths (Lakowicz, 1983). Collectively, these results suggest that the Trp in F102W resides in a relatively less polar environment when compared to cod III PV.

A nearest neighbor analysis relative to position 102 in oncomodulin and cod III parvalbumin supported the spectral results. Using the crystal structures of oncomodulin and carp pI 4.25 parvalbumin, all residues within a 6-Å radius of the CZ position of Phe-102 were located. The nearest neighbors relative to Trp-102 in cod III parvalbumin were then identified using the estimated primary sequence of cod III parvalbumin in Fig. 2. The assumption was made that the identical posi-
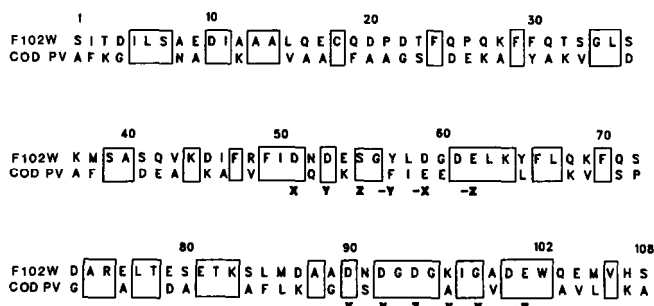
FIG. 2. **Comparison of the amino acid sequence of the oncomodulin mutant F102W (*top sequence*) and the estimated amino acid sequence of cod III parvalbumin (*bottom sequence*).** The carboxyl terminal 20-amino acid sequence of the parvalbumin was determined while the remaining tryptic fragments were placed by determination of their amino acid composition and comparison to other published parvalbumin sequences of cod III parvalbumin (Elsayed and Bennich, 1975) and whiting IIIb parvalbumin (Joassin and Gerday, 1977). The *boxes* indicate identity in both proteins, and the *one-letter amino acid code* is used (IUPAC-IUB Commission on Biochemical Nomenclature). X, Y, Z, −X, −Y, and −Z refer to the metal ion-binding ligands (Kretsinger, 1980).

FIG. 3. **Ultraviolet absorption spectra of holo (*A*) and apo (*B*) cod III parvalbumin and F102W oncomodulin mutant proteins, slits = 0.5 nm.** The proteins were in 10 mM cacodylate, 150 mM KCl, pH 7. The parvalbumin solution also contained 1 mM dithiothreitol.
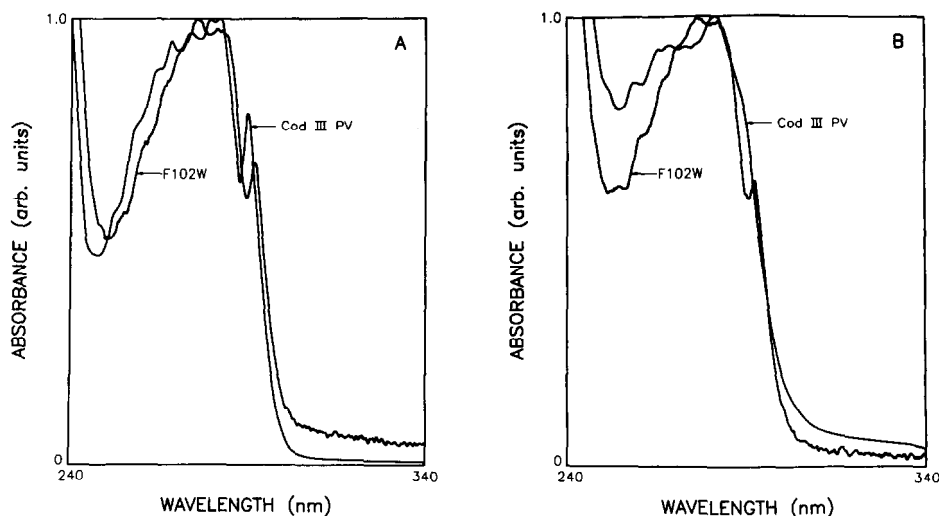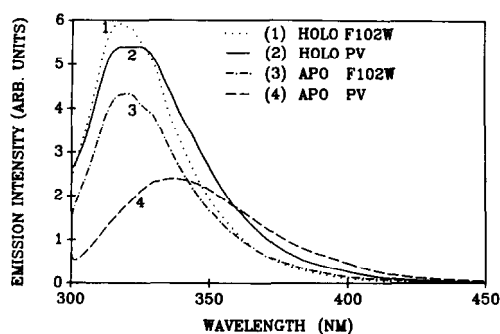
FIG. 4. **Corrected steady state fluorescence emission spectra of holo- and apo-trichloroacetic acid cod III parvalbumin and F102W oncomodulin mutant, slits = 4 nm.** The protein concentration was approximately 20 μM. λ_{ex} = 295 nm. In order to ease in the comparison of spectral shapes, the spectra of holo- and apo-trichloroacetic acid cod III parvalbumin have been multiplied by 2.

TABLE I

Nearest neighboring amino acid residues located within a radius of 6 Å relative to position 102 in oncomodulin and cod III parvalbumin

The nearest neighbors in cod III parvalbumin were identified by an examination of the crystal structure of carp pI 4.25 parvalbumin and comparison to the estimated primary sequence of cod III parvalbumin in Fig. 2. The indices of hydropathy were obtained from Kyte and Doolittle (1982). The higher total hydropathy index for oncomodulin suggests that the immediate environment of position 102 is relatively more hydrophobic when compared to the environment of cod III parvalbumin.

Oncomodulin		Cod III parvalbumin	
Residue	Hydropathy Index	Residue	Hydropathy index
Phe-30	2.8	Tyr-30	−1.3
Leu-35	3.8	Leu-35	3.8
Val-43	4.2	Ala-43	1.8
Lys-44	−3.9	Lys-44	−3.9
Ile-46	4.5	Ala-46	1.8
Phe-47	2.8	Phe-47	2.8
Ile-50	4.5	Ile-50	4.5
Leu-58	3.8	Ile-58	4.5
Phe-66	2.8	Phe-66	2.8
Leu-85	3.8	Phe-85	2.8
Ile-97	4.5	Ile-97	4.5
Total	33.6	Total	24.1

tions in carp pI 4.25 and cod III parvalbumins would function as nearest neighbors relative to position 102. These residues are listed in Table I, along with the hydropathy indices for each amino acid according to the scale of Kyte and Doolittle (1982). The 40% larger total hydropathy index of the nearest neighbors in oncomodulin supports the spectral findings which indicate the environment of Trp-102 in F102W was more hydrophobic relative to the environment in cod III parvalbumin.

A striking difference was observed for the quantum yield of fluorescence (ϕ_f), with the ϕ_f of holo-PV (0.14 ± 0.01) being only 60% that of holo-F102W (0.24 ± 0.01). As hydrogen bonding of the indole N-H represents an efficient nonradiative deactivation process (Privat *et al.*, 1979), the blue-shifted ^1L_b transition in PV argues against hydrogen bonding accounting for the difference in ϕ_f values in the two proteins. Even though F102W possesses a greater number of polar residues when compared to cod III PV, the local polar environment of the Trp may be different in terms of the proximity of one or more polar groups. Even the proximity of elements of the polar peptide bond could have an influence. However, even if the position of polar groups were completely identical in the two proteins relative to the Trp residue, differences in

the conformational heterogeneity of the proteins could alter both the collisional frequencies and the collisional geometries, resulting in large differences in the ϕ_f values (Axelsen and Prendergast, 1989). The higher ϕ_f for F102W suggests that the Trp residue in this protein is less likely to be involved in any direct physical contact with a protein moiety or solvent molecule (Schauerte and Gafni, 1989) than is the Trp in cod III PV. In any given protein, it is difficult to identify the specific interaction(s) responsible for the quenching of Trp fluorescence, given the broad range of potential quenching mechanisms that have been suggested for Trp in proteins (Creed, 1984). Despite the inability to identify the precise photophysical source of the quenching in the two proteins, the results unequivocally demonstrate differences in the local environment of the Trp.

The large difference in ϕ_f is not accounted for in the fluorescence decay measurements. Time-resolved fluorescence revealed that the fluorescence decays of both holo-PV and holo-F102W were best described by two components. The assignment of the decays to a double-exponential (two-component) model was based upon the examination of a number of statistical parameters which were generated following the comparison of the experimental decay curve (as shown in Fig. 5 for holo cod III PV) with a calculated decay curve, the latter being generated based upon a sum of discrete exponentials as described elsewhere (Willis and Szabo, 1989). Among these statistical parameters were the serial variance ratio (SVR) and the σ value, the merits of which have been well established and are explained in McKinnon *et al.* (1977). An SVR value in the range of 1.7 to 2.0 is considered to correspond to a good statistical fit of the data (O'Connor and Phillips, 1984). The adequacy of the exponential decay fitting was also judged by the inspection of weighted residual plots, which were generated based upon the difference (or residuals) between the experimental and calculated decay curves at each measured time interval. An adequate "fit" between the experimental and calculated decay curves corresponds to a randomness in the residual plots. In Fig. 6, the weighted residual plots corresponding to the decay curve in Fig. 5 are shown. In Fig. 6A, the non-random distribution of residual points, and the low SVR = 0.46, indicated that a single exponential decay function was not adequate to describe the fluorescence decay of holo-cod III PV. However, the random residuals in Fig. 6B, and the SVR = 1.94, indicated that the double exponential decay function was adequate. As only a marginal improvement was gained in fitting the data to a triple exponential function (Fig. 6C), the double exponential function was accepted.

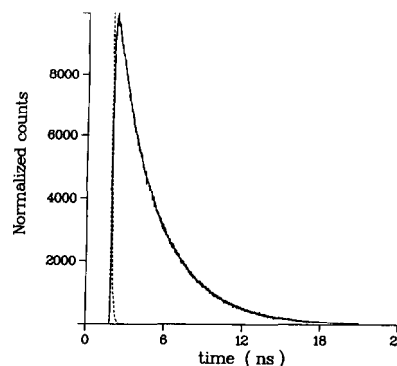The fluorescence decay curve shown in Fig. 5 was measured

FIG. 5. **Typical normalized fluorescence time intensity curves.** The instrument response function curve (*dotted line*) and the holo-cod III parvalbumin decay curve were recorded with channel width = 21.6 ps/channel, λ_{ex} = 295 nm, λ_{em} = 345 nm, and emission band pass = 4 nm.
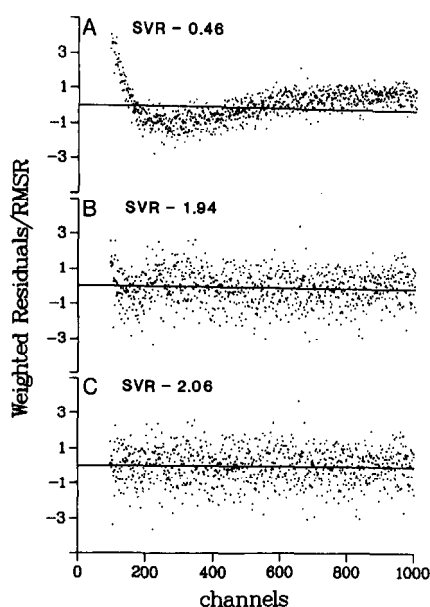
FIG. 6. **Weighted residual plots for the calculated best fit emission decay curve after deconvolution of the emission decay curve in Fig. 5.** *Panels A–C* correspond to the plots for holo-cod III parvalbumin fitted to single, double, and triple exponential decay components, respectively. The serial variance ratios (SVR) are also shown. An SVR $\simeq 2$ is considered to be a satisfactory parameter value indicative of randomness of residuals (O'Connor and Phillips, 1984).

by exciting the sample at 295 nm and measuring the emission at 345 nm. In order to improve the accuracy of estimating the pre-exponential factors and lifetime values which are determined in a discrete exponential component analysis of fluorescence decay, a simultaneous analysis of multiple fluorescence decay curves was performed (Knutson *et al.*, 1983). These different decay curves were measured at different emission wavelengths between 305 and 360 nm, all with an excitation wavelength = 295 nm. The results are summarized in Table II. Upon global analysis, the lifetime values of the two components were found to be similar for the two holoproteins (τ_1 (PV) = 3.55 ns *versus* τ_1 (F102W) = 4.19 ns; τ_2 (PV) = 1.54 ns *versus* τ_2 (F102W) = 1.89 ns). Global analysis also permitted decay-associated spectra (DAS) of the components to be obtained. Fig. 7, *A* and *B* shows that the λ_{max} for the various components are virtually identical for the two holoproteins, with λ_{max}^1 (PV) = 320 nm *versus* λ_{max}^1 (F102W) = 319 nm, and λ_{max}^2 (PV) = 316 nm *versus* λ_{max}^2 (F102W) = 313 nm. From the DAS in Fig. 7, *A* and *B*, it can be seen that the fractional fluorescences (F_i values) are virtually identical for the two proteins. For example, at 320 nm, F_1 = 0.88 for holo-PV *versus* F_1 = 0.92 for holo-F102W and F_2 = 0.12 for holo-PV *versus* F_2 = 0.08 for holo-F102W. If the reasonable assumptions are made that the radiative lifetime of each component is the same and that static quenching is absent, it is possible to calculate the relative component concentrations (c_i values) owing to their proportionality to the normalized pre-exponential terms (Hutnik and Szabo, 1989). As each lifetime component is assigned to the Trp residue in a different conformation, the relative component concentrations reflect the relative occurrence of the various conformational states. As the most highly fluorescent component may not be present in the highest concentration, this information provides some insight into the local environment of the Trp fluorophore in the various conformations. In terms of the relative component concentrations at 320 nm, both proteins are virtually identical in that the longest lived component

dominates the fluorescence and is associated with the conformation which exists in the highest concentration ($\approx 90\%$, see Table II). The only significant difference between the Trp residue in the holoproteins is the ϕ_f.

A simple relationship exists between the quantum yield of fluorescence and the singlet and radiative lifetimes, τ_s and τ_r, respectively. This relationship is as follows:

$$\phi_f = \tau_s/\tau_r \qquad (1)$$

For multiexponential fluorescence decay, it is possible to calculate a mean or intensity-weighted lifetime which can be used as an approximation of τ_s in Equation 1. The intensity-weighted mean lifetime $\langle \tau \rangle$, is given by the following equation:

$$\langle \tau \rangle = (\Sigma \alpha_i \tau_i^2)/(\Sigma \alpha_i \tau_i) \qquad (2)$$

where the α_i values are the pre-exponential terms and the τ_i values are the individual decay time components (Ross *et al.*, 1989). In the case of holo-cod III PV and holo-F102W, the 21% longer mean (intensity-weighted) lifetime of the decay parameters in F102W ($\langle \tau \rangle$ = 4.00 ns in holo-F102W *versus* $\langle \tau \rangle$ = 3.31 ns in holo-PV) is not sufficiently great enough to account for the considerably greater ϕ_f. One explanation is that the Trp in cod III PV may be involved in an interaction leading to a significant degree of static quenching which would decrease ϕ_f, but would not affect the lifetime values (Szalay and Szollosy, 1964). The specific molecular interaction leading to static quenching is not readily apparent by an examination of the crystal structure. An alternative explanation is that the radiative lifetime, τ_r, is different for the Trp in the two proteins. Similar ideas have been proposed to account for discrepancies between the apo- and holo-cod III PV fluorescence parameters (Hutnik *et al.*, 1990). If one calculates an estimate of τ_r (see Hutnik *et al.*, 1990), τ_r (holo-cod III PV) = 22 ns *versus* τ_r (holo-F102W) = 16 ns. Such differences in the radiative lifetimes may be expected if the electronic polarization, or the dipolar environment of the Trp in the two proteins, was different (Privat *et al.*, 1979). Specifically, differences may exist with respect to how the helix dipoles are oriented relative to the Trp which is situated just outside of the EF-loop at the start of the F-helix. As the interhelical angles in the two proteins appear to be virtually the same (see Table III), the different electronic/dipolar environment may originate from other local and specific differences. An examination of the crystal structures and primary sequences reveals that position 85 in the sequence differs with respect to the presence/absence of an aromatic Phe residue in close proximity to position 102. The residue in position 85 is a Leu in F102W but a Phe in cod III PV. As the residue in position 85 occurs less than 6 Å from position 102 in both proteins, it is possible that it may have an influence on the electronic interactions of Trp-102. Despite these more local electronic differences, the overall conformational heterogeneity of the two holoproteins is virtually indistinguishable in the presence of Ca^{2+}.

Spectral Comparison of Apo(Ca^{2+}-free)proteins—Treatment of the holoproteins with trichloroacetic acid resulted in greater than 96% decalcification. Atomic absorption measurements typically gave 0.03–0.08 mol of Ca^{2+}/mol of protein. Upon decalcification, dramatic changes were observed in the absorption and steady state fluorescence emission spectra of cod III PV. In Fig. 3B, it can be seen that decalcification resulted in a complete loss of the 289 nm subsidiary peak of the absorption spectrum, as well as a reduction in the defined spectral structure. The corrected fluorescence emission spectrum showed that Ca^{2+} removal prompted a substantial redshift (λ_{max} (holo) = 321 nm *versus* λ_{max} (apo) = 337 nm), a

TABLE II

Steady state and time-resolved fluorescence parameters of holo- and apo-trichloroacetic acid-cod III parvalbumin and the F102W oncomodulin mutant

λ_{ex} = 295 nm, 20 °C.

Sample	ϕ_f	λ_{max}^a	SVR[b]	τ_1^c	τ_2	τ_3	$1\ \lambda_{max}^d$	$2\ \lambda_{max}$	$3\ \lambda_{max}$	F_1^e	F_2	F_3	C_1^e	C_2	C_3	τ_r
		nm		ns	ns	ns	nm	nm	nm							ns
Holo-PV	0.14 ±0.01	321	1.92	3.55 ±0.03	1.54 ±0.02		320	316		0.88	0.12		0.76	0.24		22
Holo-F102W	0.24 ±0.01	319	1.80	4.19 ±0.01	1.89 ±0.02		319	313		0.92	0.08		0.83	0.17		16
Apo-PV	0.09 ±0.01	337	1.85	4.39 ±0.02	2.17 ±0.02	0.361 ±0.006	335	330	330	0.59	0.37	0.04	0.31	0.41	0.28	26
Apo-F102W	0.19 ±0.01	319	1.84	4.11 ±0.01	2.00 ±0.02	0.222 ±0.011	320	315	315	0.79	0.21	0.01	0.60	0.32	0.08	17

[a] λ^{ss} represents the wavelength of maximum intensity in the corrected steady state fluorescence emission spectrum. max.

[b] SVR represents the serial variance ratio. It is a statistical parameter reflecting the goodness of fit. An SVR = 2 corresponds to an ideal statistical fit of the data (McKinnon *et al.*, 1977). The SVR values shown reflect the statistics obtained upon global analysis of 10 data sets.

[c] The errors quoted for the lifetime values represent the standard errors for lifetime recovery from a given global set.

[d] λ^1_{max} represents the wavelength of the maximum intensity emission of the various components when excited at 295 nm. These values are obtained from the DAS (Figs. 7 and 10).

[e] F_1 and C_1 denotes the fractional fluorescence and relative concentrations, respectively, of the various decay time components at 320 nm. The c_1 values were calculated based upon their proportionality to the normalized pre-exponential times using equations described in detail elsewhere (Hutnik and Szabo, 1989).

[f] τ_r represents the radiative lifetime and was calculated assuming that it was the same for all of the decay components in a given protein sample. A detailed description of its calculation can be found elsewhere (Hutnik and Szabo, 1989).

FIG. 7. **Decay-associated spectra for holo-cod III parvalbumin (A), holo-F102W oncomodulin mutant (B), apo-trichloroacetic acid cod III parvalbumin (C), and apo-F102W oncomodulin mutant (D).** The spectra sum to the corresponding corrected steady state spectral intensities normalized to a value of 1 unit at the emission maximum. Standard errors are within the contours of the *plotted symbols*.
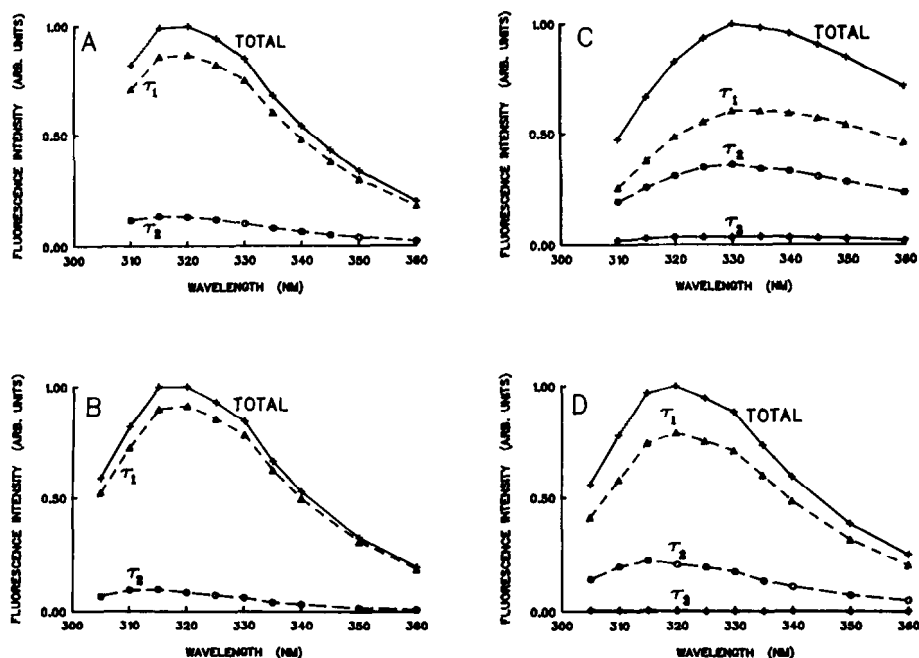
broadening of the spectrum, and a quenching of the Trp fluorescence (Fig. 4). In general, a red-shifted fluorescence emission indicates the transfer of Trp from a relatively apolar, solvent-shielded environment to a more polar, solvent-accessible environment (Van Durren, 1961). Upon decalcification, the ϕ_f decreased from 0.14 ± 0.01 to 0.09 ± 0.01 when excited at 295 nm, 20 °C.

In contrast, decalcification of F102W resulted in only minor changes in the absorption spectrum (Fig. 3B), with only a slight loss of the features associated with both Phe and Trp absorption. Unlike cod III PV, the fluorescence emission spectrum did not undergo a shape change nor a shift in the position of λ_{max} (Fig. 4). However the ϕ_f did decrease upon Ca^{2+} removal from 0.24 ± 0.01 (holo) to 0.19 ± 0.01 (apo).

Thus, the influence of decalcification on the environment of the Trp in these two proteins is markedly different. The more polar environment of the Trp in apo cod III PV may suggest that the F-helix, of which it is a part, may be better able to dissociate from the core of the molecule upon decalcification. Other studies using high resolution ^{1}H NMR and optical stopped-flow techniques have suggested that hydrophobic residues in the F-helix, especially Val-99, Phe-102, Leu-105, and Val-106 in the carp pI 4.25 β-parvalbumin, are essential in stabilization of the protein structure (Corson *et al.*, 1986). Because the F-helix of F102W contains a greater number of polar and/or charged residues (Fig. 2), it was unexpected that it experienced a *less* dramatic change upon decalcification when compared to cod III PV. In addition, the presence of a

TABLE III

A comparison done by Dr. F. Ahmed, Division of Biological Sciences, National Research Council of Canada of the interhelical angles in oncomodulin (Ahmed et al., 1989) and carp pI 4.25 parvalbumin (Kumar et al., Brookhaven Protein Data Bank)

Helices[a]	Oncomodulin	Parvalbumin
A-B	152.7°	158.6°
C-D_1	108.4°	105.6°
C-D_2	111.1°	110.2°
D_1-D_2	126.7°	129.3°
E-F	96.1°	99.1°

[a] Helix A = residues 7–18; helix B = residues 25–34; helix C = residues 40–50; helix D_1 = residues 60–65; helix D_2 = residues 65–70; helix E = residues 79–89; helix F = residues 99–108.
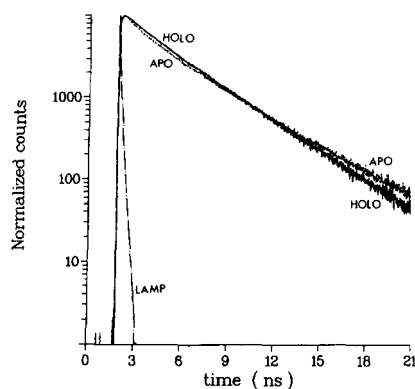

FIG. 8. **Log plots of the fluorescence decay curves of holo- and apo-cod III parvalbumin, as well as the instrument response function.** λ_{ex} = 295 nm; λ_{em} = 345 nm.
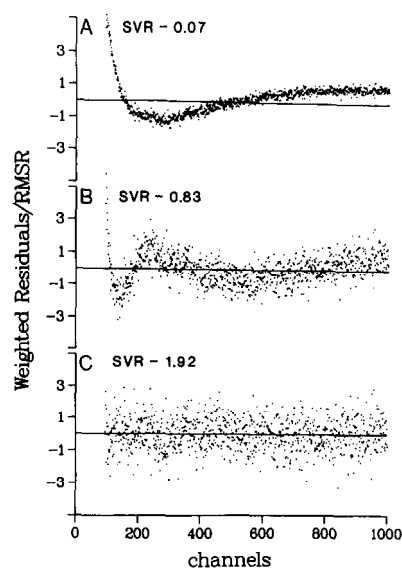

FIG. 9. **Weighted residual plots for the calculated best fit emission decay curve after deconvolution of the emission decay of apo-cod III parvalbumin.** *Panels A–C* correspond to the plots for the decay fitted to single, double, and triple exponential decay components, respectively. The SVR values are also shown.

Lys residue in position 27 of PV has been found to contribute to protein stability by formation of an ion pair bond with the carboxyl-terminal carboxyl function (Corson *et al.*, 1986). Because F102W (as well as native oncomodulin) has a Gln residue in position 27, it is unable to form this bond and, thus, should have a protein structure which is destabilized relative to PV.

Williams *et al.* (1986) found that upon decalcification the α-parvalbumins retained a large extent of both the secondary and tertiary structure typical of their holo forms. In contrast, the β-parvalbumins lost practically all of their tertiary structure and resembled random coils upon decalcification. In agreement, the fluorescence results suggest that in the absence of Ca^{2+}, the PV molecule has additional conformational heterogeneity, at least in the region probed by Trp-102. Further, cod III PV appears to experience much greater perturbations upon decalcification in the region of Trp-102 when compared to F102W. Thus, it appears that the magnitude of the conformational changes induced by Ca^{2+} may be an important difference between the two proteins. The lack of major conformational changes in the region probed by Trp-102 upon metal ion binding to, or removal from, F102W may be consistent with its putative function as a modulator protein. One would envision a modulator protein to be able to reversibly respond to ions in a manner which has a minimum entropy requirement.

Time-resolved fluorescence of the apoproteins showed that three lifetime components were required to adequately describe the decay. Log plots comparing the fluorescence decay curves of holo- and apo-cod III PV are given in Fig. 8 and the residual plots for the best fit exponential decay functions corresponding to the apoprotein decay are given in Fig. 9. The appearance of a short 200–400-ps component, which disappeared upon Ca^{2+} reconstitution, indicated that both proteins experienced a greater degree of conformational heterogeneity

in the absence of Ca^{2+}. This third component comprised only 1–4% of the total fluorescence. The values of the three lifetime components were very similar for the two apoproteins (see Table II). Despite the similarity of the lifetime values, greater differences were observed in the pre-exponential terms. The fractional fluorescence and relative concentration of the longest decay time component were significantly less for apo-cod III PV compared to apo-F102W (F_1 = 0.59 (apo-PV) *versus* F_1 = 0.79 (apo-F102W); c_1 = 0.31 (apo-PV) *versus* c_1 = 0.60 (apo-F102W) at 320 nm (Fig. 7, *C* and *D* and Table II). Upon decalcification, the middle decay time component of apo-cod III PV became relatively more prevalent. A striking difference between the apoproteins was the concentration of the third component (c_3 = 0.28 (apo-PV) *versus* c_3 = 0.08 (apo-F102W)). The results demonstrate that significantly greater conformational changes in the region probed by Trp-102 occur upon decalcification of cod III PV when compared to F102W. This is further supported by the DAS (Fig. 7, *C* and *D*) which show that the λ_{max} of all three components in apo-cod III PV experienced a 10–15 nm red-shift upon decalcification. This is in contrast to the DAS of apo-F102W which have λ_{max} occurring in the identical positions as the holoprotein (see Table II). A feature which is consistent in both apoproteins is that the DAS of component 1 is red-shifted by 5 nm relative to the other component spectra. This suggests that the conformation associated with the longest lived decay component is such that the Trp residue exists in a relatively more polar environment compared to the shorter decay time components. The same is true for the DAS of the holoproteins (Fig. 7, *A* and *B*), except that wavelength differences between the λ_{max} of the component spectra are not as great (see Table II).

Although the ϕ_f decreased for both proteins upon decalcification, a greater degree of quenching was observed for cod III PV (36% decrease of ϕ_f (PV) *versus* 21% decrease of ϕ_f (F102W)). The ϕ_f of apo-F102W (0.19 ± 0.01) was more than double that of apo-cod III PV (0.09 ± 0.01), even though the lifetime values of the three components were strikingly similar (Table II). Consistent with the rationalization suggested for the holoproteins, a change in the radiative lifetime, τ_r, best explains the data. Calculation of τ_r revealed a considerable difference between the two apoproteins (τ_r (apo-PV) = 26 ns

versus τ_r (apo-F102W) = 17 ns). In addition, when the τ_r of each apoprotein was compared to the respective holoprotein, cod III PV experienced much greater changes upon decalcification.

Addition of Ca^{2+} to the apo-trichloroacetic acid PV restored the shape, wavelength position, and greater than 96% of the fluorescence intensity (Fig. 10A, *spectrum 2*). Addition of Ca^{2+} to apo-trichloroacetic acid F102W restored 100% of the spectral features (Fig. 10B, *spectrum 1*). This suggests that the conformational changes associated with decalcification were largely reversible for both proteins. Since it is likely that conformational changes are responsible for the ability of oncomodulin to function as a modulator, then the features of these changes are that they are subtle, localized, and reversible. By contrast, the nonmodulatory PV appears to undergo much more dramatic conformational changes upon decalcification.

In Fig. 10, A and B is included the spectral response of Mg^{2+} addition to the apo forms of both cod III PV and F102W, respectively. These spectra clearly demonstrate that the Ca^{2+} specificity of the conformational changes may have little to do with the ability of a protein to function as a modulator. Addition of Mg^{2+} to apo-cod III PV had essentially no effect upon the spectral properties (Fig. 10A, *spectrum 3*). Previous work has shown how this protein may have been mistaken as a Ca^{2+}/Mg^{2+} protein (Hutnik *et al.*, 1990). In fact, F102W displayed a larger response to Mg^{2+} than did parvalbumin (Fig. 10B, *spectrum 3*) which may be due to the fact that the apoprotein was less disrupted and was more poised to bind a divalent cation with moderate affinity. Thus the feature of being able to respond to Ca^{2+} instead of Mg^{2+} may not be as important as the detailed nature of the conformational changes that are induced upon Ca^{2+} binding to these two proteins.

Ca^{2+} titration of the apoproteins revealed that the magnitude of the response of each protein to substoichiometric amounts of Ca^{2+} was different. For F102W, 1 eq of Ca^{2+}
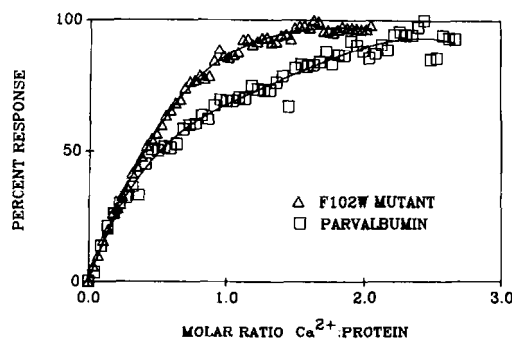
FIG. 11. Ca^{2+} titration of apo-trichloroacetic acid cod III parvalbumin (□) and -F102W oncomodulin mutant (△) plotted in terms of the fluorescence intensity change upon Ca^{2+} addition. For parvalbumin, the intensities were monitored at 325 nm, while for F102W, they were monitored at 318 nm.

produced 88% of the maximal response whereas only 73% of the total response was experienced upon addition of 1 eq of Ca^{2+} to apo-cod III PV (Fig. 11). From earlier work it was suggested that in oncomodulin the EF-site is a Ca^{2+}/Mg^{2+} parvalbumin-like site which possesses a greater affinity for Ca^{2+} compared to the weaker CD-site, which was thought to be Ca^{2+}-specific (MacManus *et al.*, 1984). It was also suggested that the CD-site was most likely involved in manifesting the putative regulatory properties of the protein. If this is true, the titration data displayed in Fig. 11 would suggest that the major conformational changes in oncomodulin are controlled by Ca^{2+} binding to the EF-site and not the CD-site. As the EF-sites of both the parvalbumins and oncomodulin are believed to have Ca^{2+}-binding affinity constants of at least 10^6 M^{-1} (Kretsinger, 1980; Williams *et al.*, 1987), it is unlikely that the Ca^{2+} in the EF-site is removed under physiological conditions. The spectral results reported here suggest the Ca^{2+} bound to the higher affinity site in oncomodulin (presumably the EF-site, Williams *et al.*, 1987) plays a strong role in sustaining the structural integrity of the protein in a similar manner to what has already been suggested for parvalbumin (Nelson *et al.*, 1976).

A major difference between the two proteins was the observable response to the second equivalent of Ca^{2+}. Binding of the second Ca^{2+} equivalent produced an additional 12% of the total response for F102W and 21% of the total response for cod III PV (Fig. 11). Examination of a number of site-specific mutants of oncomodulin revealed that the binding of the second equivalent of Ca^{2+} could produce more significant changes (44% of the total fluorescence response) only very locally in the EF-loop. This was demonstrated with the oncomodulin mutant K96W.[2] These latter results imply that *after* the binding of Ca^{2+} to the stronger EF-site, the binding of the second equivalent of Ca^{2+} to the weaker CD-site induces conformational changes in the vicinity of the distant EF-site. A complex interplay between the two Ca^{2+}-binding sites has been reported for a number of calcium-binding proteins (Sekharudu and Sundaralingam, 1988) and has specifically been suggested in oncomodulin using high resolution ^1H NMR spectroscopy and optical stopped-flow techniques (Golden *et al.*, 1989).

In contrast, Trp-102 in PV was capable of detecting conformational changes associated with the binding of the first and second equivalents of Ca^{2+} to a greater extent. However, it has been shown that the effects of the two Ca^{2+} ions are different. The binding of the first equivalent of Ca^{2+} is mainly
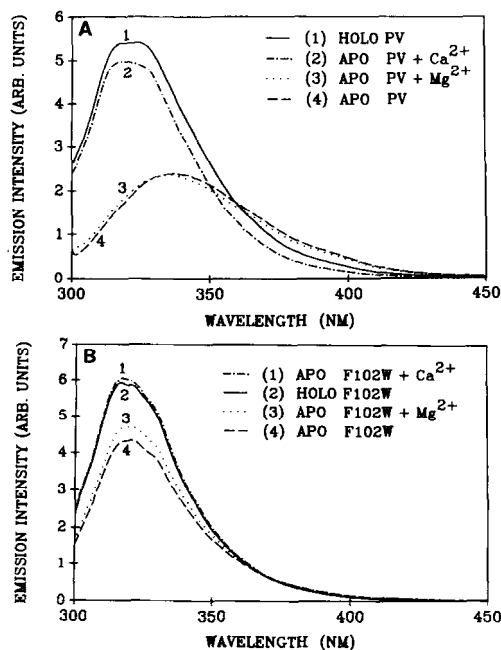

FIG. 10. Corrected steady state fluorescence emission spectra of apo-cod III parvalbumin (A) and apo-F102W oncomodulin (B) mutant showing the response of the proteins to Ca^{2+} and Mg^{2+} addition. The protein concentrations were approximately 20 μM, $CaCl_2$ was added to a final concentration of 2.5 mM, and $MgCl_2$ was added to a final concentration of 5 mM.

---

[2] C. M. L. Hutnik, J. P. MacManus, D. Banville, and A. G. Szabo, unpublished results.

responsible for the wavelength shift, while the second equivalent is important in locking the protein into its final conformation and hence restoring the full intensity of the Trp emission (Hutnik et al., 1990).

Conclusions—The data presented in this report suggest that oncomodulin is less conformationally flexible and possesses a more limited conformational response upon Ca^{2+} binding when compared to cod III parvalbumin. Differences in the absorption and fluorescence emission spectra and the radiative lifetimes, suggest that considerable electrostatic and/or dipolar differences may exist between the decalcified forms of cod III PV and F102W. This in turn suggests that the orientation and/or interactions of the tryptophanyl indole with the hydrophobic core of these two proteins are different, as well as the magnitude of the perturbations produced by Ca^{2+} removal. If the CD site of oncomodulin does possess a lower Ca^{2+} affinity constant (compared to the EF-site), it is likely that this is the site that will be exchanging Ca^{2+} under physiological conditions. The data presented in this report suggest that conformational changes associated with Ca^{2+} binding to the CD-site in oncomodulin are very subtle.

REFERENCES

Ahmed, F., Przybylska, M., Rose, D. R., Birnbaum, G. I. & MacManus, J. P. (1989) *Proceedings of the Annual Meeting of the American Crystallographic Association, Seattle, WA*, July 23–29, PB33, 112

Axelsen, P. H. & Prendergast, F. G. (1989) *Biophys. J.* **56**, 43–66

Birdsall, W. J., Levine, B. A., Williams, R. J. P., Demaille, J. G., Haiech, J. & Pechere, J.-F. (1979) *Biochimie* **61**, 741–750

Boynton, A. L., MacManus, J. P. & Whitfield, J. F. (1982) *Exp. Cell Res.* **138**, 454–458

Catalan, J., Perez, P. & Acuna, A. U. (1986) *J. Mol. Struct.* **142**, 179

Clayshulte, T. M., Taylor, D. F. & Henzl, M. T. (1990) *J. Biol. Chem.* **265**, 1800–1805

Corson, D. C., Williams, T. C., Kay, L. E. & Sykes, B. D. (1986) *Biochemistry* **25**, 1817–1826

Creed, D. (1984) *Photochem. Photobiol.* **39**, 537–562

Demaille, J. G. (1982) *Calcium Cell Funct.* **2**, 111–114

Demchenko, A. P. (1986) in *Ultraviolet Spectroscopy of Proteins*, Springer-Verlag, Berlin

Elsayed, S. & Bennich, H. (1975) *Scand. J. Immunol.* **4**, 203–208

Golden, L. F., Corson, D. C., Sykes, B. D., Banville, D. & MacManus, J. P. (1989) *J. Biol. Chem.* **264**, 20314–20319

Haiech, J., Klee, C. B. & Demaille, J. G. (1981) *Biochemistry* **20**, 3890–3897

Heizmann, C. W. (1984) *Experientia* **40**, 910–921

Hutnik, C. M. L. & Szabo, A. G. (1989) *Biochemistry* **28**, 3923–3934

Hutnik, C. M. L., MacManus, J. P. & Szabo, A. G. (1990) *Biochemistry*, in press

Ilich, P., Axelsen, P. H. & Prendergast, F. G. (1988) *Biophys. Chem.* **29**, 341–349

Joassin, L. & Gerday, Ch. (1977) *Comp. Biochem. Physiol.* **57B**, 159–161

Klee, C. B. & Vanaman, T. C. (1982) *Adv. Protein Chem.* **35**, 213–320

Knutson, J. R., Beechem, J. M. & Brand, L. (1983) *Chem. Phys. Lett.* **102**, 501–507

Kretsinger, R. H. (1980) *CRC Crit. Rev. Biochem.* **8**, 119

Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132

Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, Plenum Publishing Corp. New York

MacManus, J. P. (1979) *Cancer Res.* **39**, 3000–3005

MacManus, J. P. (1981) *FEBS Lett.* **126**, 245–249

MacManus, J. P. & Whitfield, J. F. (1983) in *Calcium and Cell Function* (Cheung, W. Y., ed.) Vol. 4, pp. 412–440, Academic Press, New York

MacManus, J. P., Watson, D. C. & Yaguchi, M. (1983a) *Eur. J. Biochem.* **136**, 9–17

MacManus, J. P., Watson, D. C. & Yaguchi, M. (1983b) *Biosci. Rep.* **3**, 1071–1075

MacManus, J. P., Szabo, A. G. & Williams, R. E. (1984) *Biochem. J.* **220**, 261–268

MacManus, J. P., Brewer, L. M. & Whitfield, J. F. (1985) *Cancer Lett.* **27**, 145–151

MacManus, J. P., Hutnik, C. M. L., Sykes, B. D., Szabo, A. G., Williams, T. C. & Banville, D. (1989) *J. Biol. Chem.* **264**, 3470–3477

McKinnon, A. E., Szabo, A. G. & Miller, D. R. (1977) *J. Phys. Chem.* **81**, 1564–1570

Moeschler, H. J., Schaer, J.-J. & Cox, J. A. (1980) *Eur. J. Biochem.* **111**, 73–78

Mutus, B., Flohr, E. J. & MacManus, J. P. (1985a) *Can. J. Biochem. Cell Biol.* **63**, 998–1002

Mutus, B., Karuppiah, N., Sharma, R. K. & MacManus, J. P. (1985b) *Biochem. Biophys. Res. Commun.* **131**, 500–506

Mutus, B., Palmer, E. J. & MacManus, J. P. (1988) *Biochemistry* **27**, 5615–5622

Nelson, D. J., Opella, S. J. & Jardetzky, O. (1976) *Biochemistry* **15**, 5552–5560

O'Connor, D. V. & Phillips, D. (1984) in *Time-correlated Single Photon Counting*, Academic Press, New York

Palmer, E. J., MacManus, J. P. & Mutus, B. (1989) *Arch. Biochem. Biophys.* **277**, 149–154

Privat, J.-P., Wahl, P. & Auchet, J.-C. (1979) *Biophys. Chem.* **9**, 223–233

Ross, J. B. A., Laws, W. R., Rousslang, K. W. & Wyssbrod, H. R. (1989) in *Fluorescence Spectroscopy, Biochemical Applications* (Lakowicz, J. R., ed.) Vol. 2, in press

Schauerte, J. A. & Gafni, A. (1989) *Biochemistry* **28**, 3948–3954

Sekharudu, Y. C. & Sundaralingam, M. (1988) *Protein Eng.* **2**, 139–146

Strynadka, N. C. J. & James, M. N. G. (1989) *Annu. Rev. Biochem.* **58**, 951–998

Szabo, A. G. & Rayner, D. M. (1980) *J. Am. Chem. Soc.* **102**, 554–563

Szalay, L. & Szollosy, L. (1964) *Acta Phys. Chem.* **10**, 3

Tanaka, T. & Hidaka, M. (1980) *J. Biol. Chem.* **255**, 11078–11080

Van Durren, B. L. (1961) *J. Org. Chem.* **26**, 2954

Williams, T. C., Corson, D. C., Oikawa, K., McCubbin, W. D., Kay, C. M. & Sykes, B. D. (1986) *Biochemistry* **25**, 1835–1846

Williams, T. C., Corson, D. C., Sykes, B. D. & MacManus, J. P. (1987) *J. Biol. Chem.* **262**, 6248–6256

Willis, K. J. & Szabo, A. G. (1989) *Biochemistry* **28**, 4902–4908

Wnuk, W., Cox, J. A. & Stein, E. A. (1982) *Calcium Cell Funct.* **2**, 243–278