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Stopped-Flow Kinetic Studies of Metal Ion Dissociation or Exchange in a Tryptophan-Containing Parvalbumin[†]

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ABSTRACT: The rates of dissociation of 2 equiv of various metal ions [Ca(II), Cd(II), Pr(III), Nd(III), Sm(III), Eu(III), Gd(III), Tb(III), Dy(III), Ho(III), Er(III), Yb(III), and Lu(III)] from the primary CD and EF metal ion binding sites of parvalbumin (isotype pI = 4.75) from codfish (*Gadus callarius L*) were measured by stopped-flow techniques. The removal or replacement of metal ions was monitored by changes in sensitized Tb(III) luminescence or in intrinsic protein tryptophan fluorescence as quenching ions [Eu(III) or Yb(III)] were bound or removed or as the apoprotein was formed. In experiments wherein the bound metal ions were removed by mixing the parvalbumin with an excess of 1,2-diaminocyclohexanetetraacetic acid (DCTA), the kinetic traces were best fit by a double exponential with k_{off} rate constants of 1.07 and 5.91 s^{-1} for Ca(II), 1.54 and 10.5 s^{-1} for Cd(II), and ~0.05 and ~0.5 s^{-1} for all of the trivalent lanthanide ions. In experiments wherein the bound metal ions were exchanged with an excess of a different metal ion, pseudo-first-order rate constants were proportional to the concentration of excess attacking metal ion for both the fast and slow processes in most experiments. In these cases, extrapolation of the rate constants to zero concentration of attacking metal ion gave values which agree well with the DCTA scavenging results. This finding demonstrates that the off rate constants do not depend on the occupancy of the neighboring site and therefore implies that there is no significant cooperativity in metal ion binding between the two sites in parvalbumin.

Calcium-modulated proteins function by responding to the increase in cytosolic Ca(II) ion concentration which occurs upon cell stimulation. The binding of Ca(II) to such proteins results in a conformational change such that the calcium-loaded forms are then capable of interacting with target proteins, for example, to trigger muscle contraction in the case of troponin C or to activate a variety of enzymatic processes in the case of calmodulin (Kretsinger, 1980). Knowledge of the kinetic processes by which metal ions, particularly Ca(II), are bound or removed from this class of proteins is vital to an understanding of their mechanisms of action. For many of the same reasons that were outlined in the introduction to the preceding paper (Breen et al., 1985), we have chosen as the initial object of our studies in this area a parvalbumin isotype from codfish (*Gadus callarius L*) which contains a single tryptophan residue. In this study, we exploit the fluorescence properties of the tryptophan and its ability to sensitize the luminescence of bound Tb(III) to measure the kinetics of metal ion replacement or removal from the two principal Ca(II) binding sites in this protein, known as CD and EF. The question of possible cooperativity between the two sites in binding metal ions is addressed in this study.

MATERIALS AND METHODS

The preparation, purification, and metal ion removal from codfish parvalbumin and the sources of all chemicals used are as given in the preceding paper (Breen et al., 1985). Solutions of metal ion loaded protein were prepared by addition of 2.0–2.2 equiv of metal ion to apoparvalbumin. Stock solutions of the piperazine buffer solution, KCl, and *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (DCTA)[1] were all stored over Chelex chelating resin to absorb all contaminating di- and trivalent metal ions.

Kinetic experiments were carried out at 24.8 °C on a stopped-flow apparatus described elsewhere (Johnson & Porter, 1983). All protein solutions were buffered to pH 5.8 with 1 mM piperazine unless otherwise indicated and contained 0.5 M KCl. The time course of the kinetic reactions was monitored by means of changes in the intensity of the tryptophan fluorescence (340-nm band-pass filter, 14-nm bandwidth). Excitation was in all cases accomplished with UV radiation into the tryptophan absorption band (280-nm band-pass filter, 11-nm bandwidth). The kinetics of metal ion removal from parvalbumin were measured by mixing the metal ion bound form of the protein (~50 μM or less in concentration) with an excess of the metal ion chelator DCTA (1–10 mM in concentration) and following the decrease in Tb(III) luminescence when that metal ion is the one removed, or changes in the tryptophan fluorescence as the apoprotein is produced from the metal ion bound species [see Figure 4 of Breen et al. (1985)]. Metal ion replacement reactions were carried out by mixing a metal ion bound form of the protein with an excess of the substituting metal ion (1–10 mM in concentration). These reactions were followed either by

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[1] Abbreviations: DCTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ln(III), trivalent lanthanide ions.

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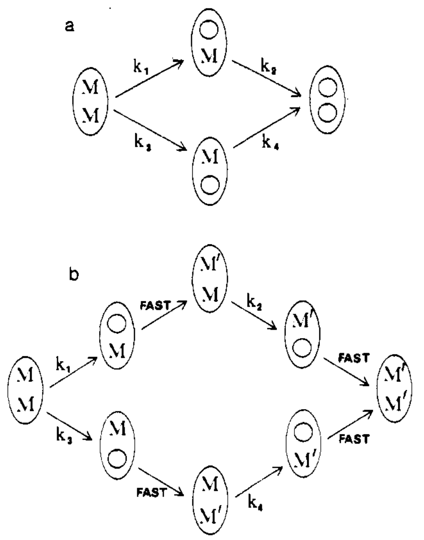

FIGURE 1: (a) Representation of fully metal-bound parvalbumin reacting with an excess of metal chelator (DCTA) to yield the metal-free protein. (b) Substitution reaction of parvalbumin bound with a metal ion M, with an excess of competing metal ion M′.

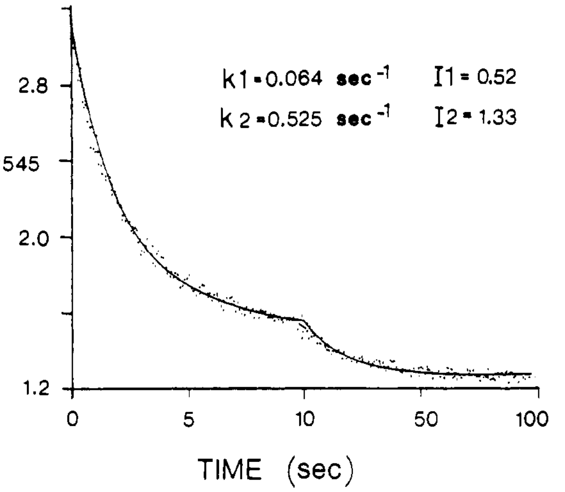

FIGURE 2: Typical kinetic trace obtained when DCTA (7 mM) is mixed with parvalbumin (19.5 μM) that is bound with 2 equiv of Tb(III) ion: λ_{ex} = 280 nm, λ_{em} = 545 nm, pH 5.8, T = 24.8 °C, 0.5 mM piperazine, 0.25 M KCl. The solid line is the fit of the data to a double exponential with the rate constants and starting intensities shown.

changes in sensitized Tb(III) luminescence when this ion was used as the attacking ion or was competed off by another ion or by the decrease or increase in tryptophan fluorescence as Eu(III) or Yb(III) was added or competed off, respectively [see Figure 5 of Breen et al. (1985)].

The kinetic traces obtained were typically double exponential in nature and were fitted by using the Marquardt (1963) method of nonlinear regression. This program is briefly described and listed elsewhere (Breen, 1984).

Theory and Calculations. Figure 1 depicts the basis for two types of experiments that were carried out on parvalbumin. Figure 1a represents the reaction sequence which occurs after mixing DCTA with the fully metal ion bound protein to yield apoparvalbumin as the end product. Figure 1b illustrates the substitution of a competing metal ion, M′, for the initially bound metal ion, M. These reactions are effectively irreversible, owing to the addition of large excesses of attacking reagent (DCTA or M′).

In the Appendix, the solutions to the differential equations describing the time dependence of each species in Figure 1 are outlined. For the case of Figure 1a, if the rate constants are independent of the occupancy of the neighboring site (no cooperativity, $k_1 = k_4$, $k_2 = k_3$), the time dependence of the observed fluorescence signal is given by

$$I(t) = \beta e^{-k_1 t} + \gamma e^{-k_2 t} + C$$

where β and γ represent the relative signal intensities associated with the two sites of parvalbumin and C represents the signal intensity from Tb(III) that is directly excited by the UV source as well as the dark current of the PM tube.

The case depicted in Figure 1b is similar to that just described for Figure 1a, except for one important detail. In Figure 1b, it is expected that $k_1 = k_4$ and $k_2 = k_3$, even if there were a cooperative interaction between sites, because the dissociation of a metal ion from one site always occurs in the presence of a metal ion in the other site. This is true because the competing metal ion will always be able to associate with an empty site much faster than the metal ion in the other site can dissociate ($k_{on} \sim 10^7$–10^8 s^{-1}) (Geier, 1965).

The fundamental difference between the two experiments illustrated in Figure 1 is that in the case of the DCTA experiment, the second metal ion is dissociating in the presence of an empty site. This does not happen in the metal substitution experiment. The only way the two experiments can yield the same rate constants, when both are fitted as double-exponential curves, is if the rate constants do not depend on the occupancy of the neighboring sites. Because cooperativity may be manifested by a change in rate constant due to the occupancy of the neighboring site, this experimental protocol provides a direct test for cooperative interactions between the sites on parvalbumin.

RESULTS

Kinetic Studies of Tb(III) Bound to Parvalbumin. When parvalbumin bound with 2 equiv of Tb(III) was mixed with a solution of either DCTA or a competing metal ion, double-exponential kinetic traces were obtained. Figure 2 shows a typical trace obtained when DCTA was mixed with parvalbumin bound with 2 equiv of Tb(III) ion (λ_{ex} = 280 nm; λ_{em} = 545 nm). The figure demonstrates the good fit that is obtained to a double exponential (solid line), the good ratio of signal to noise, and the split time base used to better resolve the two components of the double exponential. Figure 3a is a plot of the measured rate constants for the fast components of these double-exponential curves as a function of the concentration of DCTA or competing Ln(III) ion. Figure 3b exhibits plots of the corresponding values for the slow components of the double-exponential curves. The measured rate constants for Tb(III) bound to parvalbumin (Figure 3) vary directly with the concentration of competing metal ion. There is considerable scatter in the values obtained for the rate constants, due in part to the fact that they are extracted from a double-exponential signal. Linear least-squares fits of the points show, however, that the slopes are in error by typically ±12% and the y intercepts by ±14%. This margin of error is not large enough to obscure an underlying linear dependence of the rate constants on the concentration of the attacking ion M′. Linear least-squares fits to the data sets in Figure 3 converge on y-intercept values of $\sim$0.5 and $\sim$0.05 s^{-1} for the fast and slow components, respectively. These values are presumed to be the intrinsic off-rate constants, k_{off}, valid in the absence of an attacking metal ion, for the dissociation of Tb(III) from the two sites on parvalbumin. The linear increase in rate of Tb(III) dissociation from parvalbumin as a function of increasing concentration of competing metal ion, M′, implies the presence of an additional mechanism involving direct attack

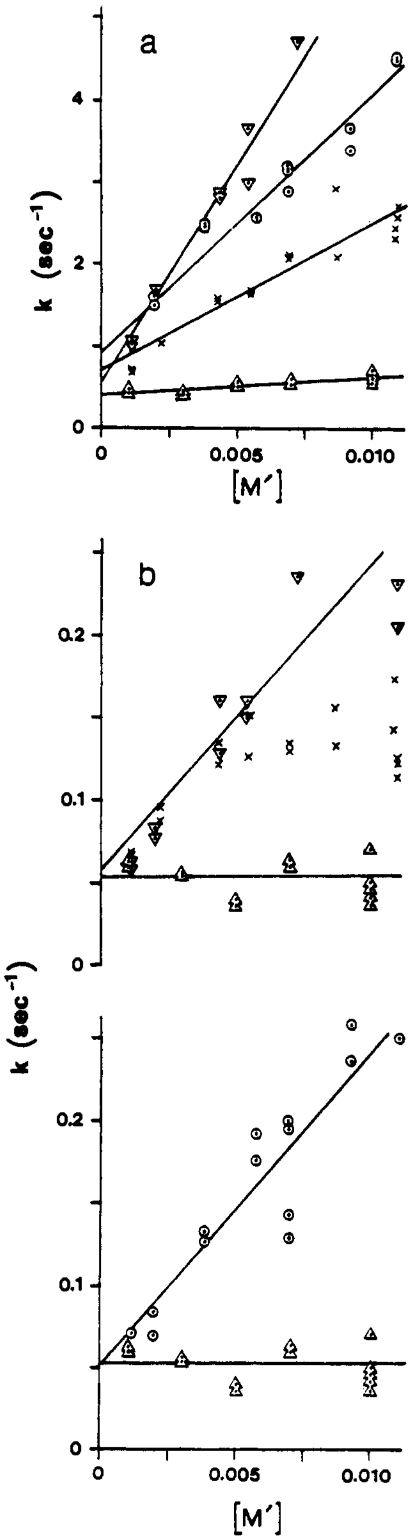

FIGURE 3: Fast (a) and slow (b) rate constants obtained from double-exponential fits of kinetic traces for the reaction of parvalbumin bound with Tb(III) ion with various concentrations of DCTA (△), Yb(III) ion (×), Gd(III) ion (○), and Nd(III) ion (▽). The lines represent least-squares fits of the data.

by the competing metal ion most likely by means of a mechanism involving a rapid equilibrium between free metal ion and metal ion bound to a subsidiary binding site on the protein. Spectroscopic and magnetic resonance evidence for binding of metal ions to a subsidiary site on parvalbumin has been presented elsewhere (Rhee et al., 1981; Cavé et al., 1979a,b). According to this model, the presence of an attacking ion in the subsidiary site has the effect of labilizing the ions in the primary CD and EF sites, leading to the observed dependence of the rate of exchange on the concentration of the attacking metal ion. The linearity of the plots [with the exception of the Yb(III) data[2] in Figure 3b] implies that the rate constant for this reaction is a pseudo-first-order rate constant, $k'[\mathrm{M}']$, which is equal to zero at the y intercept. The nonzero value at the y intercept must therefore correspond to the rate constant for dissociation of the bound metal ion from the protein in the absence of direct attack.

The rate constants calculated from the DCTA experiments show no dependence upon DCTA concentration, suggesting that DCTA is incapable of "speeding up" the Tb(III) dissociation the way that a competing metal ion can. Furthermore, the DCTA results are in good agreement with the values obtained in the metal ion substitution experiments by extrapolation to zero attacking metal ion concentration.

The dissociation of Tb(III) from parvalbumin can be followed in two different ways in a metal substitution experiment if Yb(III) or Eu(III) is used as the attacking ion. The disappearance of the sensitized Tb(III) luminescence or the quenching of the tryptophan fluorescence by either Eu(III) or Yb(III), as Tb(III) ion dissociates and is replaced by Eu(III) or Yb(III), can both be used to follow the time course of the reaction. For example, when 2.7 mM Yb(III) was mixed with parvalbumin (30 μM) fully bound with Tb(III) and the sensitized Tb(III) luminescence at 545 nm was monitored, a double-exponential decay was observed yielding rate constants of 0.120 and 0.912 s^{-1}. When, in the same experiment, the tryptophan fluorescence was monitored at 340 nm, the quenching of fluorescence as the Yb(III) bound yielded a double-exponential decay with rate constants of 0.105 and 0.888 s^{-1}. Good agreement of this type was generally observed when different means of monitoring a particular reaction were used.

An even more significant confirmation that the stopped-flow experiments provide a valid measurement of the processes indicated (Figure 1) is exemplified by data on Tb(III) removal by DCTA, monitored by two different means. For instance, in a typical experiment, a 30 μM solution of parvalbumin bound with 60 μM Tb(III) was mixed with 10 mM DCTA. Monitoring the decrease in sensitized Tb(III) luminescence at 545 nm yielded $k_{\mathrm{off}}^{\mathrm{EF}}$ and $k_{\mathrm{off}}^{\mathrm{CD}}$ values of 0.046 and 0.603 s^{-1}, respectively, while following the decrease in protein fluorescence as the apoprotein formed resulted in respective rate constants of 0.043 and 0.618 s^{-1}. Since the former measurement clearly tracks Tb(III) dissociation from the protein, while the latter presumably reflects the conformational changes which accompany metal ion removal, the agreement in the results demonstrates that the two processes occur simultaneously.

When parvalbumin bound with 2 equiv of Tb(III) was mixed with DCTA at pH 7.0, a double-exponential trace was again observed, yielding rate constants of 0.051 and 0.528 s^{-1}. These values agree well with those obtained at pH 5.8, which implies that the third metal ion binding site, with its weaker binding constant and lower quantum yield, does not interfere with the measurements (Rhee et al., 1981). The k_{off} values appear to be independent of pH in the range 5.8–7.0.

Kinetic Studies of Lu(III) Bound to Parvalbumin. Lu(III), being at the very end of the series of Ln(III) ions, was chosen

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[2] The apparent leveling off of the smaller pseudo-first-order rate constant at higher concentrations of attacking Yb(III) ion is not understood but cannot be due to saturation of the subsidiary site by this ion since this behavior is not observed for the larger rate constant. It is most likely artifactual.

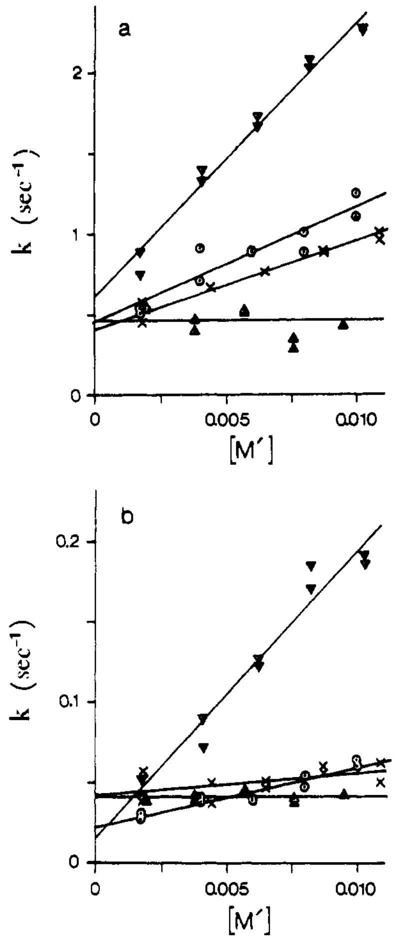


FIGURE 4: Fast (a) and slow (b) rate constants obtained from double-exponential fits of kinetic traces for the reaction of parvalbumin bound with Lu(III) ion with various concentrations of DCTA (▵), Yb(III) ion (×), Tb(III) ion (○), and Eu(III) ion (▿).

for detailed study in order to obtain a comparison with the more strongly binding Ln(III) ions in the middle of the series, such as Tb(III). The results, plotted in Figure 4 for Lu(III) bound to parvalbumin, however, are rather similar to those obtained for Tb(III). As with Tb(III), double exponentials were observed, and the measured rate constants again vary linearly with the concentration of attacking metal ion, while showing no dependency on the concentration of DCTA. As before, all plots have nearly common intercepts, with values of ~0.44 and ~0.038 s^{-1} corresponding to the fast and slow processes, respectively. The only noteworthy difference between the results for Tb(III) and Lu(III) is the finding that the measured rate constant for the slower component does not seem to be significantly affected by increased concentrations of the attacking ions Yb(III) or Tb(III) (Figure 4b). This implies that lability of the Lu(III) ion bound at the tighter EF binding site on parvalbumin is relatively unaffected by the binding of certain competing metal ions to the subsidiary site if this is, in fact, the operative mechanism. The rate constants extrapolated for Tb(III) and Yb(III) coincide quite well with those obtained by using DCTA. These values are taken to be the rate constants for dissociation of Lu(III) from parvalbumin in the absence of assistance by the attacking metal ion. The Eu(III) ion, however, appears to be fully capable of increasing the rate of the slower dissociation of Lu(III).

Kinetic Studies of Ca(II) Bound to Parvalbumin. The metal ion of greatest interest in this study is, of course, Ca(II), since this is the ion of physiological importance. The stopped-flow kinetic data (Figure 5) are interesting in that the kinetic traces observed in the metal ion substitution experiments were single exponentials for calcium-bound parvalbumin. The rate constants from these single-exponential traces increase linearly with increasing concentration of competing metal ion, M′, and the *y* intercepts of these lines also fall between the values of the rate constants obtained from the DCTA experiments, where double-exponential behavior is observed: k_{off} values of 5.9 and 1.0 s^{-1} were obtained. These values are close to those reported by White et al. (1979) for whiting parvalbumin: 5 and 2 s^{-1}.

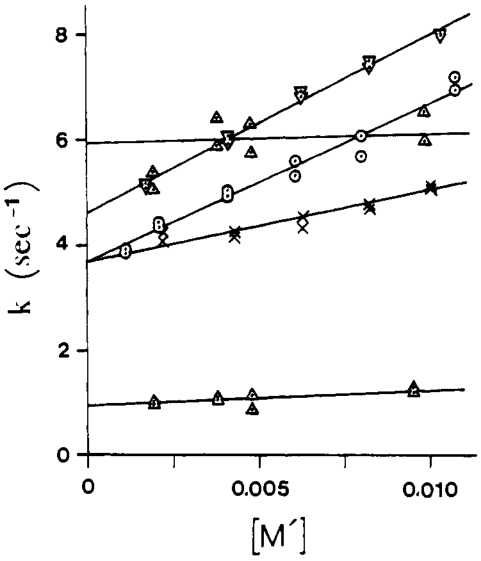


FIGURE 5: Single-exponential rate constants for the reaction of parvalbumin bound with Ca(II) ion with various concentrations of Yb(III) ion (×), Tb(III) ion (○), and Eu(III) ion (▿), along with the rate constants from double-exponential fits for reaction with DCTA (▵).

It is, however, possible to fit the traces obtained at low concentrations of attacking metal ion to two exponentials. For example, with Eu(III) at 1.7 mM used as the attacking species, a double exponential with rate constants of 4.2 and 7.4 s^{-1} nicely fits the data. As the concentration of attacking metal ion increases, however, the kinetic traces rapidly become exclusively single exponential in nature. This behavior could arise if the rate of dissociation of Ca(II) in the tighter EF binding sites on parvalbumin (slower releasing) was increased more readily by the attacking metal ion than that at the other, weaker binding site. Thus, the two rate constants may be approaching one another as the concentration of attacking metal ion increases. Since the k_{off} values in the absence of any direct attack, 5.9 and 1.0 s^{-1}, are already relatively close to each other, this explanation appears reasonable.

Kinetic Studies of Cd(II) Bound to Parvalbumin. Considering the utility of ^{113}Cd and ^{111}Cd as NMR probes of calcium-modulated proteins (Vogel et al., 1983), it was of interest to examine the kinetics of Cd(II) ion exchange and removal from parvalbumin. The results of these experiments are plotted in Figure 6. The kinetic processes observed involve two exponentials, even in the metal ion replacement cases. The rate constants for the fast and slow components obtained from the DCTA scavenging experiments are ~11 and ~2 s^{-1}, respectively, each approximately a factor of 2 greater than the corresponding value with Ca(II).

Rate Constant (k_{off}) Values from DCTA Scavenging Experiments across the Ln(III) Ion Series. The dissociation of Ln(III) ions from parvalbumin, measured by sequestering the free metal ion with DCTA, follows a double exponential in all cases. The average values obtained for all of the Ln(III)

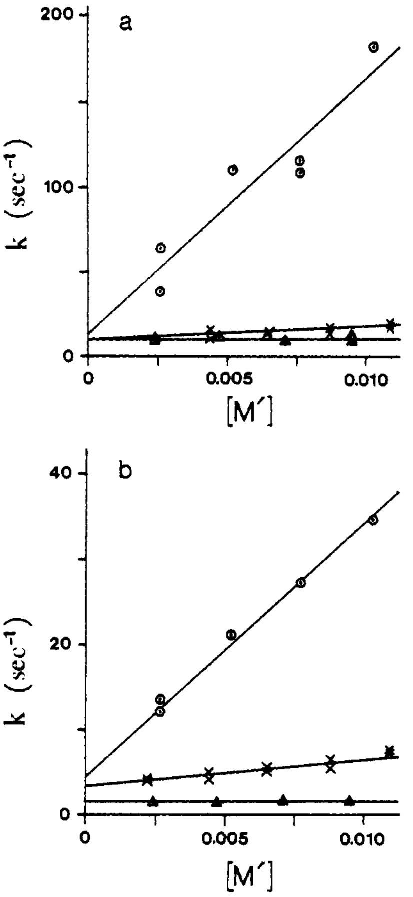


FIGURE 6: Fast (a) and slow (b) rate constants obtained from double-exponential fits of kinetic traces for the reaction of parvalbumin bound with Cd(II) ion with various concentrations of DCTA (▵), Tb(III) ion (×), and Eu(III) ion (○).

Table I: Rate Constants for Dissociation of Various Metal Ions from Codfish Parvalbumin at pH 5.8 and 24.8 °C in 1.0 mM Piperazine–0.5 M KCl

metal ion	k_1 (s^{-1})	k_2 (s^{-1})	n^a
Ca(II)	1.070 ± 0.131	5.907 ± 0.504	8
Cd(II)	1.539 ± 0.042	10.538 ± 1.585	6
Pr(III)	0.059 ± 0.004	0.468 ± 0.032	2
Nd(III)	0.055 ± 0.005	0.445 ± 0.069	4
Sm(III)	0.061 ± 0.003	0.509 ± 0.071	3
Eu(III)	0.043 ± 0.001	0.448 ± 0.018	2
Gd(III)	0.056 ± 0.005	0.629 ± 0.096	4
Tb(III)	0.050 ± 0.009	0.550 ± 0.130	17
Dy(III)	0.050 ± 0.000	0.488 ± 0.019	2
Ho(III)	0.046 ± 0.002	0.526 ± 0.011	4
Er(III)	0.043 ± 0.002	0.486 ± 0.013	2
Yb(III)	0.052 ± 0.043	0.367 ± 0.015	4
Lu(III)	0.038 ± 0.005	0.441 ± 0.078	13

[a] Total number of determinations averaged.

ions studied, along with those for Ca(II) and Cd(II), are given in Table I. The rate constants for both the fast and slow kinetic processes are essentially constant across the Ln(III) ion series at ~0.5 and ~0.05 s^{-1}, respectively. The off rates for the dipositive ions are, as expected, considerably greater than those for the tripositive Ln(III) ions.

DISCUSSION

In the studies presented here and in the preceding paper (Breen et al., 1985), we have attempted to achieve a relatively complete understanding of the nature of metal ion binding to the prototypal calcium-modulated protein parvalbumin. To do this, we have kept the system as simple as possible by exploiting the fluorescence properties of the intrinsic tryptophan residue and by using Ln(III) ions, isometric with the spectroscopically silent Ca(II) ion, as luminescent [Tb(III)] or energy-acceptor [Eu(III), Yb(III)] substitutional probes at the metal ion binding sites (Horrocks, 1982; Martin, 1983; Horrocks & Albin, 1984). These properties allow us, in most instances, to monitor a particular reaction in real time using more than one spectroscopic method. For instance, in experiments wherein parvalbumin-bound Tb(III) was replaced by Yb(III), the rate constants obtained by following the decrease in sensitized Tb(III) luminescence were identical with those found by using the decrease in tryptophan fluorescence as it was quenched by Yb(III) ions as they bound. An even more compelling comparison is the agreement of the kinetic parameters found when bound Tb(III) was removed by using DCTA to form the apoprotein. The rate of Tb(III) loss from the protein as monitored by the decrease in tryptophan-sensitized Tb(III) luminescence was the same as that obtained from the decrease in tryptophan fluorescence due to a conformational difference between metal-bound and apo forms. This important result proves that the presumed conformational changes occur at the same rates as metal ion dissociations at both sites.

The results presented in the preceding paper indicate that when apoparvalbumin is titrated with a metal ion, the EF and CD sites fill sequentially in that order. The kinetic experiments, wherein bound metal ions were removed with the chelating agent DCTA to form apoprotein, represent the reverse of the titration reaction. The finding of both fast and slow processes of metal ion release with rate constants differing by about an order of magnitude in the cases of Cd(II) and the Ln(III) ions and somewhat less for Ca(II) is consistent with the assignment of a stronger and a weaker binding site. The smaller k_{off} value is therefore associated with the tighter EF binding site, and, consequently, the CD site is assigned to the greater k_{off} rate constant.

It is interesting to note that both the $k_{\mathrm{off}}^{\mathrm{EF}}$ and $k_{\mathrm{off}}^{\mathrm{CD}}$ rate constants remain virtually unchanged across the entire Ln(III) ion series (Table I). Our results are in striking disagreement with k_{off} values for carp parvalbumin reported by Corson et al. (1983), who gave $k_{\mathrm{off}}^{\mathrm{EF}}$ values for Pr(III) to Lu(III) in the range 0.032–0.0012 s^{-1} and $k_{\mathrm{off}}^{\mathrm{CD}}$ values for Eu(III) to Lu(III) in the range 0.094–0.179 s^{-1}. Their stopped-flow experiments were carried out at pH 6.65 in the presence of free Ca(II) using carp (pI = 4.25) parvalbumin. They monitored changes in absorbance at 580 nm as a 10–20-fold excess of xylenol orange sequestered the Ln(III) ions upon release. The cause of this discrepancy is not known, but it is not a pH effect, as an experiment with Tb(III) at pH 7.0 has shown (vide supra), and it is unlikely to be an isotypic difference as preliminary experiments with carp (pI = 4.5) parvalbumin using our technique have indicated. The xylenol orange measurements are indirect and made on a more complex system without the cross-checking potential of our method.

The fact that the k_{off} values for the CD and EF sites do not vary appreciably across the Ln(III) ion series is, at first sight, incompatible with the trend in relative overall affinity for the Ln(III) ions determined in the preceding paper (Breen et al., 1985) and plotted in Figure 7a. A qualitative understanding of the relative affinity trend can be obtained with the aid of the findings of Geier (1965), who, using a T-jump method at 12 °C, measured the k_{on} rate constants for the Ln(III) ions coordinating to murexide. These values, which in effect are rate limited by the k_{off} values for H_2O from the aqua ions, are plotted in Figure 7b. The flat behavior of the protein k_{off} values across the series, combined with the k_{on} trend shown,

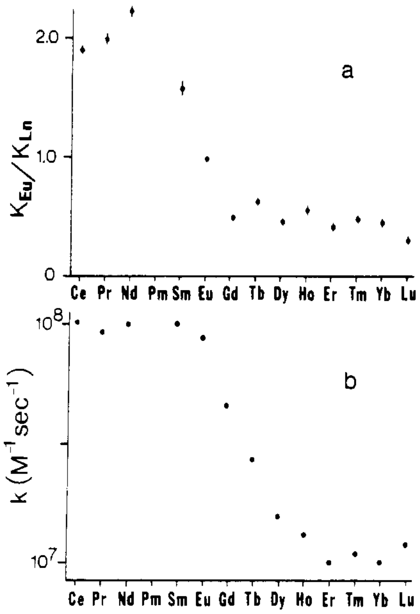


FIGURE 7: (a) Relative binding (ratio of dissociation constants) between Eu(III) and the Ln(III) ions to codfish parvalbumin, as calculated from metal ion competition experiments; pH 5.8, $T = 23$ °C. (b) Second-order rate constants reported by Geier (1965) for the binding of Ln(III) ions to murexide.

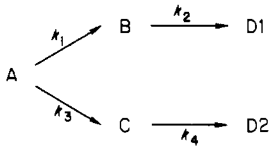
accounts nicely for the affinity trend wherein the earlier Ln(III) ions bind more strongly than the latter. The fact that the on rates measured by Geier were for a small ligand at 12 °C and that the protein on rates show a metal ion concentration dependence in some cases makes further quantitative analysis of the relative binding trends impractical at present.

The experiments wherein bound metal ions of one element were replaced by an attacking metal ion of another element under pseudo-first-order conditions reveal an interesting kinetic behavior. In many cases, particularly where the attacking ion is an early or middle member of the Ln(III) series, rate constants were obtained which were proportional to the concentration of attacking ion for both the fast and slow kinetic processes. The linear plots of observed pseudo-first-order rate constants vs. concentration of the attacking metal ion extrapolate (at zero attacking metal ion concentration) to k_{off} values quite close to those obtained from the DCTA metal ion scavenging experiments. This result suggests that there exists a bimolecular, metal ion assisted exchange pathway for metal ion replacement, in addition to a simple dissociative process. A possible explanation of this finding involves the formation of a complex where the attacking metal ion binds to a subsidiary site on the protein and, as a consequence, labilizes the ions bound to the two primary sites. Our spectroscopic observations (Horrocks & Collier, 1981; Rhee et al., 1981) of such a subsidiary site for Eu(III) at neutral pH and magnetic resonance results of others (Cavé et al., 1979a,b) lend credence to this possibility. The fact that early and middle members of the Ln(III) ion series are most effective in effecting the bimolecular mechanism may or may not be related to their greater relative overall binding affinity for the primary sites of parvalbumin.

Finally, the metal ion replacement studies provide compelling evidence against there being significant cooperativity between the CD and EF sites in metal ion binding. The fact that the $k_{off}{}^{EF}$ values, extrapolated to zero attacking metal ion concentration, agree with those obtained in the DCTA metal ion scavenging experiments implies that the rate of dissociation from the EF site is the same whether or not the CD site is occupied by a metal ion. In the substitution experiments, both the CD and EF sites are occupied when either site dissociates its ion, while in the scavenging experiments the second (EF) ion leaves while the more labile CD site is vacant. Our kinetic results reinforce the conclusions obtained in the preceding paper (Breen et al., 1985) based on sequential binding to the EF and CD sites and argue against significant cooperativity between the CD and EF binding sites of parvalbumin.

APPENDIX

The differential equations describing the time dependency of each species in Figure 1 were solved by using the representative scheme diagrammed below, depicting the two different pathways of forming D, the final product.



Once the time dependencies of D1 and D2 have been solved, they will be added together to yield the overall time dependency for D.

To begin

$$\dot{D}1 = k_2B \qquad \text{(A-1)}$$

$$\dot{B} = k_1A - k_2B \qquad \text{(A-2)}$$

$$\dot{A} = -k_1A - k_3A \qquad \text{(A-3)}$$

$$\dot{D}2 = k_4C \qquad \text{(A-4)}$$

$$\dot{C} = k_3A - k_4C \qquad \text{(A-5)}$$

$$\ddot{D}1 = k_2\dot{B} \qquad \text{(A-6a)}$$

$$\ddot{D}2 = k_4\dot{C} \qquad \text{(A-6b)}$$

By substituting eq A-2 into eq A-6a, we obtain

$$\ddot{D}1 = k_1k_2A - k_2{}^2B = k_1k_2A - k_2\dot{D}1$$

and

$$\dddot{D}1 = k_1k_2\dot{A} - k_2\ddot{D}1 \qquad \text{(A-7)}$$

If the total concentration of A, B, C, D1, and D2 is normalized:

$$A + B + C + D1 + D2 = 1$$

$$A = 1 - B - C - D1 - D2$$

then

$$\dot{A} = -\dot{B} - \dot{C} - \dot{D}1 - \dot{D}2 \qquad \text{(A-8)}$$

Substituting eq A-6a and A-6b:

$$\dot{A} = -\dot{D}1 - \frac{\ddot{D}1}{k_2} - \left(\dot{D}2 + \frac{\ddot{D}2}{k_4}\right) \qquad \text{(A-9)}$$

Substitution of eq A-2 and A-4 into eq A-5 yields

$$\dot{C} = \frac{k_3\dot{B}}{k_1} + \frac{k_2k_3B}{k_1} - \dot{D}2 \qquad \text{(A-10)}$$

Substitution of eq A-6b into eq A-10 leads to

$$\frac{\ddot{D}2}{k_4} + \dot{D}2 = \frac{k_3\dot{B}}{k_1} + \frac{k_2k_3B}{k_1} \qquad \text{(A-11)}$$

This expression can be substituted into eq A-9 to give

$$\dot{A} = -\dot{D}1 - \frac{\ddot{D}1}{k_2} - \left(\frac{k_3\dot{B}}{k_1} + \frac{k_2k_3B}{k_1}\right)$$

Solving eq A-1 and A-6a for B and $\dot{B}$ and substituting give

$$\dot{A} = -\dot{D}1 - \frac{\ddot{D}1}{k_2} - \frac{k_3\ddot{D}1}{k_1k_2} - \frac{k_3\dot{D}1}{k_1}$$

Substitution of this expression into eq A-7 yields

$$\dddot{D}1 + \ddot{D}1(k_1 + k_2 + k_3) + \dot{D}1(k_1k_2 + k_2k_3) = 0 \qquad \text{(A-12)}$$

By inspection, one root of this equation is 0, leaving

$$\ddot{D}1 + \dot{D}1(k_1 + k_2 + k_3) + D1(k_1k_2 + k_2k_3) = 0 \qquad \text{(A-13)}$$

Equation A-13 is of the form

$$\ddot{X} + a\dot{X} + bX + C = 0$$

The solution to this equation is of the form

$$D1(t) = \alpha_0 + \alpha_1e^{\lambda_1t} + \alpha_2e^{\lambda_2t}$$

where

$$\lambda_{1,2} = \frac{-a \pm \sqrt{a^2 - 4b}}{2}$$

In the case of eq A-13:

$$\lambda_{1,2} = \frac{-(k_1 + k_2 + k_3) \pm \sqrt{(k_1 + k_2 + k_3)^2 - 4(k_1k_2 + k_2k_3)}}{2} = -(k_1 + k_3) \quad \text{or} \quad -k_2$$

Thus

$$D1(t) = \alpha_0 + \alpha_1e^{-(k_1+k_3)t} + \alpha_2e^{-k_2t}$$

α_0, α_1, and α_2 must be determined from boundary conditions on D1:

at $t = 0$

$$D1 = 0 = \alpha_0 + \alpha_1 + \alpha_2 \qquad \text{(A-14)}$$

at $t = \infty$

$$D1 = \frac{k_1}{k_1 + k_3} = \alpha_0 \qquad \text{(A-15)}$$

at $t = 0$

$$\dot{D}1 = 0 = -\alpha_1(k_1 + k_3) - \alpha_2k_2 \quad \text{or} \quad \alpha_1 = \frac{-\alpha_2k_2}{k_1 + k_3} \qquad \text{(A-16)}$$

Substitution of eq A-16 and eq A-15 into eq A-14 yields

$$\alpha_1 = \frac{-k_1k_2}{(k_2 - k_1 - k_3)(k_1 + k_3)} \qquad \alpha_2 = \frac{k_1}{k_2 - k_1 - k_3}$$

Therefore

$$D1(t) = \frac{-k_1k_2}{(k_2 - k_1 - k_3)(k_1 + k_3)}e^{-(k_1+k_3)t} + \frac{k_1}{k_2 - k_1 - k_3}e^{-k_2t} + \frac{k_1}{k_1 + k_3} \qquad \text{(A-17)}$$

In a similar fashion, the time dependence of $D2$ can be obtained:

$$D2(t) = \frac{-k_3k_4}{(k_4 - k_1 - k_3)(k_1 + k_3)}e^{-(k_1+k_3)t} + \frac{k_3}{k_4 - k_1 - k_3}e^{-k_4t} + \frac{k_3}{k_1 + k_3} \qquad \text{(A-18)}$$

The sum of eq A-17 and A-18 gives the total time dependence of D, which is eq A-19:

$$D(t) = 1 - \left[\frac{k_1k_2}{(k_1 + k_3)(k_2 - k_1 - k_3)} + \frac{k_3k_4}{(k_1 + k_3)(k_4 - k_1 - k_3)}\right]e^{-(k_1+k_3)t} + \frac{k_1}{k_2 - k_1 - k_3}e^{-k_2t} + \frac{k_3}{k_4 - k_1 - k_3}e^{-k_4t} \qquad \text{(A-19)}$$

The solution describing the time dependence of B proceeds from eq A-2:

$$\dot{B} = k_1A - k_2B$$

$$\ddot{B} = k_1\dot{A} - k_2\dot{B}$$

Substitution of eq A-8 gives

$$\ddot{B} = k_1(k_2B - \dot{B} - \dot{D}2 - \ddot{D}2/k_4) - k_2\dot{B}$$

Substituting eq A-11 into this expression yields

$$\ddot{B} + \dot{B}(k_1 + k_2 + k_3) + B(k_1k_2 + k_2k_3) = 0$$

The solution to this equation again has the form

$$B(t) = \alpha_0 + \alpha_1e^{-(k_1+k_3)t} + \alpha_2e^{-k_2t}$$

From boundary conditions on B:

at $t = 0$

$$B = 0 = \alpha_0 + \alpha_1 + \alpha_2$$

at $t = \infty$

$$B = 0 = \alpha_0$$

at $t = 0$

$$\dot{B} = k_1 = -\alpha_1(k_1 + k_3) - \alpha_2k_2$$

or

$$\alpha_0 = 0 \qquad \alpha_1 = -\alpha_2 = \frac{k_1}{k_2 - k_1 - k_3}$$

which leads to eq A-20. Equation A-21 is derived in a similar manner:

$$B(t) = \frac{k_1}{k_2 - k_1 - k_3}[e^{-(k_1+k_3)t} - e^{-k_2t}] \qquad \text{(A-20)}$$

$$C(t) = \frac{k_3}{k_4 - k_1 - k_3}[e^{-(k_1+k_3)t} - e^{-k_4t}] \qquad \text{(A-21)}$$

The time dependence of A is given by eq A-22:

$$A(t) = e^{-(k_1+k_3)t} \qquad \text{(A-22)}$$

For the reaction scheme previously illustrated, the observed change in intensty, $I(t)$, corresponding to the disappearance of species A, B, and C, is given by eq A-23, where β and γ represent the relative signal intensities associated with the two sites of parvalbumin. Substitution of eq A-20, A-21, and A-22 into eq A-23 yields eq A-24, which predicts that the observed decay should contain three exponentials, with rate constants $k_1 + k_3$, k_2, and k_4. If, however, $k_1 = k_4$ and $k_2 = k_3$ (no

$$I(t) = (\beta + \gamma)A(t) + \beta B(t) + \gamma C(t) \qquad \text{(A-23)}$$

$$\text{signal}(t) = \left(\beta + \gamma + \frac{\beta k_1}{k_2 - k_1 - k_3} + \frac{\gamma k_3}{k_4 - k_1 - k_3}\right)e^{-(k_1+k_3)t} - \frac{\beta k_1}{k_2 - k_1 - k_3}e^{-k_2t} - \frac{\gamma k_3}{k_4 - k_1 - k_3}e^{-k_4t} \qquad \text{(A-24)}$$

cooperativity between sites), eq A-24 reduces to

$$I(t) = \beta e^{-k_2 t} + \gamma e^{-k_1 t} \qquad \text{(A-25)}$$

and the reaction follows double-exponential kinetics with observed rate constants corresponding to the real k_{off} values, k_1 and k_2.

When the competing metal ion is Eu(III) or Yb(III), the tryptophan fluorescence is quenched as the bound metal ion dissociates and Eu(III) or Yb(III) binds. In this case, the observed signal will behave temporally according to eq A-23 and A-25. If the competing metal ion is Tb(III), however, and the reaction is monitored by the appearance of the sensitized Tb(III) signal, the intensity will follow eq A-26, where

$$I(t) = (\theta + \phi)D(t) + \theta C(t) + \phi B(t) \qquad \text{(A-26)}$$

θ and ϕ represent the degree of sensitization of Tb(III) in the respective sites. Since $k_1 = k_4$ and $k_2 = k_3$, this leads to

$$I(t) = \theta + \phi - \theta e^{-k_2 t} - \phi e^{-k_1 t} \qquad \text{(A-27)}$$

Registry No. Ca, 7440-70-2; Cd, 7440-43-9; Pr, 7440-10-0; Nd, 7440-00-8; Sm, 7440-19-9; Eu, 7440-53-1; Gd, 7440-54-2; Tb, 7440-27-9; Dy, 7429-91-6; Ho, 7440-60-0; Er, 7440-52-0; Yb, 7440-64-4; Lu, 7439-94-3.

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Vincristine-Induced Self-Association of Calf Brain Tubulin[†]

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ABSTRACT: The vincristine-induced self-association of tubulin has been examined in a sedimentation velocity study as a function of free drug concentration in PG buffer (0.01 M NaP$_i$ and 10^{-4} M GTP, pH 7.0) at 20 °C. Analysis of the weight-average sedimentation coefficient ($\bar{s}_{20,w}$) as a function of protein concentration showed a good fit with the model of an indefinite, isodesmic self-association mechanism. Analysis of the apparent association constants in terms of the Wyman linkage relations showed a good fit to mediation of the self-association by the binding of one ligand molecule. The intrinsic association constant for dimerization of the vincristine-liganded tubulin was found to be 3.8×10^5 M^{-1}, and the intrinsic equilibrium constant for the binding of the self-association-linked vincristine molecule had a value of 3.5×10^4 M^{-1}, consistent with that measured by fluorescence in our laboratory [Prakash, V., & Timasheff, S. N. (1983) *J. Biol. Chem. 258*, 1689–1697]. Both reactions are stronger in the presence of vincristine than of vinblastine, reflecting the oxidation of a –CH$_3$ group to –CHO when going from the latter drug to the former one.

Vincristine (VCR)[1] is a vinca alkaloid derived from the periwinkle plant (*Catharanthus rosea* Linn). At very small concentrations, it is known to be an effective mitosis-arresting agent. It also has important applications in the treatment of certain neoplastic diseases (Palmer et al., 1960; Cutts, 1961; Johnson et al., 1963; Wilson et al., 1976; Owellen et al., 1976; Dustin, 1978; Skovsgaard, 1978; Redmond & Tuffery, 1979; Grush & Morgan, 1979; Zeller et al., 1979; Newlands & Bagshawe, 1979; Massa et al., 1979; Morgan & Crossen, 1980; Klein et al., 1980). Early morphological studies have indicated the destruction of spindle microtubules by VCR, leading to arrested mitosis (Cutts et al., 1960; Cutts, 1961; Cardinali et al., 1963; Johnson et al., 1963; Frei et al., 1964; George et al., 1965). Vincristine induces the aggregation of tubulin molecules both in the cytoplasm and in vitro with the formation of paracrystalline structures (Donoso et al., 1979; Dustin, 1978; Na & Timasheff, 1982). The specific binding of VCR to tubulin was first demonstrated by Owellen et al. (1972), while Lee et al. (1975) have shown that VCR binds strongly to tubulin dimers. Wilson (1970) has reported that nearly complete stabilization of the colchicine binding activity of

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[1] Abbreviations: VCR, vincristine; PG buffer, 10^{-2} M sodium phosphate and 1×10^{-4} M GTP, pH 7.0; PMG buffer, PG buffer containing 5×10^{-4} M MgCl$_2$; Gdn·HCl, guanidine hydrochloride.

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