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A Calcium-Specific Conformational Response of Parvalbumin†

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ABSTRACT: The single tryptophan containing isotype III parvalbumin from codfish (*Gadus callarius*) was purified by a modified procedure and was shown to be homogeneous by a number of biochemical techniques. Sequence analysis established the location of the single tryptophan in position 102 of the 108 amino acid primary sequence. Atomic absorption spectroscopy showed that trichloroacetic acid (TCA) precipitation was more effective in parvalbumin decalcification compared to the more commonly used method of EGTA treatment. Magnesium induced steady-state fluorescence spectral changes of the EGTA-treated, but not the TCA-treated, parvalbumin. Steady-state fluorescence and circular dichroism spectra showed that calcium, but not magnesium, induced a conformational response in the TCA-treated protein. The fluorescence decay of the calcium-loaded native (holo) cod III parvalbumin was best described by two decay time components. By contrast, three lifetime components were necessary to describe the fluorescence decay of the metal-free (apo) protein. The decay-associated spectra of each temporal component were obtained. Collectively, these results demonstrate that it is possible for a parvalbumin to display a calcium-specific response.

The parvalbumins are low molecular weight (MW ≈12000), acidic, soluble proteins which are ubiquitous in the vertebrate world (Pechère, 1974). On the basis of amino acid sequence data, as well as the X-ray crystallographic structure of carp parvalbumin, these proteins have been found to represent a subfamily of the large superfamily of calcium binding proteins (Kretsinger, 1980). The parvalbumin subfamily is further subdivided based upon two evolutionarily distinct lineages, the α-lineage and the more acidic β-lineage (Goodman & Pechère, 1977).

Over the last 15 years, these proteins and their metal binding properties have been the subject of considerable research. Interpretation of the early thermodynamic and kinetic experiments suggested that the parvalbumins were involved in muscular contraction/relaxation and were Ca^{2+}/Mg^{2+}-specific proteins (Pechère et al., 1977; Birdsall et al., 1979). A Ca^{2+}/Mg^{2+}-specific protein (1) possesses calcium affinity constants (K_{Ca}) on the order of 10^8–10^9 M^{-1} (Moeschler et

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al., 1980), (2) possesses magnesium affinity constants (K_{Mg}) on the order of 10^3–10^5 M^{-1} (Haiech et al., 1979), and (3) undergoes comparable conformational changes upon either Ca^{2+} or Mg^{2+} binding (Cox et al., 1979). A Ca^{2+}-specific protein (1) possesses $K_{Ca} \approx 10^5$–10^6 M^{-1}, (2) has $K_{Mg} \approx 10^1$–10^2 M^{-1} (Milos et al., 1986), (3) is not saturated by Mg^{2+} in the resting cell (Demaille, 1982), and (4) experiences specific conformational changes upon Ca^{2+}, but not Mg^{2+}, binding (Klee & Vanaman, 1982). The generally held belief that parvalbumins are Ca^{2+}/Mg^{2+}-specific proteins has been described by Henzl and Birnbaum (1988) as forming part of an "evolved paradigm" in which the two metal ion binding sites of the parvalbumins are believed to possess features which allow them to bind both Ca^{2+} and Mg^{2+}. However, several inconsistencies exist in the literature. Equivalent conformational responses of parvalbumin upon Ca^{2+} or Mg^{2+} binding have been reported (Cox et al., 1979; MacManus et al., 1984). However, Birdsall and co-workers (Birdsall et al., 1979) suggested global conformational changes occurred only in the presence of Ca^{2+}, while Moeschler et al. (1980) reported that the differences were confined to the vicinity of the metal binding sites. Others have suggested that parvalbumin does not bind Mg^{2+}, which is surprising for a putative Ca^{2+}/Mg^{2+}-specific protein (Cave et al., 1979b; Daures, 1977). However Mg^{2+} binding as well as the associated binding constants have been estimated (Birdsall et al., 1979; Moeschler et al., 1980). White (1988) suggested that these contradictions resulted from a variety of experimental approaches leading to conflicting and inconsistent conclusions regarding basic features of metal binding to these proteins.

Of the 40 parvalbumins of known structure (Maeda et al., 1984; Gerday, 1982; Blum et al., 1977; Closset & Gerday, 1976), only 2 contain a single tryptophan. These parvalbumins, which are members of the β-lineage, have been isolated from two members of the genus *Gadus*, namely, cod and whiting (Bhushana Rao et al., 1969; Closset, 1976). The presence of this single Trp residue has prompted a number of fluorescence studies on the isotype IIIb component of whiting (Permyakov & Burstein, 1984; Permyakov et al., 1980, 1982; White, 1988; Castelli et al., 1988) as well as on the isotype III component of cod (Permyakov et al., 1987; Eftink & Hagaman, 1985; Horrocks & Collier, 1981).

The purpose of the present study was to locate the position of the single Trp residue in highly purified cod III parvalbumin and to use it as a specific probe of protein structure and dynamics. In particular, the ability and manner in which Ca^{2+} and Mg^{2+} affect the conformation of the protein was investigated. The reported ability (Parello et al., 1979) of the frequently used soluble chelator ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)[1] to affect the metal binding was also investigated. Analysis of the time-resolved fluorescence decay and the construction of DAS (decay-associated spectra) revealed distinct conformational states in the Ca^{2+}-loaded (holo) and metal-free (apo) proteins. In contrast, Mg^{2+} binding did not influence the spectral properties of the apoprotein. Circular dichroism showed that the metal binding effects were not limited to the vicinity probed by the single Trp residue. The results demonstrate that cod III parvalbumin undergoes Ca^{2+}-specific conformational changes. The results also provide some insight into the nature of these changes.

[1] Abbreviations: NATA, *N*-acetyltryptophanamide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; *A*, absorbance; CD, circular dichroism; UV, ultraviolet; Trp, tryptophan; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; SVR, serial variance ratio; TCSPC, time-correlated single photon counting; MCA, multichannel analyzer.

Materials and Methods

Materials

Sodium dihydrogen phosphate, sodium hydrogen phosphate, sodium chloride, and potassium chloride were obtained from Fisher Scientific Co., Fair Lawn, NJ. SpectraPor dialysis tubing (MW cutoff 3500) was obtained from Spectrum Medical Industries, Los Angeles, CA. Sodium cacodylate, EDTA, piperazine, and DL-dithiothreitol were supplied by Sigma Chemical Co., St. Louis, MO. Tris buffer was obtained from Boehringer Mannheim, West Germany. Glycerol, trichloroacetic acid, calcium chloride, and magnesium chloride were purchased from Anachemia, Montreal, Quebec. Sephadex G-75SF and DEAE-Sephacel were purchased from Pharmacia Canada Ltd., Dorval, Quebec, and Whatman DEAE-52 was purchased from Mandel Scientific Co., Rockwood, Ontario. *N*-Acetyltryptophanamide (NATA) was purchased from Aldrich Chemical Co., Milwaukee, WI. Trifluoroacetic acid was obtained from Pierce, Rockford, IL. High-purity acetonitrile was purchased from Burdick & Jackson Laboratories, Inc., Muskegon, MI. Electrophoretically pure (>99.9%) acrylamide, Chelex-100 resin (100–200 mesh), Bio-Lyte 3/5 carrier ampholytes, and molecular weight standards were purchased from Bio-Rad Laboratories, Richmond, CA. The Syncropak RP-P C18 reverse-phase HPLC column was purchased from Synchrom Inc., Linden, IN. $^{45}CaCl_2$ was obtained from New England Nuclear, Boston, MA. All buffers were prepared by using reverse osmosis quality water purified by the Milli-Q Water System, Millipore Canada Ltd., Mississauga, Ontario.

Methods

Purification of Parvalbumin. Cod III parvalbumin was purified from 500 g of frozen fillets by the method of Horrocks and Collier (1981) with the following modifications. The acetone precipitation was conducted in two steps. Acetone was added to a final concentration of 54% (v/v) dropwise at 0–4 °C with stirring, followed by additional stirring for 15 min at 0 °C. This solution was centrifuged for 75 min at 9500 rpm in a Sorvall RC-5B refrigerated superspeed centrifuge. The supernatant was made 80% in acetone (v/v), and after centrifugation, the pellet was redissolved in 100 mL of 20 mM sodium phosphate buffer, pH 7.30, containing 24 mM NaCl. This solution was gently agitated overnight, centrifuged, and divided into two 50-mL portions. In two separate applications, the 50-mL protein solutions were loaded onto a Sephadex G-75SF column (100 cm × 2.5 cm), eluted at a flow rate of 3 mL/min, and collected in 5-mL fractions. The spectrophotometric absorbances of the fractions were monitored at 280 nm (the wavelength maximum of Trp/Tyr-containing proteins) to generate an elution profile. The fractions containing the low molecular weight parvalbumins were identified by both their peak position and their calcium binding activity as measured by the ^{45}Ca Chelex competition assay (Waisman & Rasmussen, 1983).

These fractions were pooled and dialyzed overnight at 4 °C against 0.0162 M piperazine buffer, pH 5.70, in preparation for DEAE-52 cellulose chromatography. After combination of the fractions from gel filtration of the two 50-mL samples, a total volume of 170 mL was applied to the ion-exchange column. The DEAE-52 column (42 cm × 3 cm) was eluted with a linear NaCl gradient (500 mL × 500 mL, 0–0.10 M NaCl), and fractions were collected. Fractions containing the

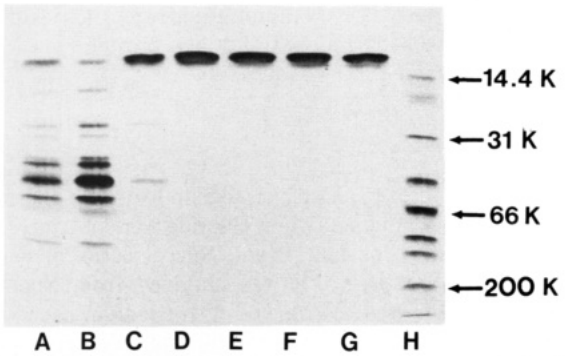

FIGURE 1: SDS–PAGE pattern of cod muscle protein extract obtained at various steps in the purification of cod III parvalbumin. Lane A, soluble extract; lane B, 54% acetone pellet; lane C, G-75 load; lane D, DEAE load; lane E, peak DEAE fraction (106); lane F, DEAE-Sephacel load; lane G, peak DEAE-Sephacel fraction; lane H, molecular weight standards.

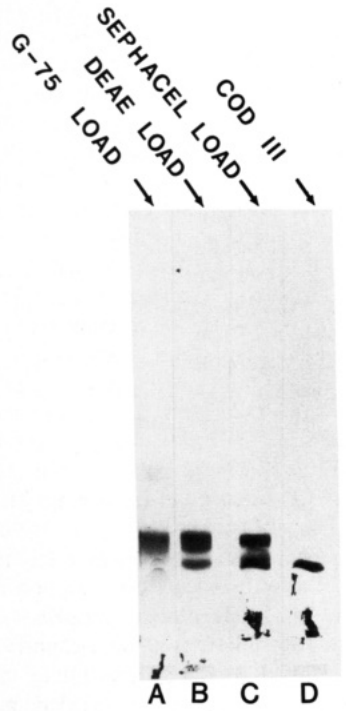

FIGURE 2: Polyacrylamide rod gels obtained upon isoelectric focusing in a gradient of pH 3–5 from top to bottom at a constant voltage of 250 V. The gels show that the additional purification step on DEAE-Sephacel was required to achieve homogeneous cod III parvalbumin.

parvalbumins were identified on the basis of their absorbance at 280 and 260 nm (to monitor differential Phe/Trp content), the protein–dye binding assay (to monitor total protein; Bradford, 1976), and the calcium binding activity.

At this stage of the purification, the protein appeared homogeneous with respect to size as established by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 8–25% gradient using the Pharmacia PhastSystem. A single band was observed as seen in Figure 1, lane E. However, upon isoelectric focusing in 7.5% polyacrylamide rod gels (Righetti & Drysdale, 1971), 5% ampholytes, pH gradient 3–5, two bands appeared in fractions across the elution profile. An example of these two bands in a fraction is seen in Figure 2, lane C. Thus, it was evident that another purification step was required. This two-component mixture of proteins was purified further as follows.

Pooled fractions from the DEAE-52 cellulose ion-exchange column were dialyzed overnight at 4 °C against 20 mM Tris,

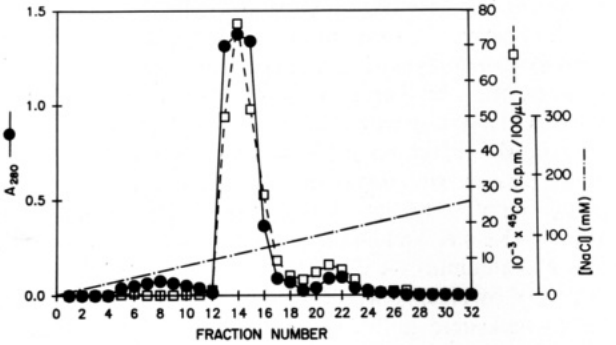

FIGURE 3: DEAE-Sephacel purification of cod III parvalbumin. The protein obtained following gel filtration and ion-exchange on DEAE-52 cellulose was applied to DEAE-Sephacel which had been preequilibrated with 20 mM Tris, pH 8, containing 1 mM dithiothreitol. The protein was eluted with a linear NaCl gradient (0–0.3 M) in the same buffer. Fractions were collected and monitored for protein by measuring A_{280}, A_{260}, and calcium binding activity. Fractions 14–16 were found to be homogeneous as judged by SDS–PAGE, isoelectric focusing, amino acid composition determination, and tryptic peptide mapping.

pH 8, containing 1 mM dithiothreitol (DTT) and applied to a DEAE-Sephacel column (30 cm × 0.9 cm) equilibrated in the same buffer. The protein was then eluted with a linear NaCl gradient (250 mL × 250 mL, 0–0.30 M NaCl); fractions were collected and monitored by measuring the A_{280} and the calcium binding activity. Of the three peaks obtained in the resulting elution profile, peak 2 in Figure 3 was found to contain homogeneous, calcium binding protein as judged by SDS–PAGE, isoelectric focusing, amino acid analysis, and tryptic peptide mapping. The fractions in this peak were pooled, and the protein concentration was estimated by using $\epsilon_{280} = 7180\ M^{-1}\ cm^{-1}$ (Closset & Gerday, 1976). From 500 g of fish, 72 mg of cod III parvalbumin was obtained.

Apoprotein Preparation. Calcium-loaded native (holo) parvalbumin was decalcified by precipitation with trichloroacetic acid (TCA) (Haiech et al., 1981). Following precipitation and centrifugation of the protein solution, the supernatant was poured off, and the sides of the Eppendorf tube were dried with tissues. The precipitated pellet was redissolved in Ca^{2+}-free buffer (atomic absorption measurements of the buffer gave <0.1 μM). Calcium removal was also accomplished by addition of an excess of EGTA at neutral pH. The EGTA-treated samples were examined spectroscopically both in the presence of the chelator and following chelator removal by G-25 Sephadex gel filtration or dialysis. Calcium content following apo (metal-free) protein preparation or metal reconstitution was measured by atomic absorption spectroscopy on a Pye-Unicam SP 191 instrument.

Amino Acid Composition and Partial Sequence Determination. Partial sequencing of holo cod III parvalbumin was performed according to methods previously described (MacManus et al., 1985). These methods involved HPLC separation of tryptic fragments of the protein on a Syncropak RP-P C18 reverse-phase column using a 0.1% trifluoroacetic acid/acetonitrile elution system. Only one Trp-containing peptide from HPLC was obtained, and hence this peptide was sequenced. The amino acid compositions of both the holo- and apo-TCA parvalbumins were determined, and the latter was used to estimate an extinction coefficient for the decalcified protein.

Instrumentation. The method of time-correlated single photon counting (TCSPC) was used in the fluorescence decay experiments. The instrumentation included a Spectra Physics sync-pumped argon ion dye laser and cavity dumper system

as the excitation source, operating at 825 kHz and with a pulse width of 10 ps. The excitation at 295 nm was vertically polarized. The channel width was typically 21.6 ps/channel, and fluorescence decay curves were collected in 1024 channels of a multichannel analyzer (MCA). To optimize the signal-to-noise ratio, a minimum of 25 000 counts was usually collected in the peak of the sample profile, giving a total of 10^6 counts in the total decay curve. A buffer blank lacking protein was also measured under identical conditions as the corresponding sample.

The fluorescence decay kinetics of the molecule were described as a sum of exponentials:

$$I_{em}(\lambda,t) = \sum_{i=1}^{n} \alpha_i(\lambda) \exp(-t/\tau_i)$$

where $\alpha_i(\lambda)$ is the preexponential term at wavelength λ and τ_i is the singlet lifetime of the ith component of the fluorescence. Adequacy of the exponential decay fitting was judged by the inspection of weighted residual plots, as well as other statistical parameters such as the serial variance ratio (SVR) and the reduced χ^2. Decay curves measured at different emission wavelengths were analyzed simultaneously by global analysis (Knutson et al., 1983). The standard error reported for the decay times upon global analysis reflect the accuracy. Combination of the time-resolved and steady-state data in the following form permitting decay-associated spectra (DAS) to be calculated:

$$I_i(\lambda) = I_{ss}(\lambda)\{\alpha_i(\lambda)\tau_i/[\sum_i \alpha_i(\lambda)\tau_i]\}$$

where $I_i(\lambda)$ is the emission intensity associated with the ith component, $I_{ss}(\lambda)$ is the total corrected steady-state intensity, and $\alpha_i(\lambda)\tau_i/[\sum_i \alpha_i(\lambda)\tau_i]$ is the fractional fluorescence of the ith component at wavelength λ (Donzel et al., 1974). Thus, DAS represent the fluorescence emission spectra associated with each individual decay component. Additional details regarding the TCSPC apparatus and data analysis can be found elsewhere (Hutnik & Szabo, 1989; Willis & Szabo, 1989).

Steady-state fluorescence measurements were performed on an SLM 8000C spectrofluorometer equipped with a Neslab Endocal refrigerated circulating bath for temperature control. Emission spectra were corrected for the wavelength dependency of the detection system and the incident light polarization (Roberts, 1981). Absorption spectra were measured on a Cary 219 spectrophotometer. Fluorescence quantum yields were determined at 20 °C using NATA (*N*-acetyltryptophanamide) in aqueous buffer, pH 7, as a quantum yield standard (ϕ = 0.14; Szabo & Rayner, 1980). The excitation wavelength was 295 nm, and the excitation band-pass and emission band-pass were both 4 nm. The absorbance of the samples was typically <0.10 at the excitation wavelength. The reported quantum yields were the mean of at least three determinations, the accuracy of which is reported as the standard error of the mean.

The circular dichroism spectra were measured on a JASCO J-600 spectropolarimeter with quartz cells (0.1- or 1.0-cm path length) at 20 °C. All spectroscopy was done with protein samples dissolved in 10 mM cacodylate buffer containing 150 mM KCl and 1 mM dithiothreitol, pH 7.0.

Results

Protein Homogeneity. A modification of the procedure of Horrocks and Collier (1981) was used to purify the isotype III component of cod parvalbumin. The major modification was the inclusion of an additional chromatographic step following ion exchange on DEAE-52 cellulose. This involved ion exchange on a DEAE-Sephacel column, with protein elution accomplished by a shallow NaCl gradient. Examination of the elution profile revealed three peaks (Figure 3) when fractions were monitored at A_{280}, with only the latter two exhibiting calcium binding activity. Electrophoresis of the major peak revealed single bands both upon SDS–PAGE (Figure 1, lane G) and upon isoelectric focusing (Figure 2, lane D). Determination of the amino acid composition yielded results identical with previous determinations (Closset & Gerday, 1976). The amino acid composition of the corresponding apo (metal-free) parvalbumin was used to determine the extinction coefficient at 280 nm. A value of ϵ_{280} = 7095 M^{-1} cm^{-1} was calculated. HPLC of tryptic fragments of the protein showed only one Trp-containing peptide to be present (based on the absorbance at 280 nm). On the basis of these criteria, the protein was considered homogeneous for spectroscopic use.

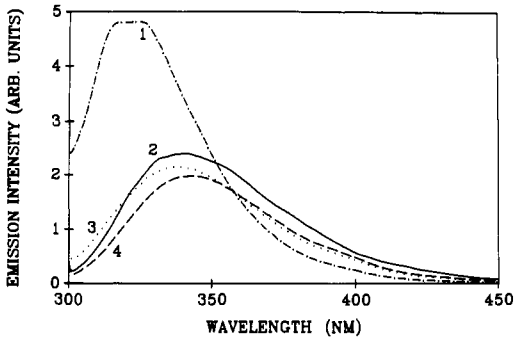

FIGURE 4: Corrected steady-state fluorescence emission spectra of EGTA-treated and untreated holo- and apo-TCA cod III parvalbumin. The protein concentration was approximately 20 μM in 10 mM cacodylate, 150 mM KCl, and 1 mM DTT, pH 7, 20 °C. λ_{ex} = 295 nm, concentration of EGTA = 1.2 mM. (1) Dashed–dotted line, holo; (2) solid line, holo + EGTA; (3) dotted line, apo; (4) dashed line, apo + EGTA.

Location of the Single Tryptophan Residue. Sequence analysis was performed on the only Trp-containing peptide found in the tryptic digest of homogeneous cod III parvalbumin. By alignment with existing parvalbumin sequences, this 20 amino acid residue peptide was found to represent the C-terminal peptide. The results revealed the following sequence: A^{88}-G-D-S-D-G-D-G-A-I-G-V-D-E-W^{102}-A-V-L-V-K^{107}. As the penultimate amino acid in the protein is lysine, the C-terminus (position 108) was cleaved by trypsin and hence was undetermined. The sequence established the location of the single tryptophan residue as position 102 of the 108 amino acid cod III parvalbumin sequence.

Spectral Results and Apoprotein Preparation. The absorption spectrum of holo native cod parvalbumin had a maximum at 280 nm and a sharp subsidiary peak at 289 nm (figure not shown). A considerable amount of structure was especially evident in the absorption spectrum below 280 nm. The corrected fluorescence emission spectrum had a λ_{max} = 321 nm (λ_{ex} = 295 nm) in the absence of added Ca^{2+}(aq) (Figure 4). TCA precipitation of holo cod III parvalbumin resulted in greater than 96% decalcification as judged by atomic absorption spectroscopy; typically, 0.03 mol of Ca^{2+}/mol of protein was measured. Concentrations of proteins were estimated by using the previously reported ϵ_{280} = 7180 M^{-1} cm^{-1} (Bhushana Rao et al., 1969) for the holoprotein or ϵ_{280} = 7095 M^{-1} cm^{-1} which was determined for the apoprotein in this study. Decalcification resulted in a complete loss of the 289-nm subsidiary peak of the absorption spectrum, as well as a reduction in spectral definition. The corrected steady-state

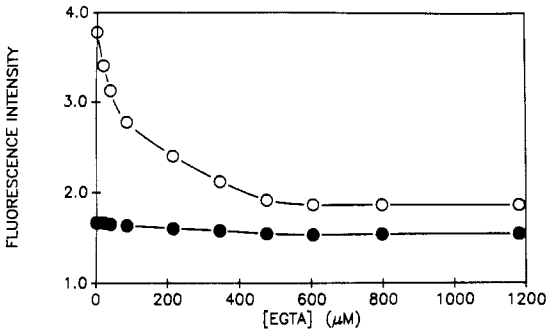

FIGURE 5: EGTA titration of holo- and apo-TCA cod III parvalbumin in 10 mM cacodylate, 150 mM KCl, and 1 mM DTT, pH 7, 20 °C. λ_{ex} = 295 nm; λ_{em} = 325 nm. (○) [holo] = 47 μM; (●) [apo] = 40 μM.

fluorescence emission spectrum showed that Ca^{2+} removal prompted a substantial red-shift (λ_{max} = 337 nm for apo; λ_{ex} = 295 nm), a broadening of the spectrum, and a quenching of the Trp fluorescence (Figure 4). Upon decalcification, the quantum yield decreased from 0.14 ± 0.01 to 0.09 ± 0.01 when excited at 298 nm, 20 °C, pH 7.

Addition of ≈30-fold molar excess of EGTA to holo cod III parvalbumin resulted in a greater red-shift of the emission spectrum compared to the spectrum of the apo-TCA protein [λ_{max} = 343 nm (holo + EGTA) vs λ_{max} = 337 nm (apo-TCA)]. The tryptophan fluorescence was quenched, but not to the same extent as that observed with the TCA-treated sample (Figure 4). When EGTA was added to an apo-TCA-treated parvalbumin, a 4–8-nm red-shift was observed (λ_{max} = 342 nm, EGTA:protein ratio = 60:1) as well as a slight decrease in the fluorescence intensity. When the holo sample was sequentially titrated with EGTA, a monophasic quenching of the Trp fluorescence was observed (Figure 5). At a EGTA:protein molar raito of 2:1, the amount of fluorescence quenching was only 57% of the maximum amount of quenching observed in the presence of a 10-fold excess of EGTA at pH 7. Following gel filtration or dialysis to remove the excess EGTA and/or Ca–EGTA complexes, the amount of Ca^{2+} remaining in the EGTA-treated samples was determined by atomic absorption spectroscopy. Results showed that 0.40 ± 0.1 mol of Ca^{2+}/mol of protein remained in the EGTA-treated sample. The fluorescence spectrum of the sample following gel filtration was the same as that observed prior to that treatment.

Metal Ion Addition to Parvalbumin. The corrected fluorescence emission spectrum of holo cod III parvalbumin showed a 5-nm blue-shift and shape change upon addition of excess Ca^{2+} (λ_{max} = 316 nm). Addition of the same excess of Ca^{2+} ion to the apo-TCA-precipitated protein restored the wavelength position of the spectrum (λ_{max} = 316 nm), narrowed the spectrum, and restored 93% of the fluorescence intensity as judged by quantum yield measurements (Figure 6). Ca^{2+} titration of an apo-TCA sample revealed progressive changes in the spectrum, with a plateau obtained at a molar ratio of 2 Ca^{2+}:1 protein (Figure 7).

Addition of excess Mg^{2+} to holo cod III parvalbumin resulted in a 4% decrease in the fluorescence intensity of the spectrum (corrected for dilution factor) and a negligible wavelength shift, or spectral broadening (Figure 8). Addition of the same excess of Mg^{2+} ion to apo-TCA parvalbumin produced a spectrum which in no way resembled that of the holo + Mg^{2+}. In fact, addition of a 250-fold excess of Mg^{2+} to apo-TCA protein caused only a slight (2–4 nm) blue-shift (λ_{max} = 335 nm) relative to the apo-TCA spectrum, no intensity changes (identical quantum yields were measured in

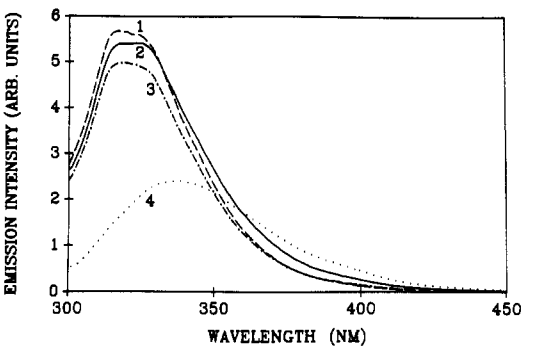

FIGURE 6: Corrected steady-state fluorescence emission spectra of holo- and apo-TCA cod III parvalbumin in the absence and presence of excess Ca^{2+} ion. The ϕ_f(holo + Ca^{2+}) = 0.14 ± 0.01 and ϕ_f(apo + Ca^{2+}) = 0.13 ± 0.01. The protein concentration was approximately 20 μM to which 1–2 μL of a concentrated $CaCl_2$ stock solution was added to yield a final concentration [Ca^{2+}] = 2.5 mM. λ_{ex} = 295 nm. (1) Dashed line, holo + Ca^{2+}; (2) solid line, holo; (3) dashed–dotted line, apo + Ca^{2+}; (4) dotted line, apo.

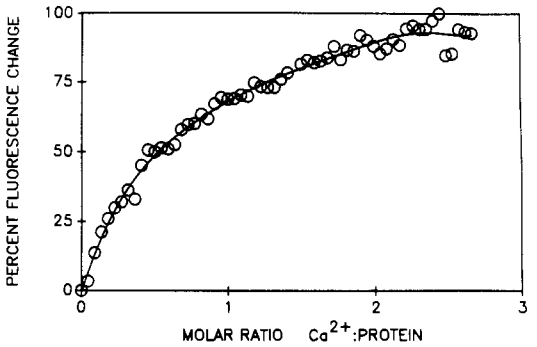

FIGURE 7: Ca^{2+} titration of TCA-treated parvalbumin plotted in terms of the difference between the fluorescence intensity at 325 nm at each metal ion ratio and the initial fluorescence intensity of the apoprotein. The concentration of the apoprotein prior to titration was 40 μM.

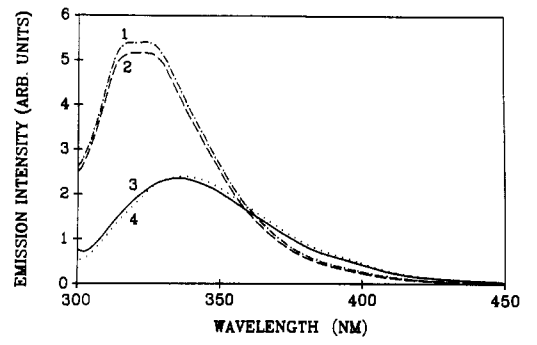

FIGURE 8: Corrected steady-state fluorescence emission spectra of holo- and apo-TCA cod III parvalbumin in the absence and presence of excess Mg^{2+} ion. The protein concentration was approximately 20 μM to which 1–2 μL of a concentrated $MgCl_2$ stock solution was added to yield a final [Mg^{2+}] = 5 mM. λ_{ex} = 295 nm. (1) Dashed–dotted line, holo; (2) dashed line, holo and Mg^{2+}; (3) solid line, apo + Mg^{2+}; (4) dotted line, apo.

the absence and presence of excess Mg^{2+}), and no spectral narrowing (Figure 8). However, addition of excess Mg^{2+} to EGTA-treated parvalbumin produced a significant blue-shift (λ_{max} = 326 nm), an increase in fluorescence intensity, and spectral narrowing (Figure 9). This EGTA-treated parvalbumin also responded to an excess of Ca^{2+} in a qualitatively similar manner as the apo-TCA-treated sample.

Circular dichroism (CD) was used as an independent method to assess the apparent inability of apo-TCA cod III parvalbumin to respond to Mg^{2+}. The CD spectra of the various apo-TCA-treated derivatives supported the fluorescence results. Both the near- and far-UV CD revealed that

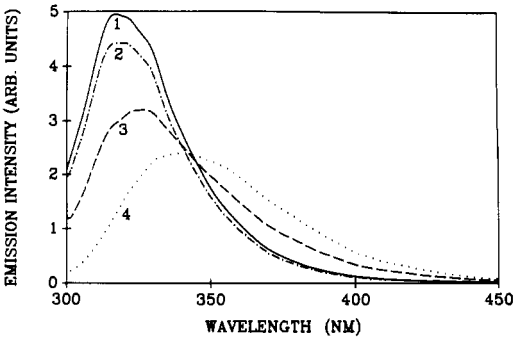

FIGURE 9: Corrected steady-state fluorescence emission spectra of holo cod III parvalbumin, holo + 1 mM EGTA, holo + 1 mM EGTA + 6 mM $CaCl_2$, and holo + 1 mM EGTA + 6 mM $MgCl_2$. The holoprotein initially was in a buffer composed of 10 mM cacodylate, 150 mM KCl, and 1 mM DTT, pH 7. The protein concentration was approximately 20 μM. λ_{ex} = 295 nm. (1) Solid line, holo + Ca^{2+}; (2) dashed–dotted line, holo + EGTA + Ca^{2+}; (3) dashed line, holo + EGTA + Mg^{2+}; (4) dotted line, holo + EGTA.

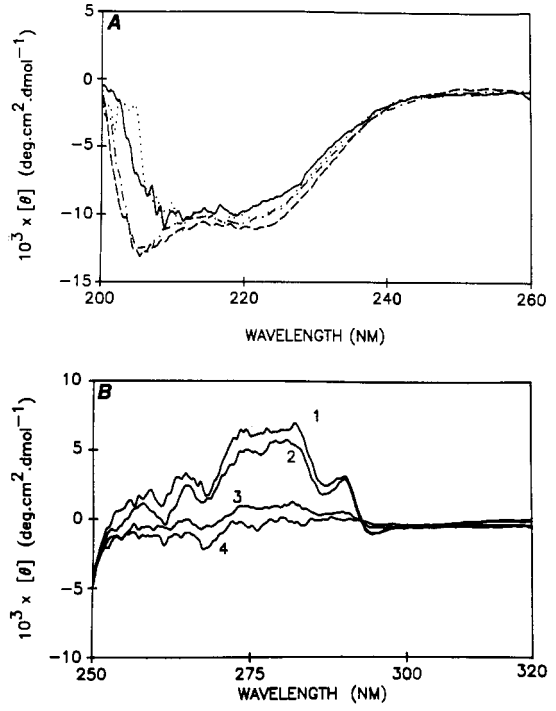

FIGURE 10: Near- and far-UV circular dichroism spectra of cod III parvalbumin holo, apo, and metal derivatives. (A) Far-UV CD; [protein] $\approx$ 20 μM. (––) holo + Ca^{2+}; (–·–) apo + Ca^{2+}; (···) apo + Mg^{2+}; (—) apo. (b) Near-UV CD; [protein] $\approx$ 150 μM. (1) holo + Ca^{2+}; (2) apo + Ca^{2+}; (3) apo + Mg^{2+}; (4) apo. The metal ions were added to a final concentration of 5 mM.

addition of Ca^{2+} to the apo-TCA-treated protein restored the majority of the spectral features associated with the holoprotein, but addition of Mg^{2+} had only a minor effect on the apoprotein spectra (Figure 10).

Time-Resolved Fluorescence. Multiexponential kinetics described the fluorescence decay of both holo and apo cod III parvalbumin. The results of decays from several emission wavelengths were analyzed by global analysis and are given in Table I. This latter analysis was possible owing to the wavelength independence of the lifetimes. The lifetime data were combined with the steady-state fluorescence data to generate decay-associated spectra (DAS, Figure 11A,B) which permitted a resolution of the contribution of each decay component to the total fluorescence. In the holo sample, two decay components were observed having lifetimes τ_1 = 3.55 ns and τ_2 = 1.54 ns. The DAS showed that the longest decay

Table I: Various Steady-State and Time-Resolved Fluorescence Decay Parameters of Holo- and Apo-TCA Cod III Parvalbumin (λ_{ex} = 295 nm; 20 °C)

sample[a]	ϕ_F	λ_{max}(ss)[b] (nm)	SVR[c]	τ_1 (ns)[d]	τ_2 (ns)	τ_3 (ns)	$\lambda_{max}(1)$[e] (nm)	λ_{max}(2) (nm)	$\lambda_{max}(3)$ (nm)	F_1[f]	F_2	F_3	C_1[f]	c_2	c_3	τ_r (ns)[g]
holo	0.14 ± 0.01	316	1.92	3.55 ± 0.03	1.54 ± 0.02		320	316		0.88	0.12		0.76	0.24		21.9
apo	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	26.1

[a] Both protein samples were in 10 mM cacodylate, 150 mM KCl, and 1 mM dithiothreitol, pH 7.0. In addition, 1 μM $CaCl_2$ was added to the holo sample to ensure complete occupancy of the metal binding sites. [b] λ_{max}(ss) represents the wavelength of maximum intensity in the corrected steady-state fluorescence emission spectrum. [c] Serial variance ratio (SVR) is a statistical parameter reflecting the goodness of fit. An SVR = 2 corresponds to a perfect statistical fit (McKinnon et al., 1977). The SVR values shown reflect the statistics obtained upon global analysis of 10 data sets. [d] The errors quoted for the lifetime values represent the standard errors for lifetime recovery from a given global set. [e] $\lambda_{max}(i)$ represents the wavelength (in nanometers) of the maximum intensity emission of the various components when excited at 295 nm. These values are obtained from the DAS (Figure 11A,B). [f] F_i and c_i denote the fractional fluorescence and relative concentrations, respectively, of the various decay time components at 320 nm. The c_i's were calculated by using equations described in detail elsewhere (Hutnik & Szabo, 1989). [g] 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 & Szabo, 1989).

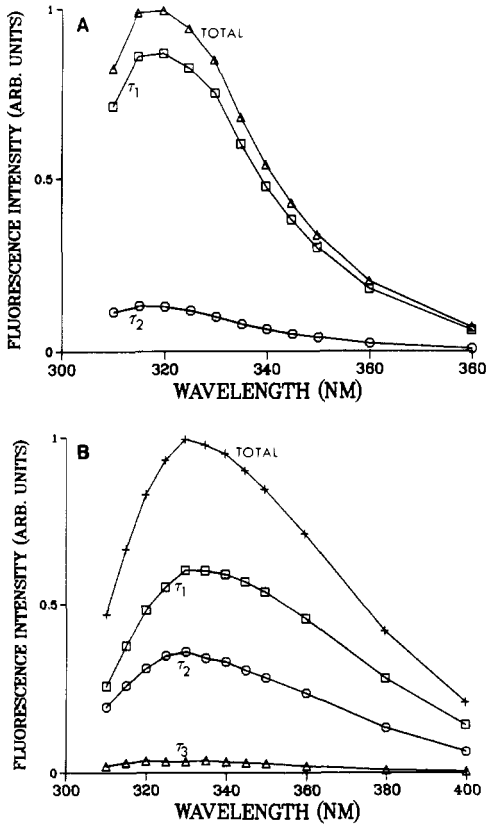

FIGURE 11: Decay-associated spectra for (A) holo cod III parvalbumin and (B) apo-TCA cod III parvalbumin. Spectra sum to the corresponding corrected steady-state spectral intensities normalized to a value of 1 unit at the emission maximum. Standard errors are within the contours of the plotted symbols.

component dominated the fluorescence (≈90%), and calculations revealed that the relative concentration of this component was 76% (see Table I). The relative component concentrations are proportional to the normalized preexponential terms if it is reasonably assumed that the radiative lifetime of each component is the same and static quenching is absent (Hutnik & Szabo, 1989). For holoparvalbumin, the λ_{max} of the spectrum of the longest decay time component (τ_1 = 3.55 ns) was 320 nm, while the spectrum of the shorter decay time component (τ_2 = 1.54 ns) had a λ_{max} of 316 nm (Figure 11A). Decalcification resulted in an increase in the lifetimes of the two components, τ_1 and τ_2, and a substantial red-shift of the spectra corresponding to both the longest (τ_1 = 4.39 ns; λ_{max} = 335 nm) and middle (τ_2 = 2.17 ns; λ_{max} = 330 nm) decay time components. Also, an additional short 361-ps component (λ_{max} = 330 nm), with a relative concentration of 28%, but only a fractional fluorescence of 4%, was required to adequately describe the decay kinetics of the apoparvalbumin. The relative concentrations of the other two decay components experienced a significant alteration upon decalcification (see Table I), with a substantial decrease in the concentration of the 4-ns decay component (31% apo vs 76% holo) and an almost 2-fold increase in the 2-ns component (41% apo vs 24% holo). Addition of Ca^{2+}(aq) to the apo-TCA parvalbumin resulted in decay kinetics indistinguishable from those of the original native holoprotein.

Discussion

Protein Homogeneity. In order to avoid ambiguous interpretation of the spectroscopic data, it is essential to ensure protein homogeneity. A frequently used method by which protein homogeneity is judged is that of SDS–PAGE, but it

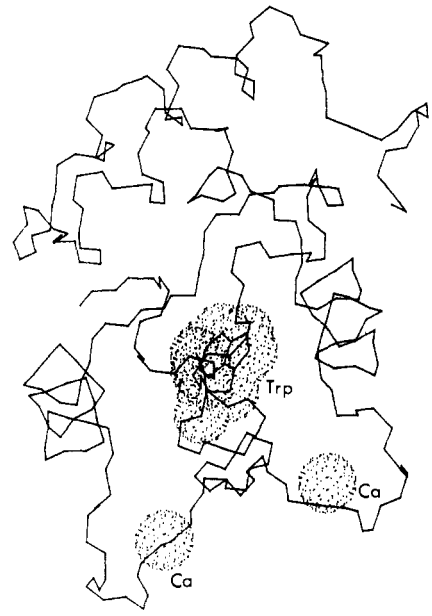

FIGURE 12: X-ray crystallographic structure of carp p*I* 4.25 parvalbumin in which phenylalanine in position 102 has been replaced with a tryptophan. The atomic spheres of the Trp and the two Ca^{2+} are also shown.

is inadequate as a definitive test. Although molecular weight homogeneity may be indicated, SDS–PAGE permits no assessment of purity based upon molecular charge. In the purification of the isotype III component of cod parvalbumin, isoelectric focusing indicated that the protein which appeared homogeneous by SDS–PAGE [Figure 1, lane E (single band)] still required another purification step [Figure 2, lane C (two bands)]. After DEAE-Sephacel chromatography, a rigorous assessment of protein purity was made by SDS–PAGE, isoelectric focusing, determination of the amino acid composition, and tryptic peptide analysis. Atomic absorption spectroscopy detected 2.1 ± 0.1 mol of Ca^{2+}/mol of protein in the final purified sample.

Location of the Single Tryptophan Residue. Previous amino acid composition determination (Closset & Gerday, 1976) and absorption spectroscopy (Bhushana Rao et al., 1969) revealed that the isotype III of cod parvalbumin contained tryptophan. The only reported attempt to sequence this protein placed the single Trp residue in position 109 of an anomalously long 113-residue protein (Elsayed & Bennich, 1975). Subsequently, the amino acid sequence of the only other parvalbumin known to contain Trp, whiting IIIb, was determined which located the Trp in position 102 (Joassin & Gerday, 1977). On the basis of inference, the Trp in cod III parvalbumin was assigned to position 102 but had never been established (Horrocks & Collier, 1981; Eftink & Hagaman, 1985). In order to be more definite in the interpretation of the fluorescence data, a Trp-containing, 20 amino acid residue segment of the homogeneous cod III parvalbumin was sequenced, and the location of the single Trp was established to be position 102. In Figure 12, the approximate location of the single Trp residue relative to the two Ca^{2+} binding sites is shown based upon the coordinates of the crystal structure of carp parvalbumin (Kumar, Lee, and Edwards, Brookhaven Protein Data Bank). The indole ring of the Trp is located approximately 11.6 Å equidistant from the two Ca^{2+} ions and is surrounded by a number of phenylalanine residues within the hydrophobic core.

Apoprotein Preparation and Problems with EGTA. Treatment of calcium binding proteins with the soluble che-

lators EGTA and EDTA has frequently been employed to create "apo" (Ca^{2+}-free) proteins, despite several reports of the method's inefficiency. Although some have reported greater than 95% decalcification of parvalbumins with EDTA at pH 7 (Cox et al., 1979), others have reported that the calcium cannot be removed unless the pH is above 8 (the apparent binding constant of Ca^{2+} to EDTA is approximately 100-fold higher at pH > 8 compared to the highest reported affinity constant of Ca^{2+} in any parvalbumin; Birdsall et al., 1979; White, 1988). At neutral pH, others have found that EGTA/EDTA are effective in complete protein decalcification only in the presence of 8 M urea (Sykes, personal communication).

Treatment of cod III parvalbumin with excess EGTA at neutral pH did not remove all of the Ca^{2+} from the protein solution. Atomic absorption spectroscopy revealed 0.4 ± 0.1 mol of Ca^{2+}/mol of protein remained after gel filtration or dialysis to remove Ca–EGTA complexes. It has been suggested that at neutral pH, EGTA can selectively remove one of the Ca^{2+} ions of parvalbumin (Nelson et al., 1976) and that under these conditions, it is the Ca^{2+} bound to the EF-loop. It was reasoned that this Ca^{2+} existed closer to the surface and had a water molecule as one of its liganding groups (Kretsinger & Nockolds, 1973). Because of the uncertainty regarding the extent of decalcification of the protein by EGTA treatment, EGTA–fluorescence titration curves, such as the one shown in Figure 5, cannot be used to provide quantitative information on the Ca^{2+} binding affinity of the two Ca^{2+} binding sites. In the absence of a direct measurement of Ca^{2+} content by atomic absorption spectroscopy, it is virtually impossible to relate the fluorescence change observed at each EGTA:protein ratio to the Ca^{2+} content of the protein. In Figure 5, it can be seen that, at pH 7 and at a ratio of 2 mol of EGTA:1 mol of protein, only a fraction of the total quenching response was apparent.

The fact that atomic absorption of this EGTA-treated protein detected 0.4 ± 0.1 mol of Ca^{2+}/mol of protein, even after gel filtration and/or dialysis, deserves comment. The error of the atomic absorption results was shown to be no more than 5% when measuring calcium standards or the native holo cod III protein. The best rationalization of the results was that the Ca^{2+} had been removed from both metal binding sites but Ca–EGTA complexes remained associated with the protein despite gel filtration and dialysis. Thus, the 0.40 mol of Ca^{2+}/mol of protein is a measure of the amount of Ca–EGTA complexes associated with the protein structure. Other workers have shown that apoparvalbumins interact strongly with these chelators (Lehky et al., 1977; Parello et al., 1979; Cave et al., 1979a). This problem of a tight association between EGTA/EDTA and protein is not limited to the parvalbumins as Kronman and Bratcher (1983) found that ^{14}C-labeled EDTA bound to α-lactalbumin and modified the equilibrium between different conformational states. In addition, the association of protein with EGTA is not dependent upon the presence of Ca^{2+} because further alteration of the spectral properties was observed upon EGTA addition to a sample of completely decalcified TCA-treated cod III parvalbumin.

The evidence presented above for the formation of a Ca–EGTA–protein ternary complex has important implications for the interpretation of the Ca^{2+} binding data presented by others. The interaction of EGTA with the protein makes it exceedingly difficult to obtain an accurate measurement of binding constants. The use of an EGTA–Ca^{2+} buffer system is one of the few methods available to achieve solution concentrations of Ca^{2+} below 10^{-8} M. Such low concentrations of Ca^{2+} are necessary to accurately measure high affinity binding constants. However, the interaction of EGTA with the protein alters the binding properties in such a way to make any measurements invalid. Indeed, in the type of data presented in this report, it is difficult to relate the metal ion status of the protein to its spectral properties if EGTA is present, and no direct measurements of Ca^{2+} content are performed.

Metal Ion Addition to Parvalbumin. The results in the present report obtained with EGTA and Mg^{2+} provide a clear example of the large influence the use of the chelator can have upon experimental results and conclusions. The absorption, CD, and fluorescence emission spectra revealed that decalcification resulted in drastic changes in the Trp region of the protein and, further, that greater than 93% of these changes were reversible upon Ca^{2+}, but not Mg^{2+}, addition. It is proposed that the ability of the EGTA-treated protein to respond to Mg^{2+}, as reported by Permyakov et al. (1987), was most likely due to Ca^{2+}, as opposed to Mg^{2+} binding. The addition of excess Mg^{2+} to a solution containing 20 μM protein and 1 mM EGTA would cause a displacement of Ca^{2+} from the Ca–EGTA complexes. The free Ca^{2+} ions would then be available to bind to the metal binding sites of the protein and promote the spectral changes observed. This explanation is preferred over the possibility that trace amounts of Ca^{2+} still present in the protein could permit a Mg^{2+} response in the presence of an excess of the latter. When excess Mg^{2+} was added to apo-TCA samples containing 0, 0.5, 1, and 2 mol of Ca^{2+}/mol of protein, no response to Mg^{2+} was observed. Clearly, in the absence of chelator, only Ca^{2+} induced distinct and reversible changes in the conformation of this parvalbumin. Therefore, it is suggested that reports that this protein responds to Mg^{2+} are the result of an experimental artifact due to the existence of an EGTA–Ca–protein complex.

Ca^{2+} titration of apo-TCA cod III parvalbumin (Figure 7) showed that fluorescence intensity changes occurred over the range of 0–2 mol of Ca^{2+}/mol of protein, with a plateau occurring upon the addition of 2 equiv of Ca^{2+}. Upon titration, it was found that at a molar ratio of 1:1, virtually the full wavelength shift (from 337 to 316 nm) was complete, even though the intensity changes were not. Thus, depending upon which fluorescence parameter is selected, different conclusions can be derived. In the absence of a direct measurement of Ca^{2+} content, the spectroscopic data can lead to ambiguous interpretations.

It is interesting that substoichiometric levels of Ca^{2+} could produce spectral changes typically interpreted as being indicative of a conformational change leading to a transfer of the single Trp residue from a rigid hydrophobic interior to the protein surface (Permyakov et al., 1985). On the basis of the work of others (Nelson et al., 1976; Coffee et al., 1974; Iio & Hoshihara, 1984), removal of Ca^{2+} from the EF-loop is thought to induce the bulk of the conformational changes associated with parvalbumin. In addition, it has been suggested that the EF binding site is much more flexible than the CD site, with the former being able to expand or contract relative to the Ca^{2+}-coordinating positions (Williams et al., 1984). The results reported here with cod parvalbumin support this fact insofar that full Ca^{2+} reconstitution is not required to recover the majority of the spectral changes. Although no attempt was made in the present study to estimate the binding constants in cod parvalbumin, the results suggest that the affinities of Ca^{2+} for the two sites are different. The full wavelength shift was complete when only 1 equiv of Ca^{2+} was present, which would imply that binding to one site was responsible for this effect, while binding of the second equivalent of Ca^{2+} was

Table II: Comparison of Fluorescence Lifetime Data of Tryptophan-102 in Cod III Parvalbumin and Whiting IIIb Parvalbumin

protein	temp (°C)	λ_{max}(ss)[b] (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	F_1	F_2	F_3	ref
cod III PV[a]	20								
holo		316	3.55	1.54		0.88	0.12		
apo[b]		337	4.39	2.17	0.361	0.59	0.37	0.04	Hutnik et al. (this report)
cod III PV[c]	25								
holo		315	4.20	1.71		0.604	0.396		Eftink & Hagaman (1985)
cod III PV[d]	25								
holo		316	4.61	2.22		0.83	0.17		
apo[e]		336	4.21	1.43		0.72	0.28		Eftink & Wasylewski (1989)
whiting IIIb PV[f]	20								
holo		320	3.5	2.0		0.80	0.20		
apo[g]		346	4.0	1.0		0.55	0.45		Permyakov et al. (1985)
whiting IIIb PV[h]	20								
holo		330[j]	4.580						
apo[i]		348[j]	4.26	1.28		0.86	0.14		Castelli et al. (1988)

[a] In 10 mM cacodylate buffer containing 150 mM KCl and 1 mM dithiothreitol, pH 7 (holoprotein with 1 μM $CaCl_2$). [b] Prepared by TCA precipitation (see Methods). [c] In phosphate buffer, pH 7. [d] In 50 mM Tris-HCl, pH 8.0 (holoprotein with 10 mM $CaCl_2$). [e] Prepared by addition of 10 mM EDTA to approximately 3×10^{-5} M protein. [f] In 50 mM Tris-HCl, pH 8.0. [g] Prepared by addition of excess EGTA. [h] In 20 mM borate buffer containing 1 M KCl and 0.1 mM dithiothreitol, pH 9.0. [i] Prepared by addition of EGTA to a final concentration equal to 3–4.5 times the protein concentration. [j] λ_{max} measured in the presence of 1 M KI, 20 mM borate, and 0.1 mM dithiothreitol, pH 9.0.

required to achieve the final conformation, and hence the final fluorescence intensity. The two binding constants estimated in whiting parvalbumin have been found to be different by 2 orders of magnitude (Permyakov et al., 1980), and thus it is possible similar differences may exist in cod parvalbumin owing to the virtual identity of the amino acid compositions.

Time-Resolved Fluorescence. Time-resolved fluorescence of the holo and apo forms of cod III parvalbumin permitted a detailed assessment of the conformational differences. The data of both holo- and apo-TCA parvalbumin revealed that the tryptophan residue, and hence the protein, existed in more than one conformationally distinct state. In Table II, the time-resolved fluorescence results of the single Trp residue in cod parvalbumin are compared to the results of other groups who have measured the fluorescence of both cod and whiting parvalbumins. Although the data base is small, most results (including those presented in this report) suggest that the fluorescence of Trp-102 in the holo (Ca^{2+}-bound) parvalbumin decays in a double-exponential manner. The only contradictory report is that of Castelli et al. (1988), who found the decay of whiting parvalbumin to be monoexponential. Although we cannot rule out the possibility that pH and ionic strength may be accounting for the discrepancy [as has already been suggested by Eftink and Wasylewski (1989)], it is also possible that differences in instrumentation may be responsible for different resolution capabilities. Although the lifetimes which we measure for holo cod III parvalbumin are among the shortest (τ_1 = 3.55 ns; τ_2 = 1.54 ns), there is relatively good agreement among the various reported values for both the lifetimes (τ_i's) and fractional fluorescences (F_i's). Perhaps the differences are reflecting the nature of the instrumentation used, as well as different buffer solutions and degrees of protein homogeneity.

The results reported herein distinctly show that upon decalcification three, instead of two, components were required to adequately describe the measured fluorescence decay. This is consistent with the idea that Ca^{2+}-free loops have a greater degree of conformational freedom compared to the Ca^{2+}-bound state (Herzberg et al., 1987). The decay-associated spectra (DAS) of the various components show that in the absence of Ca^{2+}, the spectra of both the longest and middle decay components were substantially red-shifted relative to the same two components of holoparvalbumin. Similar results were reported by both Permyakov et al. (1985) and Eftink and Wasylewski (1989). However, unlike Eftink and Wasylewski (1989), our results, similar to those of Permyakov et al. (1985), show a substantial change in the fractional contribution of the first and second components upon decalcification. The second component becomes relatively more dominant in the absence of Ca^{2+} (F_2 = 37% in apo; 12% in holo). In addition, the cod parvalbumin results in this report reveal a 4% contribution to the total fluorescence by a third component with a lifetime of only 361 ps. The DAS revealed that both the apo- and holoproteins possess conformational heterogeneity. In the case of the holoprotein, the two components possessed spectra typical of Trp located in a relatively hydrophobic, less solvent-exposed environment, with the short decay component having the most blue spectrum. This is supported by the sharp subsidiary peak at 289 nm in the absorption spectrum. The DAS of the apoparvalbumin revealed two conformational states in which the Trp was relatively more solvent-exposed (supported by the loss of the 289-nm peak in the absorption spectrum). Thus, the presence or absence of Ca^{2+} does not, by itself, impart conformational heterogeneity in the protein, but definitely determines the conformational distribution of the various states as judged by the degree of exposure of the Trp to the external solvent. In the absence of Ca^{2+}, additional conformational freedom is evident as judged by the appearance of an additional component which disappears upon Ca^{2+} reconstitution. This latter observation suggests that the third component does not reflect an irreversibly denatured form of the protein resulting from experimental conditions.

Some differences do exist between the various reports. In both the apo and holo forms of cod parvalbumin, the DAS revealed that the second component (τ_2) has a blue-shifted spectrum relative to τ_1 (see Figure 11 and Table I). Similar results were reported with the phase-resolved spectra of cod parvalbumin (Eftink & Wasylewski, 1989). This would suggest that the Trp conformation corresponding to τ_2 was in a relatively more solvent-shielded environment (blue-shifted spectrum) compared to τ_1. However, the opposite situation was reported for whiting parvalbumin (Permyakov et al., 1985). Using external quenchers such as Cs^+ and I^-, both Permyakov et al. (1985) and Castelli et al. (1988) found that the component with the shorter lifetime was more readily quenched than the component with the longer lifetime. In addition, they report that the short lifetime component had a red-shifted spectrum. This suggests that the DAS are capable of detecting differences between the cod and whiting parvalbumins which is surprising in view of the near-identity in amino acid composition and the identical position of the tryptophan residue. It is interesting that unlike Castelli et al.

(1988) and Permyakov et al. (1985), the cod III parvalbumin time-resolved data reported herein showed no dependence of lifetime upon emission wavelength. This condition has been shown to be necessary to render global analysis valid (Knutson et al., 1982).

The removal of Ca^{2+} resulted in a quenching of the Trp fluorescence quantum yield from 0.14 to 0.09. However, decalcification resulted in an increase in the lifetime values of both the first and second decay components, as well as the detection of a third short component. Permyakov et al. (1985) also found a quantum yield decrease despite an increase in the mean lifetime of the single Trp in whiting parvalbumin. This contrasts with the results of Castelli et al. (1988), who reported a 45% decrease in quantum yield, and a 35% *decrease* in the weighted fluorescence lifetime. Reasons for discrepancies in the lifetime data have been discussed above. However, in all cases, it is clear that changes in the quantum yield upon decalcification cannot be accounted for in the lifetime data. Given that $\phi_f = \tau_s/\tau_r$, the results can be rationalized in two different ways. One possibility is that the Trp in parvalbumin may be experiencing a greater degree of static quenching in the absence of Ca^{2+} which would decrease the ϕ_f. A second possibility is that the radiative lifetime, τ_r, is changing upon decalcification. This proposal has also been suggested by Castelli et al. (1988) for parvalbumin and by Hutnik and Szabo (1989) for azurin. It is possible to calculate the τ_r from the measured fluorescence parameters (Hutnik & Szabo, 1989) by using the equation:

$$\phi_f = c_1(\tau_1/\tau_r) + c_2(\tau_2/\tau_r) + c_3(\tau_3/\tau_r) \quad (1)$$

where ϕ_f is the total quantum yield of fluorescence, c_i is the relative concentration of the ith component, τ_i is the singlet lifetime of the ith component, and τ_r is the radiative lifetime, which is assumed to be the same for all components. Upon calculation, the following results are obtained: τ_r(holo) = 22 ns and τ_r(apo) = 26 ns. The longer radiative lifetime in apoparvalbumin is consistent with studies on model indole components. It was found that the radiative rate constant decreased (and hence τ_r increased) upon transfer of indole from a nonpolar to a more polar environment (Privat et al., 1979). Such a situation is suggested for the Trp in parvalbumin upon decalcification. The single Trp in cod parvalbumin is situated at the start of the F-helix, lying just outside of the EF-loop. On the basis of the crystal structure of troponin C, it has been suggested that decalcification of EF-hand structures in calcium binding proteins results in an increase in interhelical angles within a given binding domain (Herzberg & James, 1985). Thus, it is possible that in cod III parvalbumin the electronic environment of the indole nucleus of Trp-102 may be significantly perturbed by the interaction with the dipole of either the F- or the E-helix, the orientation of which is expected to change upon decalcification.

Conclusions

A Mg^{2+}–Ca^{2+} exchange is believed to underly the putative role of parvalbumin as a soluble relaxation factor in muscle contraction (Pechère et al., 1977). This would make the Ca^{2+} and Mg^{2+} binding properties of these proteins central to their function. However, considerable disagreement in the literature exists concerning the Ca^{2+} and Mg^{2+} binding properties of these proteins. Two important sources of these discrepancies are the use of soluble chelators for removal of Ca^{2+} and a lack of appreciation for differences that may exist among different parvalbumin molecules. The latter suggests that the formulation of detailed generalizations is difficult when discussing members of the parvalbumin subfamily.

With cod III parvalbumin from the β-lineage, the results of this report suggest that the completely decalcified protein could only regain its initial conformation upon Ca^{2+}, but not Mg^{2+}, binding. Hence, this parvalbumin exhibited a Ca^{2+}-specific conformational response. This presents some doubt regarding the generally accepted notion that identical perturbations result upon Ca^{2+} and Mg^{2+} binding to parvalbumins.

The observation of dramatic Ca^{2+}-specific conformational changes in a nonmodulatory parvalbumin leads to the question of whether in fact large Ca^{2+}-specific conformational changes are at the root of the observed modulatory properties of other proteins such as calmodulin and oncomodulin (Henzl & Birnbaum, 1988; MacManus et al., 1984). The combination of spectroscopy with protein engineering may offer some answers to the questions concerning metal specificity, conformational change, and the function of these and other calcium binding proteins (MacManus et al., 1989).

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Registry No. Ca, 7440-70-2; Mg, 7439-95-4; Trp, 73-22-3.

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