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# Evidence that deprotonation of Serine-55 is responsible for the pH-dependence of the parvalbumin $Eu^{3+}$ $^7F_0 \rightarrow {}^5D_0$ Spectrum

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The Eu(III) $^7F_0 \rightarrow {}^5D_0$ excitation spectra of the parvalbumins are highly pH-dependent. Below pH 6.0, they exhibit a sharp, partially resolved doublet centered near 5,795 Å. However, as the pH is raised, the spectrum becomes increasingly dominated by a much broader signal near 5,784 Å. This behavior has been traced to the Eu(III) ion bound at the CD site, but the identity of the moiety undergoing deprotonation remains uncertain. Site-specific mutagenesis studies on the parvalbumin-like protein known as oncomodulin now suggest that the species in question is a liganding serine hydroxyl group. Specifically, replacement of serine-55 by aspartate (the residue present at the corresponding position in the EF site) affords a protein that retains two functional lanthanide binding sites, but fails to undergo the pH-dependent spectral alteration. By contrast, replacement of aspartate-59 by glycine (the corresponding EF site residue) fails to abolish the pH-dependent behavior

Parvalbumin; Oncomodulin; Lanthanide; Europium; $Ca^{2+}$-binding protein

## 1. INTRODUCTION

Parvalbumins are small ($M_r \approx 12{,}000$), vertebrate-specific proteins containing two functional $Ca^{2+}$-binding domains [1,2]. Both domains exhibit the archetypal 'calmodulin fold' [3], consisting of a central metal ion-binding loop flanked by short α-helices. The two $Ca^{2+}$-binding sites in parvalbumin are commonly referred to as the CD and EF sites, a reference to the helical segments flanking the ion-binding loops. In general, they belong to the 'high-affinity' or '$Ca^{2+}/Mg^{2+}$' category – exhibiting substantial affinity for both $Ca^{2+}$ and $Mg^{2+}$ at physiological pH and ionic strength [4,5].

Similar to $Ca^{2+}$ in terms of size and coordination properties, $Eu^{3+}$ is a useful spectroscopic probe of calcium-binding proteins [6,7]. The $Eu^{3+}$ $^7F_0 \rightarrow {}^5D_0$ transition [8] has shown particular utility. Since ground-and excited states are both non-degenerate, this transition is immune to ligand-field splitting, resulting in a one-to-one correspondence between the number of binding sites and the number of spectral features. The $^7F_0 \rightarrow {}^5D_0$ transition has been used to study the ion-binding domains in various calcium-binding proteins.

The parvalbumin $^7F_0 \rightarrow {}^5D_0$ spectrum is noteworthy for its striking pH-dependence. At pH 5.0, the spectrum consists of a partially resolved doublet near 5,795 Å, reflecting the contributions from the $Eu^{3+}$ ions bound at the CD and EF sites. At higher pH, the doublet is replaced by a much broader spectrum centered at 5,784 Å. The $pK_a$ for this spectral alteration is 8.2 for parvalbumins from pike [9], rat [10], and chicken [11]. First observed more than a decade ago [12], the precise cause of this phenomenon remains uncertain. We now have evidence, based on $Eu^{3+}$ luminescence studies with site-specific variants of the parvalbumin-like protein called oncomodulin, that deprotonation of a coordinating serine hydroxyl is responsible for the behavior.

## 2. MATERIALS AND METHODS

$EuCl_3$ and $TbCl_3$ were purchased from Aldrich Chemical Co. $^{45}CaCl_2$ was purchased from DuPont-New England Nuclear. All other chemicals were reagent grade or better.

Site-specific variants of rat oncomodulin were produced by methods described previously [13–15]. Protein concentrations were determined with the Pierce protein assay reagent, employing wild-type oncomodulin of known concentration as a standard. Residual metal ions were removed from the protein preparations by passage over EDTA-derivatized agarose as described elsewhere [13]. Lanthanide solutions were standardized by titration with EDTA at pH 6.0, employing Xylenol orange as the indicator [16]. $Ca^{2+}$ dissociation constants were determined by flow-dialysis [17], as described previously [13], fitting the data to a two-site Scatchard equation.

$Eu^{3+}$ $^7F_0 \rightarrow {}^5D_0$ excitation spectra were obtained between 576.0 and 580.5 nm with the instrumentation described previously [9,10]. Emission was monitored at 618 nm, employing an effective band pass of

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*Abbreviations* MES, 2-(*N*-morpholino)ethane-sulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The following one-letter amino acid abbreviations appear in this manuscript: D, aspartate; E, glutamate; G, glycine; S, serine.


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1.6 nm. $Tb^{3+}$ titrations were performed on a Fluorolog II spectrofluorimeter (SPEX Industries, Edison, NJ).

## 3. RESULTS AND DISCUSSION

Oncomodulin is a parvalbumin-like protein that was first observed in extracts of a rat hepatoma [18,19]. Normal expression of oncomodulin is restricted to the fetal placenta [20], but it frequently reappears upon tumorigenesis [21]. Despite greater than 50% sequence identity with rat muscle parvalbumin, the CD site in oncomodulin is of the 'low-affinity' or '$Ca^{2+}$-specific' type [13,22]. Like other parvalbumins, the oncomodulin $^7F_0 \rightarrow {}^5D_0$ spectrum is pH dependent; however, the observed $pK_a$ value for the spectral alteration is 6.0 rather than 8.2 [10].

Originally, the parallel decrease in both signals of the low-pH doublet suggested that both ion-binding sites participate in the phenomenon and led Henzl et al. [9] to speculate that deprotonation of $Eu^{3+}$-coordinated water molecules was responsible for the pH-dependence. However, detailed analysis of the oncomodulin site-specific variant known as D59E recently revealed that the pH-dependent behavior in oncomodulin, and other parvalbumins by extension, is confined to the CD binding site [23]. This finding has forced us to reconsider involvement of an ionizable amino acid side chain in the spectral alteration. In attempting to identify this residue, we have focused on the sequence differences between the CD and EF binding loops.

The coordination geometry within the calmodulin fold is pseudo-octahedral, and the six liganding groups are denoted +X, +Y, +Z, −Y, −X and −Z, beginning at the N-terminal end of the binding loop [2,24]. The parvalbumin CD and EF sites differ most notably at the −X and +Z positions [2]. In the CD site, the −X residue is glutamate or aspartate; in the EF site, the −X residue is glycine. The pH-dependent behavior of the CD site $^7F_0 \rightarrow {}^5D_0$ signal is quite sensitive to the identity of the −X ligand. For example, replacement of aspartate-59 in oncomodulin with glutamate (to yield D59E) raises the observed $pK_a$ value for the spectroscopic alteration from 6.0 to 6.9 [13], while replacement of aspartate-59 with serine (to produce D59S) lowers the $pK_a$ to 5.4 (data not shown). Moreover, both mutations alter the position and appearance of the high-pH signal. However, replacement of aspartate-59 in oncomodulin by glycine (to produce D59G) fails to eliminate the pH-dependent behavior (Fig. 1A). Although the affinity of the CD site in D59G for $Ca^{2+}$ ($K_{Ca} = 7.7 \times 10^{-6}$ M) is reduced by an order of magnitude, relative to the wild-type protein ($K_{Ca} = 8.0 \times 10^{-7}\ M^{-1}$), the low- and high-pH $^7F_0 \rightarrow {}^5D_0$ spectra are largely unchanged.

The +Z ligand in the parvalbumin CD site is an invariant serine hydroxyl; in the EF site, it is an aspartyl carboxylate [2]. Replacement of serine-55 in oncomodulin by aspartate (to afford S55D) shifts the position of the low-pH CD signal to lower wavelength, resulting in the appearance of a shoulder in the $^7F_0 \rightarrow {}^5D_0$ spectrum near 5,792 Å (Fig. 1B). More significantly, the mutation eliminates the pH-dependent spectral alteration. Although the intensity of the spectrum is decreased slightly at pH 8.0, there is no hint of the high-pH signal near 5,781 Å.

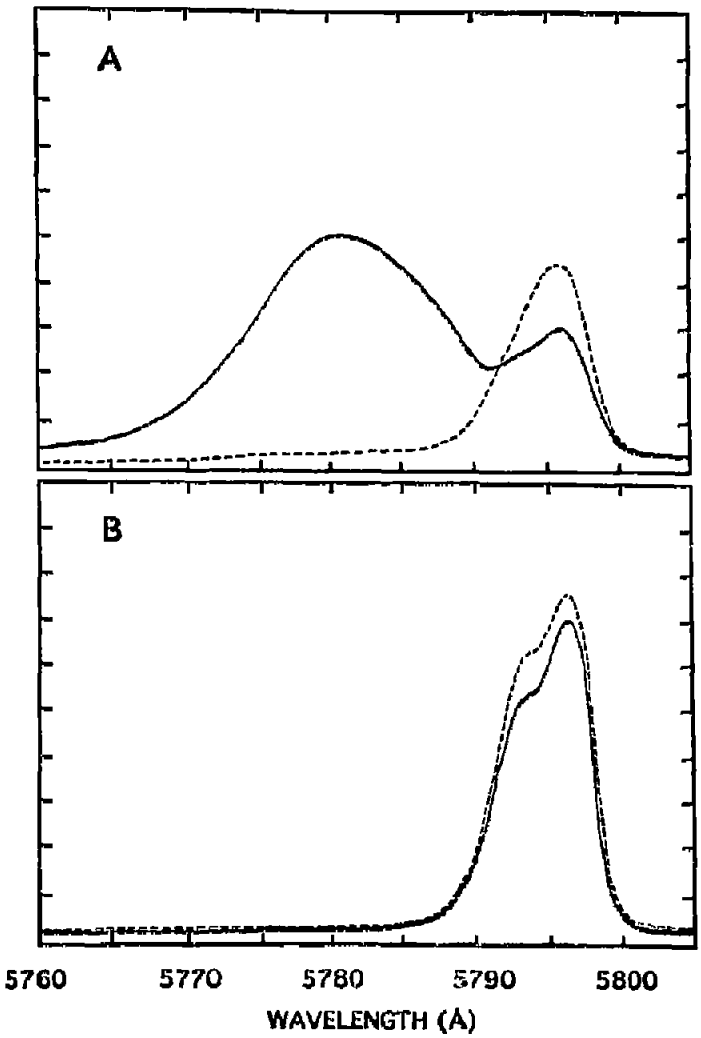


Fig. 1. $Eu^{3+}$ $^7F_0 \rightarrow {}^5D_0$ excitation spectra of $Eu_2$-D59G and $Eu_2$-S55D at pH 5.0 and pH 8.0. (A) Two equivalents of $Eu^{3+}$ were added to a $5.0 \times 10^{-5}$ M solution of the D59G variant at pH 5.0, in 0.15 M NaCl, 0.020 M MES, 0.020 M HEPES. After acquiring the $^7F_0 \rightarrow {}^5D_0$ spectrum (dashed line), the pH was raised to 8.0 with 1 M NaOH, and the spectrum was reacquired (solid line). (B) The identical experiment performed with the S55D variant.

The absence of pH-dependent behavior is not simply due to disruption of the CD binding domain by the introduction of an additional carboxylate. If a sample of the S55D variant is titrated with either $Tb^{3+}$ or $Eu^{3+}$, the lanthanide luminescence increases linearly until two molar equivalents of the ion have been added (Fig. 2). This result indicates that S55D retains two functional metal ion-binding sites.

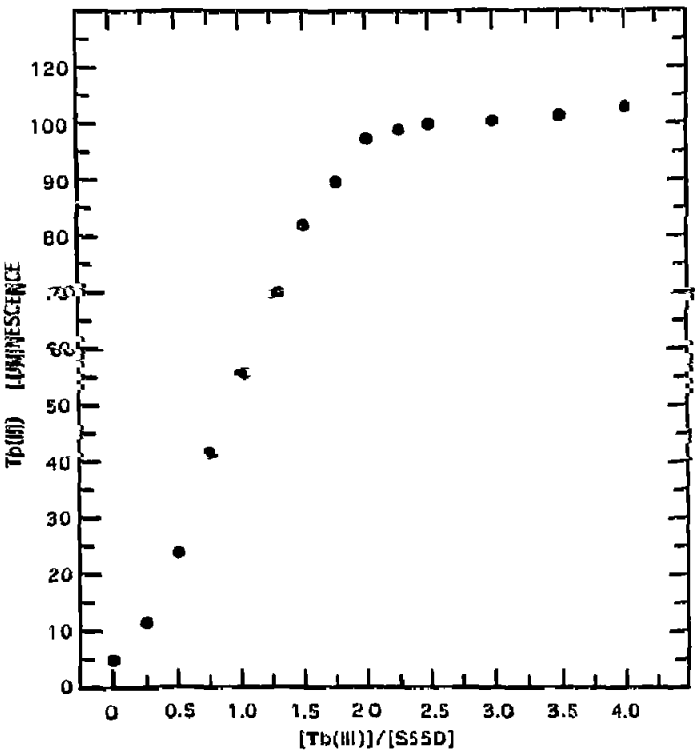


Fig. 2. Titration of S55D with $Tb^{3+}$. Aliquots of $Tb^{3+}$ were added to a $3.0 \times 10^{-5}$ M solution of the oncomodulin variant S55D, in 0.15 M NaCl, 0.025 M MES, pH 6.0. Following each addition, the sample was irradiated at 275 nm in an Aminco spectrofluorimeter, and the $Tb^{3+}$ luminescence (due to resonance energy transfer from adjacent tyrosine residues) was monitored at 545 nm. The excitation and emission bandpasses were approximately 5.5 and 11 nm, respectively. A cuvet having a 1.0 cm path length was employed for the experiment.

The insensitivity of the S55D spectrum to pH suggests that deprotonation of the serine-55 hydroxyl is responsible for the pH-dependent alteration of the parvalbumin $^7F_0 \rightarrow {}^5D_0$ spectrum. In general, alkyl hydroxyl groups are very weak acids, with typical $pK_a$ values between 13–16 [25]. Thus, the $pK_a$ values associated with the appearance of the high pH signals of oncomodulin (6.0) and other parvalbumins (8.2) might seem too low to reflect deprotonation of a serine side chain. However, proximity to highly charged metal ions can dramatically increase acidity. For example, although the $pK_a$ for water is 15.8, the hydrolysis constant ($pK_h$) for $Fe^{3+}$ is 3.05 [26]. In effect, coordination to ferric ion increases the acidity of the water molecule by a factor approaching $10^{13}$. The metal-dependent acid phosphatases have evolved to exploit this phenomenon, relying on $Fe^{3+}$ to generate a nucleophilic hydroxide ion at the active site under acidic conditions [27].

There is precedent, in the alkaline phosphatases, for the metal ion-facilitated deprotonation of serine at physiologically relevant pH values. It has been suggested [28] that the serine residue responsible for the initial attack on the phosphoester bond actually exists as a 'coordinated serine alkoxide' – its $pK_a$ value drastically reduced by coordination to one of the two zinc ions at the active site. Estimates of the hydrolysis constant for $Eu^{3+}$ range from 7.3 to 8.9 [29]. These values are significantly lower than that of $Zn^{2+}$ ($pK_h = 9.6$) [30], suggesting that the lanthanide ion should likewise be capable of facilitating deprotonation of a coordinated serine hydroxyl.

In the oncomodulin variant called D59S, aspartate-59 is replaced by serine. Interestingly, the $^7F_0 \rightarrow {}^5D_0$ spectrum of this variant at pH 8.0 exhibits prominent peaks at 5,775 Å and 5,782 Å (Fig. 3). Serine residues occupy two of the coordinating positions within the CD-binding loop of this protein. The presence of two distinct signals in the high-pH spectrum suggests that both side-chain hydroxyls may be capable of undergoing deprotonation. X-ray crystallographic data for the $Ca^{2+}$-bound forms of parvalbumin [31] and oncomodulin [32] indicate that the serine-59 hydroxyl would be too distant for direct coordination to the bound calcium ion. However, it is far from certain that the characteristic pentagonal bipyramidal geometry is retained in the $Eu^{3+}$-bound CD site at high pH. The pronounced shift in peak position and the enormous increase in linewidth (from 5 Å to 22 Å [18]) argue that the deprotonation event provokes a substantial change in coordination geometry.

In general, negatively charged ligands shift the $Eu^{3+}$ $^7F_0 \rightarrow {}^5D_0$ transition to lower energy, a trend that was attributed by Albin and Horrocks [33] to a *nephalauxetic* effect. In other words, the increase in volume resulting from ligand–ligand repulsion reduces the interelectron repulsion associated with pairing two 4f electrons in the $^5D_0$ excited state, thereby decreasing the separation between the F and D terms. The parvalbumin CD site, however, exhibits flagrant disregard for this general trend. First, as Albin and Horrocks noted, the position of the low-pH CD signal in carp and rabbit parvalbumins is more typical of a site having just two negatively charged ligands rather than four. Moreover, the nephalauxetic hypothesis predicts that replacement of serine-55 by aspartate (to give S55D) should shift the low-pH signal about 10 $cm^{-1}$ to *lower* energy. In reality, the signal is shifted to *higher* energy. Deprotonation of a coordinating hydroxyl group should likewise shift the signal about 10 $cm^{-1}$ to lower energy. Instead, the high pH signal appears roughly 40 $cm^{-1}$ higher in energy. These inconsistencies may reflect the uncharacteristic rigidity of the parvalbumin CD site.

Sykes and co-workers first remarked on the 'cryptand-like' nature of the CD site, as revealed by its interactions with the members of the lanthanide series [34]. In contrast to the parvalbumin EF site, which binds most tightly to the smaller lanthanides, the CD site displays greater affinity for larger members of the series. Sykes proposed that the flexibility of the CD ion-binding loop is limited by global protein folding considera-

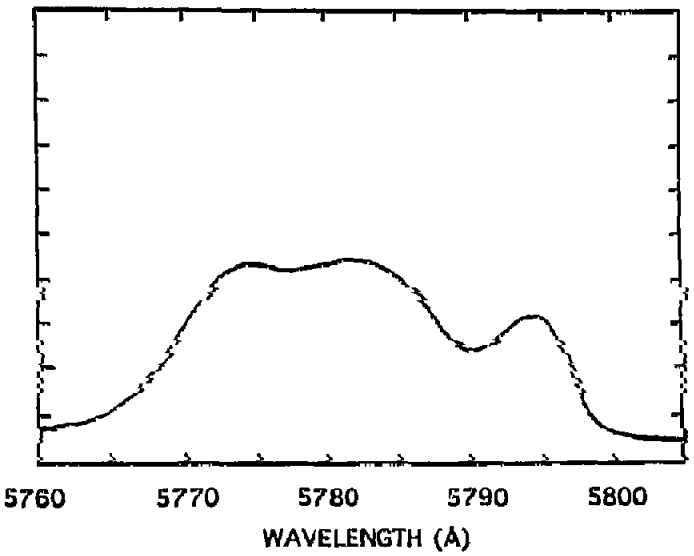


Fig. 3. $Eu^{3+}$ $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of the $Eu_2$-D59S at pH 8.0. Two molar equivalents of $Eu^{3+}$ were added to a $5.0 \times 10^{-5}$ M solution of the oncomodulin variant known as D59S, in 0.15 M NaCl, 0.020 M MES, 0.020 M HEPES. The pH was then adjusted to 8.0 with 1 M NaOH, and the $^7F_0 \rightarrow {}^5D_0$ spectrum was obtained.

tions, resulting in constrained metal–ligand bond distances. Whereas the EF loop can expand or contract to accommodate ions of variable size, the CD loop apparently defines a cavity of relatively fixed volume.

Nephalauxetic considerations are valid only when ligands are free to reorganize in response to alterations in charge. If metal–ligand distances are fixed by the folding of a polypeptide chain, an increase in ligand charge might actually accentuate the increased interelectron repulsion that accompanies the $^7F_0 \rightarrow {}^5D_0$ transition, increasing the F-D term separation and shifting the transition to higher energy. This explanation may account for the anomalous shifts in the CD site signal that accompany either replacement of the neutral hydroxyl ligand with a carboxylate or the proposed deprotonation of the coordinating hydroxyl. The relative magnitudes of the spectral shift are consistent with this hypothesis, since deprotonation of the hydroxyl would introduce substantially greater charge density into the inner coordination sphere than replacement of the hydroxyl by a monodentate carboxylate [35].

Of course, the data presented here do not rule out other possible explanations for the pH-dependence – e.g., an anomalously high carboxyl $pK_a$ value. However, any viable explanation of the phenomenon must be reconciled with its relative rarity. The pH-dependent behavior discussed herein is not generally observed with EF-hand proteins; rather, it appears to be restricted to the parvalbumins. An appealing aspect of the hydroxyl deprotonation hypothesis is that serine is relatively uncommon at the +Z position in members of the calmodulin superfamily [2,24]. Although additional studies will be required to verify that deprotonation of the serine-55 hydroxyl is responsible for the pH-dependence of the parvalbumin $^7F_0 \rightarrow {}^5D_0$ transition, the elimination of this behavior by replacement with a carboxylate argues that serine-55 is directly involved in the phenomenon.

Finally, it should be noted that both $Ca^{2+}$ and $Mg^{2+}$ have substantially lower charge densities than $Eu^{3+}$, as reflected by their hydrolysis constants – 12.7 and 11.4, respectively [36]. Therefore, we predict that neither ion would perturb the hydroxyl $pK_a$ sufficiently to promote ionization of serine-55 at pH values near neutrality. In all likelihood, the proposed deprotonation event would require occupation of the CD site by a lanthanide ion *having a charge density comparable to that of* $Eu^{3+}$.

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