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Site-specific Mutants of Oncomodulin

^{1}H NMR AND OPTICAL STOPPED-FLOW STUDIES OF THE EFFECT ON THE METAL BINDING PROPERTIES OF AN $Asp^{59} \rightarrow Glu^{59}$ SUBSTITUTION IN THE CALCIUM-SPECIFIC SITE*

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High resolution ^{1}H nuclear magnetic resonance spectroscopy and optical stopped-flow techniques have been used to study the metal binding properties of a site-specific mutant of bacterial recombinant oncomodulin in which glutamate has replaced a liganding aspartate at position 59 in the CD calcium-binding site. In particular we have followed the replacement of calcium by lutetium in bacterial recombinant oncomodulin and D59E oncomodulin to provide a measure of the protein's preferences for metal ions of different ionic radii. The result of the Asp → Glu substitution is to make the mutant oncomodulin more similar to rat parvalbumin in terms of its relative CD- and EF-domain affinities for lutetium(III), that is to increase its affinity for metal ions with smaller ionic radii. This finding supports the original hypothesis that the presence of Asp at sequence position 59 is an important factor in the reduced preference of the CD site of oncomodulin for smaller metals such as magnesium (Williams, T. C., Corson, D. C., Sykes, B. D., and MacManus, J. P. (1987) *J. Biol. Chem.* 262, 6248–6256). However, our studies show that *both* the CD and the EF sites are affected by this single residue substitution suggesting that many factors play a role in the metal binding affinity and interaction between the two sites.

A family of calcium-binding proteins genetically related to calmodulin has been recognized that have in common repeated domains of approximately 30 residues comprising a helix:calcium binding loop:helix motif (Baba *et al.*, 1984; Kretsinger, 1980; Van Edkik *et al.*, 1982; Grand, 1985). This family includes proteins with four such domains such as calmodulin or troponin C, which are calcium-dependent modulators capable of binding to enzymes (Grand, 1985; Klee *et al.*, 1980; Potter and Johnson, 1982). Other members are smaller proteins with three domains such as parvalbumin or two domains such as calbindin-D_{9K}. However, these smaller proteins have not been shown to bind to other target proteins (Demaille, 1982; Heizmann, 1984; Wnuk *et al.*, 1982). In addition, the two functional loops on parvalbumin and the two C-terminal loops on troponin C are capable of binding magnesium. It is not known what confers calcium specificity or calcium-dependent protein-protein interaction to various members of the family.

Oncomodulin is a member of this family, sharing 50% identity of amino acid sequence with rat parvalbumin (MacManus and Whitfield, 1983). It is unique in being expressed during prenatal development in the placenta, but not in the fetus (MacManus and Whitfield, 1983; Brewer and MacManus, 1985; MacManus *et al.*, 1987). In addition, it is noteworthy in not having been detected in normal adult human or rodent tissues. However, following neoplastic transformation by viruses or chemicals, oncomodulin is found in a majority of tumors (MacManus and Whitfield, 1983; Brewer and MacManus, 1985; MacManus *et al.*, 1987). This apparent *de novo* activation of oncomodulin expression in the adult is in contrast to other calcium-binding proteins whose concentrations in a particular tissue are merely increased above normal following neoplastic transformation (Watterson *et al.*, 1976; Dorin *et al.*, 1987; Heizmann and Berchtold, 1987).

The function of oncomodulin during normal development or its role in carcinogenesis is unknown. Despite its similarity to parvalbumin in primary structure, oncomodulin is also noteworthy in apparently having a calcium-specific site (MacManus *et al.*, 1984; Henzl *et al.*, 1986; Williams *et al.*, 1987), positive cooperativity of binding between the two sites (Williams *et al.*, 1987), and the capability of activating some enzymes such as phosphodiesterase or calcineurin in a calcium-dependent manner (MacManus 1981; Mutus *et al.*, 1985; Mutus *et al.*, 1988). Since oncomodulin is relatively small, studies to define what delineates a calcium-specific site from a calcium-magnesium site or what confers the ability to interact with other proteins should be easier using oncomodulin than the larger four domain proteins.

We have previously used ^{1}H NMR[1] spectroscopy to follow the titration of oncomodulin and several parvalbumins with metal ions (Corson *et al.*, 1983a, 1983b; Williams *et al.*, 1984, 1986, 1987). In particular, we have followed the displacement of magnesium by calcium and by various members of the lanthanide series of metal ions, which vary in ionic radius from the largest (lanthanum) to the smallest (lutetium). These displacement titrations yield the relative affinity of a given site for the two metals and thereby provide a measure of the preference of the site for ions of different ionic radii. In a previous lutetium titration of the calcium-saturated form of

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[1] The abbreviations used are: NMR, nuclear magnetic resonance; Onco, oncomodulin; brOnco, bacterial recombinant oncomodulin; D59E brOnco, the site-specific mutant (Asp→Glu) in position 59 of bacterial oncomodulin; D_2O, deuterium oxide; HDO, hydrogen deuterium oxide; UV, ultraviolet; CD and EF, designations of calcium-binding loops between C and D and E and F helices, respectively (Kretsinger (1980)).

oncomodulin, Williams *et al.* (1987) showed that while one site bound Lu(III) more than two orders of magnitude stronger than Ca(II), the other site did not prefer Lu(III) over Ca(II). This was also observed using Eu(III) as a probe (Henzl and Birnbaum, 1988). This is in contrast to rat parvalbumin, and other parvalbumins, for which both the CD and EF Ca(II)-Mg(II) sites preferred the smaller Lu(III) over Ca(II). On this basis it was concluded that the putative Ca(II)-specific site in oncomodulation, the CD site, achieved its specificity by discriminating (in a relative sense) against smaller metals such as Mg(II) and Lu(III). Because all parvalbumins sequenced to date from carp, cod, coelacanth, frog, hake, pike, rabbit, rat, ray, and whiting have Glu in the -*X* calcium coordinating position equivalent to position 59 in oncomodulin which is Asp, it was postulated that this ligand was at least in part responsible for the specificity. Fluorescence studies on this same mutant showed it to have a sensitivity to Mg not evident in the native protein (MacManus *et al.*, 1989). In this paper we have used ^{1}H NMR spectroscopy to follow the Lu(III) titration of a site-specific mutant of bacterial recombinant oncomodulin which has Glu in place of Asp in position 59. The same mutant has been studied by Hapak *et al.* (1989) using fluorescence techniques.

EXPERIMENTAL PROCEDURES

Materials—Rat oncomodulin was purified from Morris hepatoma 5123tc (MacManus, 1980). Bacterial recombinant oncomodulin was expressed in *Escherichia coli* and purified as described by MacManus *et al.* (1989). The site-specific mutant D59E bacterial recombinant oncomodulin was produced according to the method described by MacManus *et al.* (1989). BrOnco and D59E brOnco were dialyzed against 1 mM $CaCl_2$ to ensure the Ca(II)-saturated form of the protein was present followed by a second dialysis against distilled/deionized water to remove any excess Ca(II). Protein concentrations were determined by UV spectrophotometry using a Varian DMS 200 spectrophotometer. Absorbance measurements were made with 1-cm quartz cuvettes. The molar extinction coefficient used to calculate the protein concentrations was $E^{1\%}_{276} = 2.7$ (MacManus, 1982).

Lutetium chloride hexahydrate was of standard high grade and was obtained from Alfa Products Ventron Division. The lanthanide solution was made up by weight and then standardized against EDTA using xylenol orange (Terochem Laboratories Ltd.) as an indicator (Birnbaum and Sykes, 1978).

^{1}H NMR Methods—BrOnco in the calcium(II)-saturated form (0.74 mM protein, 150 mM KCl in D_2O, pH 6.9, 40 °C) was titrated with microliter aliquots of a standardized $LuCl_3$ solution (Birnbaum and Sykes, 1978). D59E brOnco in the calcium(II)-saturated form (0.49 mM protein, 150 mM KCl in D_2O, pH 6.9, 40 °C) was also titrated as above. ^{1}H NMR spectra of brOnco were obtained with a Varian VXR 500 spectrometer with the following parameter settings: spectral width, 6000 Hz; pulse width, 15.0 μs (90°); acquisition time, 1.6 s. Homonuclear decoupling was employed for presaturation (2 s) of the HDO resonance. Post-acquisition processing involved a Lorentzian to Gaussian line shape transformation. Spectra are referenced relative to the methyl resonance of 4,4′-dimethyl-4-silapentane-1-sulfonate. This protocol was duplicated for D59E brOnco.

Overlapping ^{1}H NMR resonances were analyzed to determine the area of the contributing resonances using a curve-fit analysis program (CRVFIT) developed for the Varian software by Robert F. Boyko (University of Alberta). The resulting distribution of species plots was then used to calculate binding constants and coefficient of

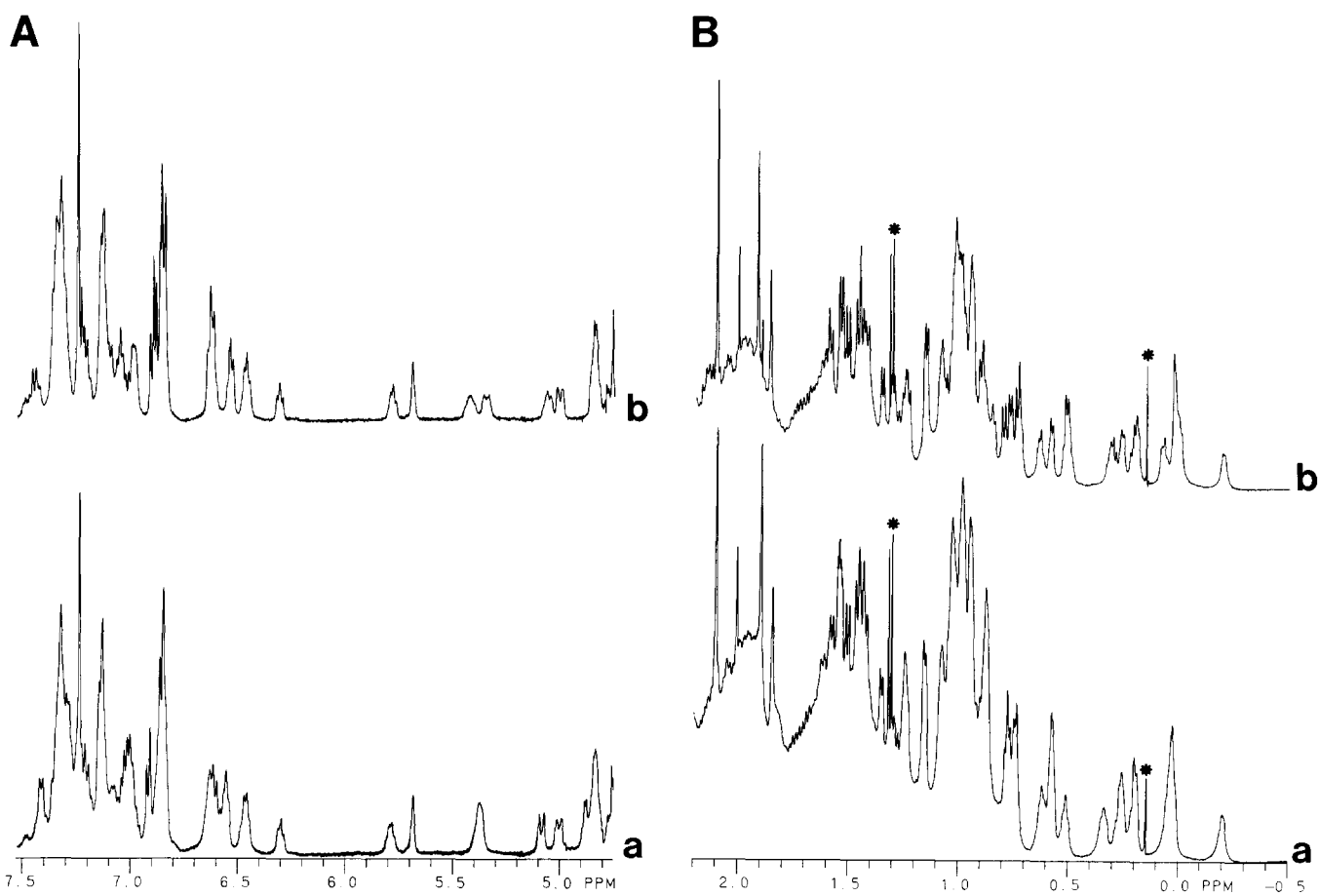

FIG. 1. 500 MHz ^{1}H NMR spectra of 0.7 mM brOnco (*bottom*) and 0.5 mM D59E brOnco (*top*) in 150 mM KCl, 40 °C, pH 6.9, in D_2O: *A*, aromatic and downfield-shifted α-CH region; *B*, aliphatic region from Met ε-CH_3 protons to upfield-shifted CH_3 protons. Resonances denoted by * are impurities.

cooperativity values (Corson *et al.*, 1983b; Williams *et al.*, 1984, 1986, 1987).

Kinetics Methods—Onco, brOnco, and D59E brOnco were prepared for kinetic analysis by two different methods: 1) by the direct addition of stoichiometric amounts of Lu(III) to the calcium-saturated protein or 2) by the addition of a 100–500-fold excess of Lu(III) followed by extensive dialysis *versus* H_2O. The off-rate of lutetium from oncomodulin, brOnco, and D59E brOnco were determined by optical stopped-flow methods. The technique used follows the increase in optical absorption at 570 nm caused by the binding of the metal chelating dye xylenol orange to lutetium following its release from the protein using the techniques fully described in Corson *et al.* (1983a). These experiments were done with protein concentrations from 0.5 to 1.5 × 10^{-6} M and at xylenol orange concentration of 45 × 10^{-6} M. All stopped flow work was done at 23.5 ± 0.5 °C.

RESULTS

In a previous paper (MacManus *et al.*, 1989) we showed that the ^1H NMR spectra of oncomodulin and brOnco were very similar with the exception of the chemical differences at the N terminus (oncomodulin is *N*-acetylated whereas brOnco is approximately 50% *N*-formyl methionine, and 50% unmodified). 500 MHz 1H NMR spectra of brOnco and D59E brOnco are presented in Fig. 1, *a* and *b*, respectively. These show the similarity between these two proteins. This is especially clear for regions of the spectrum that contain residues shifted well outside of the normal range for the particular amino acid because of their specific environment within the protein. These include the phenylalanine ring and aliphatic methyl residues buried in the hydrophobic core of the protein that are shifted as far upfield as 5.8 and −0.2 ppm, respectively, and the α-CH residues which are in the small section of β-sheet between the two Ca(II)-binding loops and shifted as far downfield as 5.7 ppm (Williams *et al.*, 1987).

Lu(III) Titration of Ca(II)$_2$-BrOnco and Ca(II)$_2$-D59E BrOnco—Shown in Fig. 2*A* for brOnco and in Fig. 2*B* for D59E brOnco are selected regions of the ^1H NMR spectra taken during the titration of the calcium-saturated forms of these proteins with Lu(III). The ^1H NMR spectral region between 2.2 and 1.8 ppm contained the ε-methyl resonances of Met-38 -86, and -105 and the *N*-formyl methionine residue which are sharp and well resolved throughout the entire titration, and therefore the most useful in following the Lu(III) titration. These resonances have been assigned previously (MacManus *et al.*, 1989) as follows: Met-38 CH_3 at 2.10 ppm, *N*-formyl Met CH_3 at 2.01 ppm, Met-105 CH_3 at 1.90 ppm, and Met-86 CH_3 at 1.85–1.86 ppm in Fig. 2*A*(*a*) and *B*(*a*). Similar changes occurred throughout the spectrum (data not shown), and some of these were also used to monitor the Lu(III) titration. In the titration of Ca(II)$_2$-brOnco with Lu(III), one can see the CH_3 proton resonances of Met-105 (partially obscured by an impurity) and Met-86 disappear and be replaced by new resonances shifted slightly upfield during the first stages of the titration ([Lu(III)]/[brOnco] = 0 → 1).

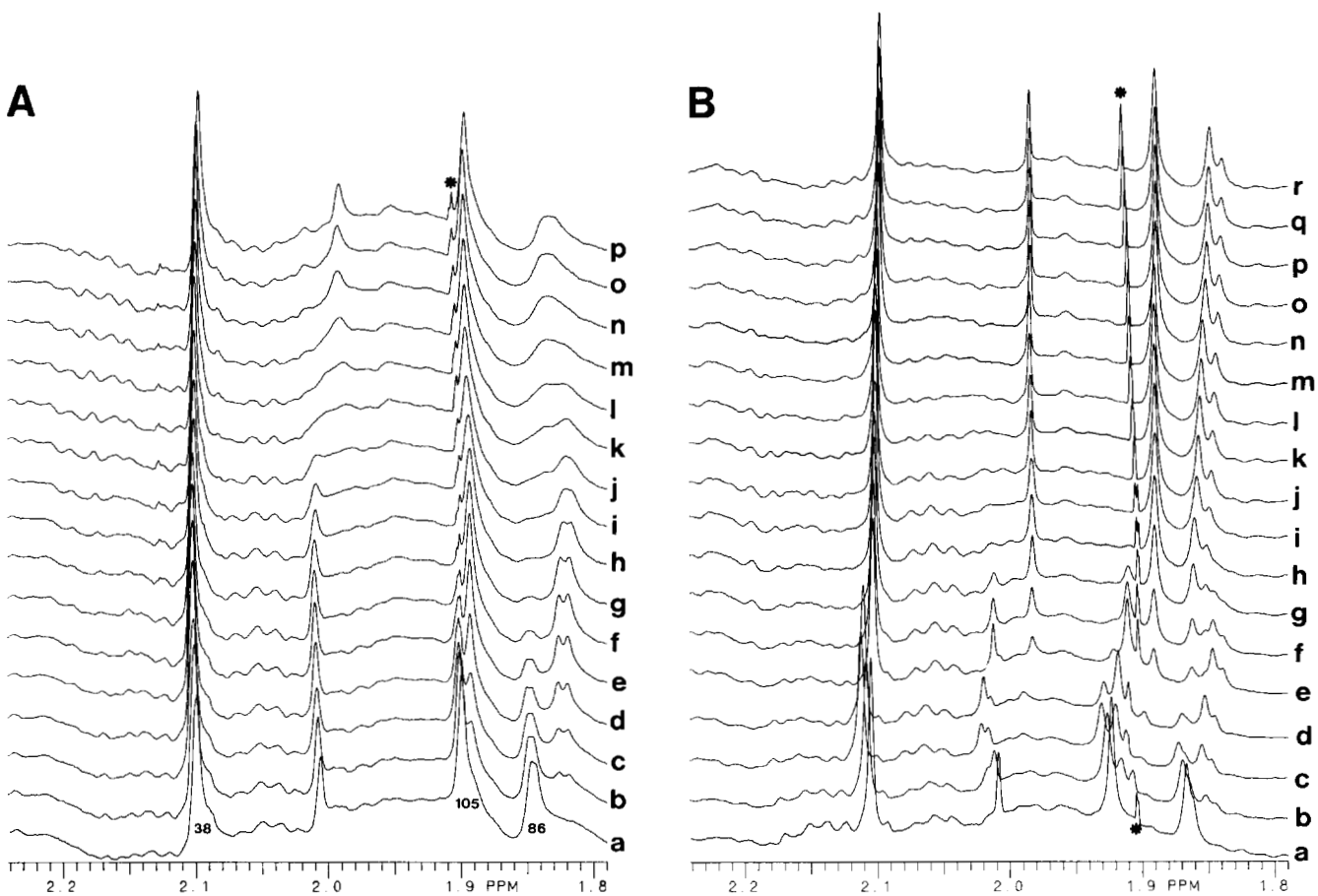

FIG. 2. **Partial aliphatic region (2.2–1.8 ppm) of the 500 MHz ^1H NMR spectrum of brOnco (*A*) and D59E brOnco (*B*) during Lu(III) titration of the Ca(II)-saturated forms of the proteins.** Sample conditions are the same as for Fig. 1. Lu:protein ratios for the spectra are as follows: for *A*, 0.00 (*a*), 0.20 (*b*), 0.40 (*c*), 0.62 (*d*), 0.81 (*e*), 1.01 (*f*), 1.20 (*g*), 1.43 (*h*), 1.62 (*i*), 1.83 (*j*), 2.03 (*k*), 2.22 (*l*), 2.42 (*m*), 2.65 (*n*), 2.84 (*o*), 3.04 (*p*); and for *B*, 0.00 (*a*), 0.22 (*b*), 0.45 (*c*), 0.68 (*d*), 0.89 (*e*), 1.12 (*f*), 1.33 (*g*), 1.56 (*h*), 1.77 (*i*), 2.02 (*j*), 2.25 (*k*), 2.46 (*l*), 2.68 (*m*), 2.89 (*n*), 3.16 (*o*), 3.38 (*p*), 3.59 (*q*), 3.80 (*r*). Resonances denoted by * are impurities.

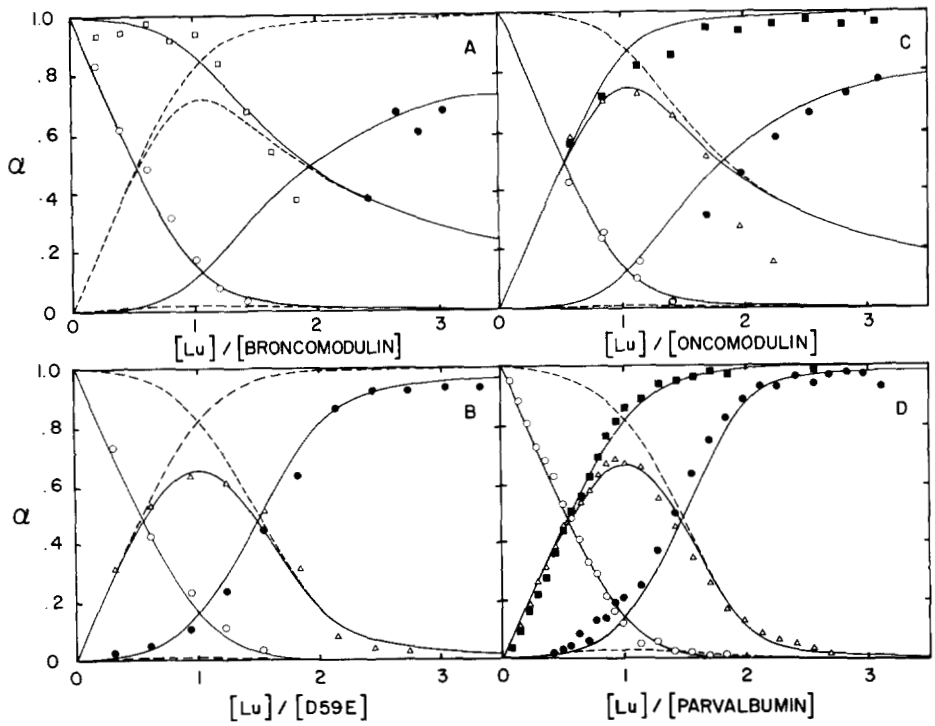

FIG. 3. **Plots of the normalized concentrations of various metal-bound forms of brOnco (*A*), D59E brOnco (*B*), Onco (*C*), and rat parvalbumin (*D*) as determined by ^{1}H NMR during Lu(III) titration of the Ca(II)-saturated proteins.** The results for brOnco and D59E brOnco are from this study. Results for oncomodulin are adapted from Fig. 3 of Williams *et al.* (1987). Results for rat parvalbumin are adapted from Fig. 10*c* of Williams *et al.* (1986). The symbols used for the data have been standardized for each panel and represent the fractional concentrations of the following protein forms: ○, $Ca(II)_2$ form; □, $Ca(II)_2$ plus Ca(II)Lu(III) forms; △, Ca(II)Lu(III) form; ■, Ca(II) Lu(III) plus $Lu(III)_2$ forms; ●, $Lu(III)_2$ form. Calculated curves corresponding to all these forms or sums of forms have been drawn in all panels; *dashed curve* data indicates not present for that form. The values of $^{rel}\beta_{CD}$, $^{rel}\beta_{EF}$, and cooperativity used to calculate all the curves in each panel are presented in Table I. The particular resonances observed in this study are: *A*: ○, average of α-CH resonances at 5.1 and 5.07 ppm; □, *N*-formyl Met CH_3 resonances at 2.0 ppm; ●, *N*-formyl Met CH_3 resonance at 1.99 ppm. *B*: ○, the average of Met CH_3 resonances at approximately 2.01 and 1.93 ppm; △, the average of Met CH_3 resonances at approximately 2.02 and 1.92 ppm; ●, the average of Met CH_3 resonances at approximately 1.99 and 1.90 ppm.

In contrast the CH_3 resonance of the *N*-formyl Met is not shifted until later in the titration ([Lu(III)]/[brOnco] = 1 → >2). This implies that the CH_3 resonances of Met-86 and -105 primarily reflect the addition of the first Lu(III) to the EF site followed by the replacement of Ca(II) in the CD site by the second Lu(III), whereas the shift of the *N*-formyl Met CH_3 resonance primarily reflects addition of the second Lu(III) to the CD site. Fractional intensity measurements for three resonances (the *N*-formyl Met CH_3 resonance of $Ca(II)_2$-brOnco at 2.01 ppm, the *N*-formyl Met CH_3 resonance of $Lu(III)_2$-brOnco at 1.99 ppm, and the average of two α-CH resonances at 5.10 and 5.07 ppm (data not shown)) were evaluated using the CRVFIT program and plotted as a function of the [Lu(III)]:[brOnco] ratio in Fig. 3*A*.

For the titration of D59E brOnco, the pattern of the titration is different. For the CH_3 resonances of the *N*-formyl Met and Met-105 (again partially obscured by an impurity), each resonance is first replaced by a second resonance which in turn disappears and is replaced by a third resonance as the titration proceeds from 0 to 1 to ≥2 mol of Lu(III) per mol of protein. These resonances represent in turn the $Ca(II)_2$ form, the Ca(II)Lu(III) form, and the $Lu(III)_2$ form of the protein. Six resonances were followed in this spectral region of D59E brOnco (Fig. 2*B*). These resonances consisted of three pairs of peaks moving in a correlated fashion at 2.01 and 1.93 ppm, 2.02 and 1.92 ppm, and 1.99 and 1.90 ppm. Each singlet resonance was analyzed to determine normalized peak area using the CRVFIT program. Resulting intensity measurements were summed for each pair and plotted as a function of the [Lu(III)]:[D59E brOnco] ratio in Fig. 3*B*.

The NMR data presented in Fig. 3 reflect the fractional concentration of various species that occur during the titration or sums of the various species. The possible species are the $Ca(II)_2$ form, the Ca(II)Lu(III) form, the Lu(III)Ca(II) form, and the $Lu(III)_2$ form of either protein where the first metal listed is in the CD site and the second metal listed is in the EF site. The NMR resonance can reflect the sum of two or more species if the chemical shift of the resonance followed is not changed for the forms involved. The solid theoretical lines drawn in Fig. 3 represent the fractional concentrations of the forms or sum of forms which most closely match the data. These are indicated in the legend to Fig. 3. These curves were calculated for various values of the relative affinities of the CD and EF sites ($^{rel}\beta_{CD}$ and $^{rel}\beta_{EF}$, respectively) and the cooperativity (*c*) between the two sites (see Williams *et al.* (1987) for mechanism). The best fit values for $^{rel}\beta_{CD}$, $^{rel}\beta_{EF}$, and *c* are listed in Table I, along with the previously determined values for oncomodulin (Williams *et al.*, 1987) and rat parvalbumin (Williams *et al.*, 1986).

Lutetium Kinetic Measurements—The proteins prepared by extensive dialysis after the addition of excess Lu(III) showed only two kinetic phases of equal intensity (the off-

TABLE I

Relative affinities of Lu(III) versus Ca(II) for and coefficient of cooperativity between the CD and EF sites of Onco, brOnco, D59E brOnco, and rat parvalbumin

	$^{rel}\beta_{CD}$	$^{rel}\beta_{EF}$	
Onco[a]	1.2	175	5
brOnco	2.5	100	1.3
D59E	10	715	4.5
Rat parvalbumin[b]	120	2500	(1)[c]

[a] Williams *et al.* (1987).
[b] Williams *et al.* (1986).
[c] Cooperativity fixed at 1.0 in Williams *et al.* (1986).

TABLE II

Rate constants for lutetium dissociation from the CD and EF sites of Onco, brOnco, D59E brOnco, and rat parvalbumin

	k_1	k_2	k_3
		s^{-1}	
Lu_2 and CaLu[a] protein			
Onco	3.9 ± 1.3	0.059 ± 0.007	0.0034 ± 0.0003
D59E brOnco	1.4 ± 0.24	0.021 ± 0.004	0.0007 ± 0.0002
Rat parvalbumin[b]	0.33	No reaction[c]	0.001
Lu_2 protein			
Onco/brOnco[d]	3.9 ± 1.6	0.049 ± 0.005	No reaction
D59E brOnco	1.9 ± 0.7	0.024 ± 0.004	No reaction

[a] First metal listed in CD site, second listed in EF site.
[b] This data from Williams *et al.* (1987).
[c] These experiments were done with excess calcium present. The high affinity of parvalbumin's CD site for Ca(II), together with very fast on rates of Ca(II) for this site, ensure that the CD site is immediately occupied by calcium after Lu(III) dissociates.
[d] Average of Onco and brOnco results.

rate constants are listed in Table II). Since the protein prepared by this method should have both metal binding sites occupied with Lu(III) and no free Ca(II) or Lu(III) in solution, we attribute the faster of the off-rate constants (k_1) to the off-rate of Lu(III) from the weaker affinity CD site with the EF site occupied by Lu(III), and the slower off-rate constant (k_2) to the off-rate of Lu(III) from the EF site with the CD site unoccupied. Both of these off-rate constants were decreased slightly for D59E brOnco to 1.4 and 0.024 s^{-1}, respectively (see Table II).

The protein prepared by the direct stoichiometric addition of 2 mol of lutetium per mol of Ca(II)-saturated protein yielded three kinetic phases with roughly equal amplitudes (see Table II for off-rate constants). Because of the micromolar concentrations used for these experiments, along with the relatively weak binding constants of the CD site for lutetium and calcium and high binding constant of the EF site for Lu(III),[2] we expect the EF site, in this case, to be totally occupied with Lu(III) but the CD site to be only partially occupied with Lu(III), with the remainder of the CD sites to be occupied by Ca(II). Therefore we would expect three off-rates under these conditions, *i.e.* the off-rate of Lu(III) from the CD site with the EF site occupied with Lu(III) (k_1); the off-rate of Lu(III) from the EF site with the CD site unoccupied (k_2); and the off-rate of Lu(III) from the EF site, with the CD site occupied by Ca(II) (k_3). Because the off-rates from the EF site are so much slower than those of the CD site, protein in the $Lu(III)_2$ form will have the CD site unoccupied for most of the EF site Lu(III) dissociation reaction. Of the three off-rate constants measured under these circumstances, two are the same as measured above; we therefore attribute the third constant (0.0034 ± 0.0003 s^{-1}) to the off-rate of Lu(III) from the EF site with the CD site occupied by Ca(II). Again this off-rate is slower in the D59E form (0.0007 s^{-1} ± 0.0002).

DISCUSSION

The ^1H NMR spectra of hepatoma oncomodulin (Onco), bacterial recombinant native oncomodulin (brOnco), and oncomodulin mutated at Asp-59 (D59E brOnco) show all three proteins to be very similar in structure. Hundreds of resonances are present in the NMR spectra of these proteins, and the chemical shifts, linewidths, and other parameters such as coupling constants of these resonances reflect the details of the structures. Similar conclusions about the structural similarity of Onco, brOnco, and D59E brOnco have been made by MacManus *et al.* (1989) and Hapak *et al.* (1989).

The focus of this paper is the metal binding properties of these proteins. We have measured the relative affinities of the two metal binding sites for the smaller (and higher charged) lutetium with respect to calcium, and the cooperativity between the two sites. The ^1H NMR spectrum provides an ideal way to monitor the Ca(II) → Lu(III) titration since the many resolved resonances can reflect the different species present in the titration. The results of the titrations of brOnco and D59E brOnco are presented as species distribution plots in Fig. 3 along with the previously published results for Onco (Williams *et al.*, 1987) and rat parvalbumin (Williams *et al.*, 1986). The quantitative relative affinity constants and degree of cooperativity obtained from theoretical fitting of these results is presented in Table I. Even without looking at the quantitative results it is evident from Fig. 3 that brOnco is very similar to Onco and that the Asp to Glu substitution in position 59 ($-X$ position in metal binding loop) has changed Onco into a protein very similar in its metal binding properties to rat parvalbumin. In these curves, identical resonances have not been followed in all cases so that different combinations of the metal-bound forms were used to calculate the values of $^{rel}\beta_{CD}$, $^{rel}\beta_{EF}$, and c; but the overall binding profiles for all forms can be calculated from these parameters and show the similarities described above. Similar conclusions based upon the Eu(III) luminescence spectra have been made by Hapak *et al.* (1989). A fluorescence study also showed that D59E had a sensitivity to Mg as does rat parvalbumin but not Onco (MacManus *et al.*, 1989).

Quantitatively, we see that Onco and brOnco are similar with the most striking result in this regard, compared with all other parvalbumins, that the CD site does not prefer Lu(III) to any great extent ($^{rel}\beta_{CD} \approx 1$–3), whereas the EF site is more normal ($^{rel}\beta_{EF} \approx 100$–200). For all these proteins the cooperativity is low ($c \approx 1$–5). The Asp to Glu substitution increases the relative affinity of *both sites* by a factor of ≈5, bringing the numbers closer to those for rat parvalbumin.[3]

These changes in the affinities are paralleled by changes in the off-rate constants for Lu(III) from the various protein-metal species involved measured by optical stopped-flow

[2] A binding constant of Ca(II) for the CD site ($\approx 10^5$ M^{-1}) can be calculated from the data of MacManus *et al.* (1984). The binding constant of Ca(II) for the EF site is shown to be at least 10 times stronger. Williams *et al.* (1987) estimate Ca(II) binding constants of 1.5×10^5 and 1.3×10^6 M^{-1} for the CD and EF sites, respectively, from Lu(III)-Ca(II) displacement titrations and Lu(III) kinetic data. These two sets of data are in agreement. Given $^{rel}\beta_{CD} \approx 1$–3 and $^{rel}\beta_{EF} \approx 100$–200, binding constants of $\approx 10^5$ and $\geq 10^8$ M^{-1} can be estimated for Lu(III) binding to the CD and EF sites, respectively.

[3] The results for rat parvalbumin were analyzed without taking cooperativity into account. Since the binding is nearly sequential, the results are best compared by relating the product $c \times {}^{rel}\beta$ for D59E brOnco to $^{rel}\beta$ for rat parvalbumin (Williams *et al.*, 1987).

methods.[4] Here again we found Onco and brOnco to be virtually identical, whereas all rate constants for D59E brOnco are slower by a factor of ≈2–5.

The difficulty in the further interpretation of these results is that one does not know the effects on the absolute individual affinities for Ca(II) or Lu(III). For example, for the D59E substitution, the Ca(II) affinity could have remained unchanged and the Lu(III) affinity increased, or both have increased with the effect being greater for Lu(III). Possibly most surprising is that the effect was felt equally at both sites. This effect, coupled with the fact that the off-rate constants also all changed by about the same amount and the fact that the cooperativity was left relatively unchanged, suggests that any explanation based solely on the effects of the D59E substitution on the detailed ligation of Ca(II) or Lu(III) at the CD site would be incomplete and that a more general explanation is appropriate. One partial explanation is that the D59E substitution has destabilized the apo form of the protein relative to all metal-bound forms. Another part of the explanation may lie in an understanding of long range electrostatic interactions in these proteins. For example, the recent studies by Forsen and co-workers (Linse *et al.*, 1987; Forsen *et al.*, 1988) on the effects on metal binding affinity in calbindin-D_{9K}, caused by substitution of non-liganding Asp residues by Asn residues, indicate the influence of subtle changes in charge or charge position within the protein.

So, the original postulate (Williams *et al.*, 1987) that Asp in the $-X$ position might be the reason for the difference between the metal binding properties of the CD sites of oncomodulin and parvalbumin appears to be vindicated. By the simple change of one residue in the CD site, the D59E mutant acquired an approximate 5-fold increased affinity for Lu(III) (this study), increased affinity for Eu(III) (Hapak *et al.* (1989), and for Tb(III)[5] in both the CD and EF sites. The understanding of the reason for this global effect of a single-site change awaits specific mutagenesis of many more residues in both the CD and EF loops of oncomodulin.

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[4] The interpretation of the stopped-flow results in this manuscript is different from that presented in Williams *et al.* (1987) given the additional results presented herein. The metal-bound protein for the experiments in the previously published paper was prepared by the direct addition of a 2.3-fold excess of Lu(III) over $Ca(II)_2$ Onco; thus the Ca(II)Lu(III) form was present and three off-rate constants were measured (17.5 ± 2.5 s^{-1}; 0.060 s^{-1}; 0.0036 s^{-1}). The fastest was assigned to the off-rate of Lu(III) from the CD site, 0.066 s^{-1} we assigned as the off-rate of Lu(III) from the EF site with Ca(II) in the CD site; and 0.0036 assigned as the off-rate of Lu(III) from the EF site with Lu(III) in the CD site. However, the present results prove that the slowest off-rate measured is Lu(III) leaving the EF with Ca(II) in the CD site, and the intermediate reaction is Lu(III) leaving the EF with the CD site unoccupied. Also in the previous work the off-rate of Lu(III) from the CD site was not measured directly by stop-flow but calculated from the line shape analysis of specific ^1H NMR resonances from various points of the Lu(III) titration of calcium-saturated oncomodulin. This work was done at 40 °C which would have the effect of increasing the calculated off-rate constant a few-fold over the CD off-rate constant measured in the present work at 23.5 °C.

[5] J. P. MacManus, C. Hogue, and A. G. Szabo, manuscript in preparation.

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