Probing the Metal-Binding Sites of Cod Parvalbumin Using Europium(III) Ion Luminescence and Diffusion-Enhanced Energy Transfer†

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ABSTRACT: Excitation spectroscopy of the $^7F_0 \rightarrow {}^5D_0$ transition of Eu^{3+} and diffusion-enhanced energy transfer are used to study metal-binding characteristics of the calcium-binding protein parvalbumin from codfish. Energy is transferred from Eu^{3+} ions occupying the CD- and EF-binding sites to the freely-diffusing Co(III) coordination complex energy acceptors: $[Co(NH_3)_6]^{3+}$, $[Co(NH_3)_5H_2O]^{3+}$, $[CoF(NH_3)_5]^{2+}$, $[CoCl(NH_3)_5]^{2+}$, $[Co(NO_2)_3(NH_3)_3]$, and $[Co(ox)_3]^{3-}$. In the absence of these inorganic energy acceptors, the excited-state lifetimes of Eu^{3+} bound to the CD and EF sites are indistinguishable, even in D_2O; however, in the presence of the positively charged energy acceptor complexes, the Eu^{3+} probes in the cod parvalbumin have different excited-state lifetimes due to a greater energy-transfer site from Eu^{3+} in the CD site than from this ion in the EF site. The observation of distinct lifetimes for Eu^{3+} in the two sites allows the study of the relative binding site affinities and selectivity, using other members of the lanthanide ion series. Our results indicate that during the course of a titration of the metal-free protein, Eu^{3+} fills the two sites simultaneously. Eu^{3+} is competitively displaced by other Ln^{3+} ions, with the CD site showing a preference for the larger Ln^{3+} ions while the EF site shows little, if any, competitive selectivity across the Ln^{3+} ion series.

Parvalbumin is an acidic, low molecular mass (~12 000 Da) calcium-binding protein found mainly in the muscle tissue of vertebrates (Müntener et al., 1987; Gerday, 1988; Leberer et al., 1987). It is believed that parvalbumin plays a role in muscle relaxation, although no enzymatic function is known (Müntener et al., 1987; Gerday, 1988; Leberer et al., 1987; Haiech et al., 1979). Parvalbumins, in general, have 106–111 amino acids with high phenylalanine to tyrosine or high phenylalanine to tryptophan ratios (Kretsinger, 1980); cod parvalbumin specificially contains 107 residues (Hutnik et al., 1990). Parvalbumins have six α-helices, labeled A–F, connected by loops. The two Ca(II) ion binding sites are in the loops between the C and D helices (the CD site) and between helices E and F (the EF site).

The X-ray crystal structures have been determined for the carp p*I* = 4.2 (Moews & Kretsinger, 1975a; Kumar et al., 1990) and pike I (Declercq et al., 1988) isotypes; other isotypes are thought to be isostructural (Kretsinger, 1980). Although the structure was determined for the Ca(II)-bound form, there is evidence that Ln(III)[1] ions may occupy these sites if they are present (Moews & Kretsinger, 1975b; Sowadski et al., 1978; Corson et al., 1983; Breen et al., 1985a,b). Ln(III) ions are able to replace the calcium in the sites because they have similar ionic radii, coordination numbers, and preferences for oxygen donor ligands (Martin & Richardson, 1979; Horrocks, 1982). The fact that the Ln(III) ions effectively replace the spectroscopically-silent Ca(II) ion in the binding sites permits the exploitation of Ln(III) ion spectroscopic properties in the study of metal ion binding. (Nieboer, 1975; Martin & Richardson, 1979; Rhee et al., 1981; Horrocks & Albin, 1984; Henzl et al., 1985).

This laboratory has developed methods based on the unique properties of Eu(III) in the study of calcium-binding proteins (Horrocks & Sudnick, 1979, 1981; Rhee et al., 1981; Horrocks & Collier, 1981; Breen et al., 1985a,b; Horrocks et al., 1987; Horrocks & Tingey, 1988; McNemar & Horrocks, 1990). Eu(III) has a nondegenerate ground ($^7F_0$) and a nondegenerate photoemissive excited state ($^5D_0$). Exciting the $^7F_0 \rightarrow {}^5D_0$ transition (at about 580 nm) requires a powerful excitation source due to the low molar absorptivity of this transition, a result of its being Laporte-forbidden. One useful characteristic of this transition is that each different Eu(III) environment, in principle, produces a separate peak in the excitation spectrum. In the case of the very similar CD and EF sites of parvalbumin, however, only a single, asymmetric peak is observed (Breen et al., 1985a; Horrocks & Collier, 1981; Rhee et al., 1981; McNemar & Horrocks, 1990), making it difficult to study ion binding at the individual sites. Additionally, the excited-state lifetimes of the two classes of Eu(III) ions are indistinguishable (Horrocks & Collier, 1981; Rhee et al., 1981); this makes time-resolution (Horrocks et al., 1983; Horrocks & Tingey, 1988) impossible.

Diffusion-enhanced energy transfer has been used previously as a technique to study biological systems [see, for example, Wensel and Meares (1983), Wensel (1984), Horrocks et al. (1987), and McNemar et al. (1988)]. For this technique to be effective, the system must be in the "rapid diffusion limit" wherein the energy donor undergoes many collisions with the energy acceptor during the average lifetime of its excited state. Eu(III) and Tb(III) ions with relatively long lifetimes (on the order of milliseconds) are ideal luminescent energy donors for this technique, which is described in detail, with examples, elsewhere (Stryer et al., 1982).

The studies of cod parvalbumin presented here involve the use of Eu(III) ions as probes of the Ca(II)-binding sites where they serve as luminescent energy donors for the diffusion-enhanced energy-transfer experiments. A major objective of this study is to demonstrate the differential perturbation of the excited-state lifetimes of the Eu(III) ions in the two sites, allowing them to be resolved and studied individually. The energy acceptor complexes used were $[Co(NH_3)_6]^{3+}$, [Co-

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[1] Abbreviations: ox, oxalate ion ($C_2O_4^{2-}$); EDTA, ethylenediaminetetraacetate; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetate; Ln(III), any trivalent lanthanide series ion.

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$(NH_3)_5(H_2O)]^{3+}$, $[CoF(NH_3)_5]^{2+}$, $[CoCl(NH_3)_5]^{2+}$, $[Co(NO_2)_3(NH_3)_3]$, and $[Co(ox)_3]^{3-}$. Having resolved the excited-state lifetimes of Eu(III) in the two sites, traditional metal ion binding studies and competition experiments using various Ln(III) ions and Eu(III) can be performed. Additionally, we sought to elucidate the photophysical mechanism of energy transfer.

Energy transfer from excited Eu(III) to Co(III) complexes can occur by either the mechanism of Förster (1948) or the exchange-transfer mechanism proposed by Dexter (1953). Förster's theory entails a through-space weak dipole–dipole coupling nonradiative energy-transfer mechanism, capable of transfer over relatively long distances, reportedly up to 80 Å, depending on the nature of the acceptor (Stryer et al., 1982). If certain conditions are met, Förster-type energy transfer in the rapid-diffusion limit can be used to estimate the distance of closest approach of the energy donor to the acceptor. The exchange mechanism involves a collisionally-induced transfer of energy through an overlap of the wavefunctions of the donor and acceptor. This type of energy transfer occurs, predictably, over much smaller distances and is not easily related to the distance of closest approach.

Prior to the present studies, there were still questions concerning whether metal ion binding at the two sites is simultaneous or sequential. Cooperativity has been observed for Ca(II) binding [for example, see White (1988) and Castelli et al. (1988)], but earlier research with Ln(III) ions did not support the cooperativity model (Breen et al., 1985b). Also, there is evidence for both equality of binding constants (Parello et al., 1978; Cavé et al., 1979) and inequality of binding constants [for example, see Breen et al. (1985a) and Tanokura et al. (1986)]. We here report experiments performed using Ln(III) ions competing with Eu(III) to provide information concerning the relative binding affinities of the individual sites which may be compared with results obtained from NMR experiments on the carp pI = 4.2 isotype (Williams et al., 1983; Corson et al., 1983).

MATERIALS AND METHODS

Cod parvalbumin was purified from codfish filets as described elsewhere (Breen et al., 1985a). Protein solutions were made apo by stirring the solution with Chelex resin at pH 9–10 for about 12 h at 5 °C. All concentrations were determined by UV absorbance measurements using a molar absorptivity of 7180 M^{-1} cm^{-1} at 280 nm (McNemar, 1988). All Ln(III) ion concentrations were determined by titration with EDTA using an arsenazo indicator (Fritz et al., 1958). Concentrations of the cobalt complexes were determined using visible spectroscopy and experimentally verified molar absorptivities at the absorbance maxima. The UV and visible spectra were taken using a Varian-Cary 210 UV–visible spectrophotometer.

The excitation source used was a Quantel International (now Continuum) Nd:YAG pumped-pulsed (10 Hz, 60–90 mJ/pulse) tunable dye laser; the entire system is described in detail elsewhere (Tingey, 1987). The excitation wavelength was 579.3 nm unless otherwise noted. Luminescence at 614 nm was measured at 90° to the incident beam, the wavelength corresponding to the $^5D_0 \rightarrow {}^7F_2$ Eu(III) emissive transition. The protein samples (6–8 μM parvalbumin in 15 mM piperazine buffer, pD 6.3) were irradiated in 1-cm Uvonic quartz cuvettes. Although the separation of the excited-state lifetimes may be accomplished in H_2O, with similar results, D_2O solutions were used to minimize the OH oscillator effect in the Eu(III) deexcitation process (allowing for longer excited-state lifetime values). This aided the analysis of the excited-state decays and ensured that the systems were in the rapid-diffusion limit.

The excited-state lifetimes and the amplitudes of the individual exponential decay components were analyzed using a BASIC computer program, written for an IBM 9000 laboratory computer, which utilizes the Marquardt nonlinear regression algorithm (Marquardt, 1963); this program is listed and described elsewhere (McNemar, 1988). The experimental decays were fit to the equation:

$$I = I_0^1 \exp(-\tau_1^{-1} t) + I_0^2 \exp(-\tau_2^{-1} t) \quad (1)$$

where I is the intensity at time t, I_0 is the intensity at $t = 0$, τ_0^{-1} represents the decay rate constant, and superscripts 1 and 2 refer to each of the two components comprising the experimental decay curve; details of the resolution program may be found elsewhere (McNemar & Horrocks, 1989). By resolving the experimental decay using eq 1, the energy-transfer rates (change in τ^{-1} values as a function of energy acceptor concentration) corresponding to each of the two sites were obtained by determining the slope of the linear plots of reciprocal lifetime vs concentration acceptor, using a linear least-squares algorithm.

In the competition studies, the displacement of Ln^{3+} ions from the binding sites by Eu^{3+} ions was studied and analyzed in terms of the displacement equilibrium equations:

$$\mathrm{LnCD} + \mathrm{Eu}^{3+} \rightleftharpoons \mathrm{EuCD} + \mathrm{Ln}^{3+} \quad (2)$$

$$\mathrm{LnEF} + \mathrm{Eu}^{3+} \rightleftharpoons \mathrm{EuEF} + \mathrm{Ln}^{3+} \quad (3)$$

These two equilibria exist simultaneously and are assumed to be independent of each other since at all times during the experiment both sites are completely filled. The equilibrium constants of eq 2 and 3 may be determined using the equations and methodology presented by Albin et al. For example, for the equilibrium described by eq 2:

$$K_{dis}^{CD} = \frac{(I_0^{CD}/\alpha_{CD})\{[\mathrm{Ln(III)}]_{tot} - [\mathrm{CD + EF\ sites}] + (I_0^{tot}/\alpha_{tot})\}}{\{[\mathrm{CD\ site}] - (I_0^{CD}/\alpha_{CD})\}\{[\mathrm{Eu(III)}]_{tot} - (I_0^{tot}/\alpha_{tot})\}} \quad (4)$$

where the I_0, values are from eq 1, I_0^{CD} is the intensity at $t = 0$ which corresponds to the Eu(III) in the CD site, and α_{CD} is a constant relating the emission intensity of Eu(III) in the CD site to concentration; I_0^{tot} and α_{tot} refer to the respective values for the sum of the CD and EF sites.

RESULTS AND DISCUSSION

Resolution of Eu(III) Excited-State Lifetimes. An excitation spectrum of Eu(III) bound to cod parvalbumin, computer-resolved into its two component peaks, is shown in Figure 1. The peaks correspond to the two bound Eu(III) probes; the peak at the lower wavelength (579.1 nm) is assigned to the CD site, and the peak at the higher wavelength (579.4 nm) is assigned to the EF site. These assignments, discussed by McNemar and Horrocks (1990), are based on compelling Eu(III) spectroscopic evidence involving a comparison of rat oncomodulin and rat parvalbumin and the product of genetic engineering at the CD site in which a more parvalbumin-like oncomodulin in formed (Henzyl & Birnbaum, 1988; Hapak et al., 1989). Moreover, this assignment is consistent with the results of a variety of studies involving optical stopped-flow ^1H and ^{113}Cd NMR (Lee & Sykes, 1981; Corson et al., 1983, 1982; Williams et al., 1984; Vogel et al., 1983).

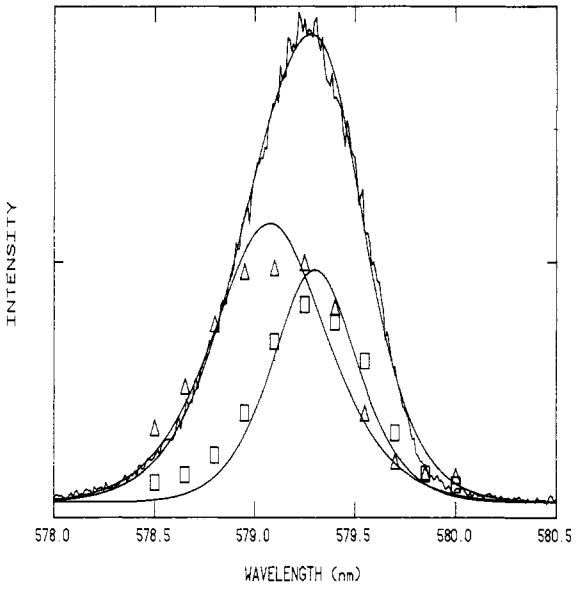

FIGURE 1: Computer-resolved excitation spectrum of $Eu(III)_2$–cod parvalbumin in the absence of an inorganic energy acceptor combined with a plot of the individual I_0 values for each of the two bound Eu(III) ions from a time-resolved study (△, CD site; □, EF site). The time-resolution is possible only in the presence of the positively charged inorganic energy acceptors (in this case, approximately 0.3 mM $[Co(NH_3)_6]^{3+}$).

Although the excitation spectrum is resolvable into two components, the excited-state decay curve is accurately fit by a single-exponential function, even in D_2O. However, upon addition of certain energy acceptor complexes (e.g., $[Co(NH_3)_6]^{3+}$), the decay curve becomes resolvable into two components, as depicted in Figure 2, which shows the experimental excited-state decay of Eu^{III}_2–parvalbumin in the presence of $[Co(NH_3)_6]^{3+}$.

As the energy acceptor complex is added to the Eu(III)-loaded protein solution, the reciprocal lifetimes, τ^{-1}, increase. The difference in the τ^{-1} values in the presence and absence of acceptor, plotted as a function of the concentration of energy acceptor, follows Stern–Volmer kinetics, as described by eq 5 where k_T is the observed energy-transfer rate and τ_c^{-1} is the

$$k_T = (\tau_c^{-1} - \tau_0^{-1})/c \qquad (5)$$

reciprocal lifetime at an energy acceptor concentration of c. These changes are exemplified by the inset of Figure 2 which shows a plot of the τ^{-1} values for Eu(III) in each of the sites vs the concentration of the $[Co(NH_3)_6]^{3+}$ energy acceptor. Energy-transfer rate values are obtained by linear least-squares fits of the plots of τ^{-1} vs concentration of acceptor.

Time-Resolution of the Excitation Spectrum. Figure 1 shows the individual I_0 values (eq 1) of Eu(III) in the CD (△) and EF (□) sites obtained at various wavelengths across the excitation spectrum superimposed on the computer-resolved excitation spectrum. Although this type of time-resolution analysis has been used successfully in this laboratory previously with other systems [for example, see Horrocks and Tingey (1988)], it must be stressed that this is possible with the cod parvalbumin only because of the presence of the inorganic energy acceptor. The presence of the acceptor does not appear to affect significantly the excitation spectrum of the protein; the points corresponding to the time-resolved spectrum conform well to the computer-resolved components of the spectrum. This indicates that the presence of the acceptor ion does not cause any significant distortion of the Eu(III) ion binding sites.

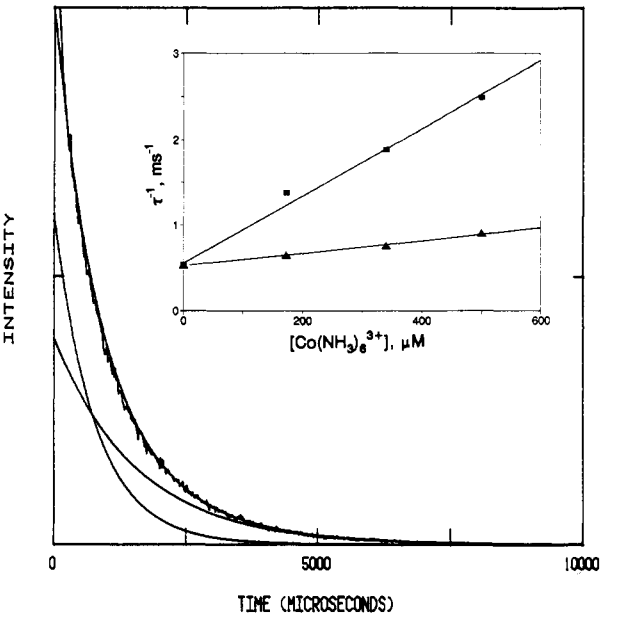

FIGURE 2: Excited-state luminescence decay for $Eu(III)_2$–cod parvalbumin in the presence of a positively charged inorganic energy acceptor which allows the decay to be resolved into two component single-exponential functions. The shorter lifetime component corresponds to the CD site while the longer lifetime component corresponds to the EF site. Inset: Stern–Volmer plots of the reciprocal lifetimes of the Eu(III) probes vs the concentration of the inorganic energy acceptor. The acceptor, $[Co(NH_3)_6]^{3+}$, accepts energy from the excited state of the Eu(III) in the CD site (■) more effectively than the Eu(III) in the EF site (▲), enabling the resolution of the two lifetimes. The change in the reciprocal lifetime with respect to the concentration of the $[Co(NH_3)_6]^{3+}$ acceptor is the diffusion-enhanced energy-transfer rate (CD site, 3800 ± 690 ms^{-1} M^{-1}; EF site, 600 ± 76 ms^{-1} M^{-1}).

The time-resolved spectrum (Figure 1) indicates that the peak at the lower wavelength corresponding to the Eu(III) in the CD site has the greater energy-transfer rate to the $[Co(NH_3)_6]^{3+}$ complex. The transfer rate for the EF-site Eu(III) ions closely resembles the rate obtained from the carp (pI = 4.2) isotype in preliminary studies performed in this laboratory. When the amino acid sequences for the two binding sites in the two isotypes (Kretsinger, 1980), shown in Table I, are compared, the only differences are in the CD-binding loop in which the codfish isotype contains two more negatively charged groups (Glu-52 and Glu-55) than does the carp isotype. The extra negative charge in the CD loop may be responsible for the increased energy transfer to the positively charged acceptor.

Energy Acceptor Studies. In order to examine the effect of the nature of the energy acceptor on the energy-transfer rate and to determine the mechanism of energy transfer, studies were carried out using a number of Werner-type cobalt(III) complexes. The complexes employed, $[Co(NH_3)_6]^{3+}$, $[Co(NH_3)_5(H_2O)]^{3+}$, $[CoF(NH_3)_5]^{2+}$, $[CoCl(NH_3)_5]^{2+}$, $[Co(NO_2)_3(NH_3)_3]$, and $[Co(ox)_3]^{3-}$, have different spectral properties and vary in charge, ranging from 3+ to 3–. The energy-transfer rates from the Eu(III) probes in parvalbumin to these Co(III) complexes are listed in Table II, where the EF site is the one containing the Eu(III) ion having the lower transfer rate (e.g., filled triangles in the inset of Figure 2) and the CD site is the one having the higher transfer rate. It is interesting to note that the excited-state decay can be resolved into two exponential decay curves (as shown in Figure 2) only with the positively charged acceptors.

A comparison is made between the transfer rates obtained for the cod parvalbumin and those obtained for the Eu(III)

Table I: Amino Acid Sequences of Parvalbumin-Binding Sites[a]

CD site	51	52	53	54	55	56	57	58	59	60	61	62
cod	Asp	Glu	Asp	Lys	Glu	Gly	Phe	Ilu	Glu	Glu	Asp	Glu
carp B[b]	Asp	Gln	Asp	Lys	Ser	Gly	Phe	Ilu	Glu	Glu	Asp	Glu
EF site	**90**	**91**	**92**	**93**	**94**	**95**	**96**	**97**	**98**	**99**	**100**	**101**
cod	Asp	Ser	Asp	Gly	Asp	Gly	Lys	Ilu	Gly	Val	Asp	Glu
carp B[c]	Asp	Ser	Asp	Gly	Asp	Gly	Lys	Ilu	Gly	Val	Asp	Glu

[a] Taken from Kretsinger (1980). [b] Underlined residues are coordinated to the metal ion in the crystal structure (Moews & Kretsinger, 1975a; Swain & Amma, 1989; Kumar et al., 1990). [c] Crystal structure indicates a coordinated water molecule in the EF site.

Table II: Diffusion-Enhanced Energy Transfer from Eu(III) in Cod Parvalbumin and Eu(III) Chelates to Cobalt(III) Acceptors

			energy-transfer rate constants (M^{-1} ms^{-1})			
acceptor	R_0[a]	J[b]	site CD[c]	site EF	$EuEDTA^-$	EuHEDTA
$[Co(NH_3)_6]^{3+}$	11.0	9.90	3800 (690)	660 (76)	11200	1800
$[Co(NH_3)_5(H_2O)]^{3+}$	12.6	22.8	6600 (440)	840 (100)	20400	3700
$[CoF(NH_3)_5]^{2+}$	13.5	34.4	4200 (400)	630 (24)	37400	3500
$[CoCl(NH_3)_5]^{2+}$	16.2	99.6	4900 (70)	990 (56)	12000	2600
$[Co(NO_2)_3(NH_3)_3]$	13.3	31.2		1200[d] (75)	3400	1700
$[Co(ox)_3]^{3-}$	26.2	1840		1100[d] (100)	7100	8700

[a] $R_0^6 = (8.78 \times 10^{-25})\kappa^2\Phi\eta^{-4}J$, where $\kappa^2 = 2/3$, Φ = quantum yield, η = refractive index, and R_0 is in angstroms. [b] The overlap integral $J = \int\epsilon(\nu)F(\nu)\nu^4\,d\nu/\int F(\nu)\,d\nu$ where $\epsilon(\nu)$ is the molar absorptivity, $F(\nu)$ is the luminescence intensity, and ν is the frequency. J has units of 10^{-18} cm^6/mol. [c] Standard deviation of the mean value is listed in parentheses. [d] Values arbitrarily placed in the EF-site column.

chelates $[Eu(EDTA)]^-$ and [Eu(HEDTA)], also listed in Table II (Cronce & Horrocks, 1990). This comparison reveals that $[Co(NH_3)_5(H_2O)]^{3+}$ is the best of the acceptors at resolving the lifetimes on the basis of the fact that the difference between the two rates is the largest among all acceptors. Although $[CoCl(NH_3)_5]^{2+}$ has a larger transfer rate constant for the EF site, its rate constant for the CD site is roughly 25% less than the corresponding rate for $[Co(NH_3)_5(H_2O)]^{3+}$. Surprisingly, the $[CoF(NH_3)_5]^{2+}$ acceptor, which exhibits very large transfer rate constants in the Eu(III) chelate systems, is only an average energy acceptor in the protein system. In general, however, there is no apparent relationship between the parvalbumin energy-transfer rates and those for either $[Eu(EDTA)]^-$ or [Eu(HEDTA)], nor is there a correlation between the protein energy-transfer rates and the values of the spectral overlap integrals or R_0 values (see footnotes to Table II).

An examination of the amino acid sequences for the two sites in the cod parvalbumin, listed in Table I, reveals eight negatively charged residues in the CD site compared with five in the EF site. This extra negative charge density in the CD site attracts the positive acceptors while repelling the negative $[Co(ox)_3]^{3-}$ ion. The neutral acceptor, having no net attraction to or repulsion from the negative metal-binding site environment, is likewise incapable of differential energy transfer.

Using eq 6, which assumes a Förster energy-transfer mechanism and no electrostatic bias, and the experimental transfer rates, k_T, listed in Table II, the distances of closest approach

$$a = (2.523R_0^6\tau_0^{-1}/k_T)^{1/3} \qquad (6)$$

were calculated. These calculated distances for the positive and neutral acceptors are in the range of 1–3 Å, obviously too small to be realistic. Even the calculated distance for $[Co(ox)_3]^{3-}$, 7 Å, is very likely too small. Even when the electrostatic influences are accounted for by using a potential of mean force factor (Cronce, 1991), the percentage of Förster-type energy transfer in the observed rate is still very small. For example, the amount of Förster-type energy transfer calculated for the $[Co(NH_3)_6]^{3+}/[Eu(EDTA)]^-$ system increases nearly 8-fold when electrostatic attraction is considered, but the energy-transfer rate obtained for this system, assuming the Förster-type energy-transfer mechanism, increases only from 3.49 to 27.7 M^{-1} ms^{-1}, a minor percentage of the observed 11 200 M^{-1} ms^{-1} rate constant. Similar findings occur for the other acceptor/donor systems presented in Table II and are not expected to be much different for the Eu(III)-substituted parvalbumin. These results imply that the exchange mechanism, as opposed to the Förster mechanism, accounts for the vast majority of energy transfer in both the protein and small-molecule systems.

The characteristics of these energy-transfer rate experiments can be explained in terms of electrostatics and the mechanism of energy transfer. The negative and neutral acceptors are not able to resolve the lifetimes because they have no net attraction to the metal-binding site which is in a negative environment. The positive acceptors, having a distribution favoring the CD site because of the electrostatics, collide more frequently with the protein than the neutral or negative acceptors, thus accounting for the ability of these positive acceptors to separate the two excited-state lifetimes and enable the luminescence decay to be resolved into the two components (eq 1). Since energy transfer occurs mainly through an exchange mechanism, the rates of this energy transfer are not expected to correlate well with the spectral overlap integrals or the R_0 values, and they do not.

Europium(III) Ion Binding to Cod Parvalbumin. The fact that the excited-state lifetimes are resolvable using the energy-acceptor complexes allows the two binding sites to be examined individually. Figure 3 is a plot of both the individual and total I_0 values vs equivalents of Eu(III) added to the apoprotein in the presence of 0.3 mM $[Co(NH_3)_6]^{3+}$ with excitation at 579.5 nm where the EF site contributes more to the intensity than does the CD site. The filled symbols are the intensities of the individual sites, and the open squares are the total intensities. This graph indicates that the sites are filling simultaneously up to a ratio of 2 mol of Eu(III)/mol of parvalbumin, after which no further binding is observed. This result is consistent with Eu(III) excitation spectra taken at various Eu(III) to protein ratios where the excitation peak shows no apparent shape changes during the course of a titration (McNemar, 1989). These observations are consistent

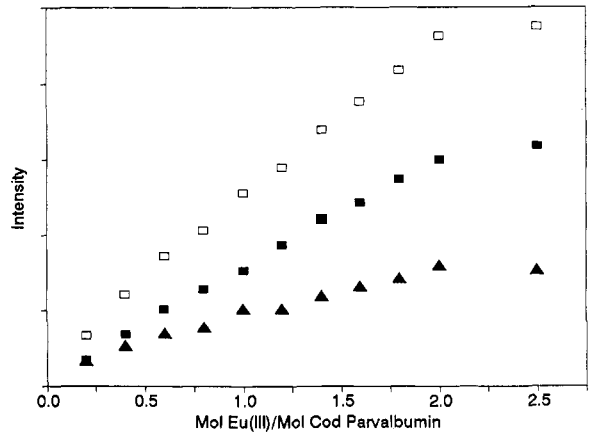

FIGURE 3: Plots of the I_0 values for the two resolved lifetimes, as determined from eq 1 (▲, CD site; ■, EF site), and the total I_0 (□) vs equivalents of Eu(III) added to metal-free parvalbumin. The titration was carried out in the presence of 0.3 mM energy acceptor, $[Co(NH_3)_6]^{3+}$, with excitation at 579.5 nm, where the EF site contributes more to the emission intensity than does the CD site.

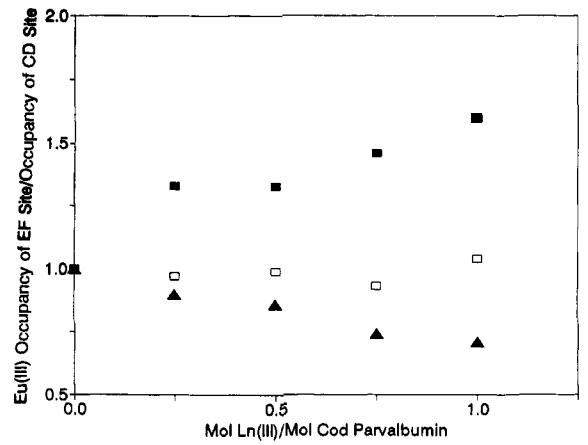

FIGURE 4: Plot of the ratios of the occupancies by Eu(III) of the EF site to those of the CD site vs moles of Ln(III) ion (■, La; □, Gd; ▲, Lu) added to parvalbumin already containing 2 mol of Eu(III)/mol of protein. The occupanies were determined from the I_0 values for Eu(III) emission from the individual sites.

with a positive cooperative binding model or with the binding affinities of the two sites for Eu(III) being very similar.

Lanthanide Competition Studies. In order to examine further and characterize the metal ion binding sites, and to help confirm the previously mentioned CD and EF assignments, metal-binding competition studies between the Eu(III) probes and other Ln(III) ions were performed. La(III), Gd(III), and Lu(III) were chosen because of their relative positions in the lanthanide series and also because of their electronic structure, which allows no energy transfer to them from the excited Eu(III).

Corson et al. (1983) used optical stopped-flow and proton NMR techniques to assign dissociation constants for La(III) and Lu(III) to the CD and EF sites of the carp (pI = 4.2) isotype. Their results indicate that La(III) and Lu(III) have equal dissociation constants for the EF site and that the dissociation constant for Lu(III) is greater than that of La(III) for the CD site. These authors conclude that the early Ln(III) ions have higher dissociation constants for the EF site than for the CD site, the later Ln(III) ions have lower dissociation constants for the EF site than for the CD site, and the middle Ln(III) ions have approximately equal dissociation constants for the two sites.

In the present study, changes in the Eu(III) emission intensity upon addition of Ln(III) ions are used to determine the relative affinities of the various Ln(III) ions compared to Eu(III). Cod parvalbumin containing 2 mol of Eu(III)/mol of parvalbumin, in the presence of 0.3 mM $[Co(NH_3)_6]^{3+}$, was titrated with the competing Ln(III) ions; the ratios of I_0 values (I_0^{EF}/I_0^{CD}), determined from eq 1, were used to determine the ratios of Eu(III) ion occupancy of the two sites. These ratios are plotted as a function of the amount of competing Ln(III) added, as shown in Figure 4. An increasing ratio indicates that Eu(III) is being displaced predominantly from the CD site; hence, the affinity of the CD site for Eu(III) is less than the corresponding affinity for the competing Ln(III) ion. Conversely, a decreasing ratio indicates that Eu(III) is being displaced predominantly from the EF site; hence, the affinity of the EF site for Eu(III) is less than the corresponding affinity for the competing Ln(III). An unchanging ratio indicates similar affinities of the two sites for both the Eu(III) and the competing Ln(III).

The results (Figure 4) show that the ratios increase for La(III), decrease for Lu(III), and remain relatively constant for Gd(III). Therefore, the affinity of the CD site for Eu(III)

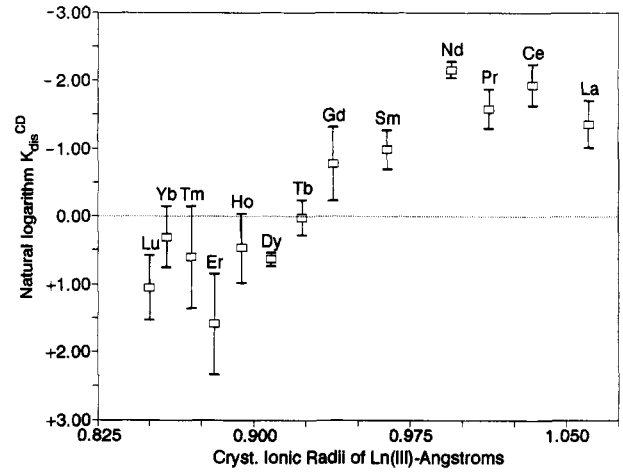

FIGURE 5: Plot of the natural logarithms of the equilibrium constants for the displacement (K_{dis}) from the CD site of the various Ln(III) ions by Eu(III) (eq 2) vs the crystal ionic radii.

is less than that of La(III), roughly equal to that of Gd(III), and greater than that for Lu(III). The present studies are in agreement with those of Corson et al. (1983), who used optical stopped-flow kinetics and proton NMR.

The present studies were extended to the other members of the Ln(III) ion series in order to confirm the above trends. Using changes in the I_0 values from eq 1, the equilibrium constants for the displacement of Ln(III) ions by Eu(III) ions (see eq 2 and 3) were determined for each of the sites. The results are shown in Figures 5–7. Figure 5 plots the displacement equilibrium constants for the displacement of Ln(III) by Eu(III) at the CD site, Figure 6 shows the analogous equilibrium constants for the EF site, and Figure 7 shows the ratios in the displacement equilibrium constants for the two sites all plotted against crystal ionic radii of the Ln(III) ions. These graphs show that the differences in the displacement equilibrium constants for the Ln(III) ions are due to the variation in the equilibrium constants for the CD site, favoring the larger Ln(III) ions (Figure 5); the EF site shows little, if any, change in these constants (Figure 6). Figures 5 and 6 reflect trends similar to those observed by Williams et al. (1983) and support the general conclusions made by Corson et al. (1983) concerning the carp (pI = 4.2) isotype.

The trends shown in Figures 5 and 6 reveal that the CD and EF sites represent two different binding site classes. Figure 6 reflects a binding site class (EF site) which does not

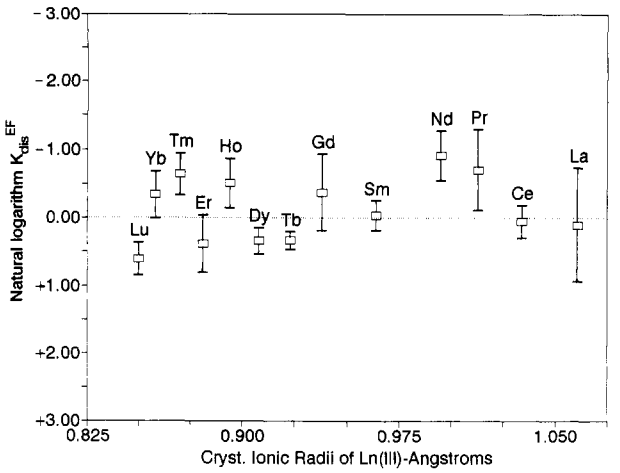

FIGURE 6: Plot of the natural logarithms of the equilibrium constants for the displacement (K_{dis}) from the EF site of the various Ln(III) ions by Eu(III) (eq 3) vs the crystal ionic radii.

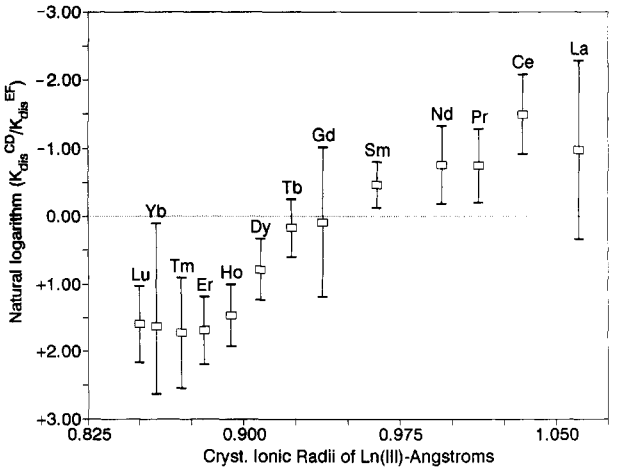

FIGURE 7: Plot of the differences in the natural logarithms of the displacement equilibrium constant (K_{dis}) values, $\ln K_{dis}^{CD} - \ln K_{dis}^{EF}$, across the Ln(III) ion series.

discriminate among metal ions on the basis of their size; Figure 5 reveals a binding site class (CD site) which selectively favors larger metal ions over smaller ones.

Conclusions. These results show that diffusion-enhanced energy transfer from excited-state Eu(III) ions occupying calcium-binding sites in parvalbumin to Werner-type cobalt(III) complexes in solution occurs via an exchange mechanism. This phenomenon causes the separation of two otherwise indistinguishable Eu(III) ion excited-state lifetimes, allowing for the individual examination of each of the two metal-binding sites. With systems like cod parvalbumin, diffusion-enhanced energy transfer may be used as a powerful analytical tool in probing questions of metal binding and protein electrostatics.

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Registry No. Eu^{3+}, 7440-53-1; Ca, 7440-70-2.