^{113}Cd NMR Study of Bovine Prothrombin Fragment 1 and Factor X†

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ABSTRACT: The interaction of Cd^{2+} with bovine prothrombin fragment 1, prothrombin intermediate 1, factor X, and a modified (Gla-domainless) factor X has been studied with ^{113}Cd NMR. All the ^{113}Cd resonances observed in this study were in the chemical shift range expected for oxygen ligands, suggesting that cadmium is binding at the same sites where calcium binds. Both fragment 1 and factor X displayed two major resonances, one near 10 ppm from $^{113}Cd^{2+}$ that did not exchange rapidly with unbound $^{113}Cd^{2+}$ (the high-affinity, or H, resonance) and one near −15 ppm from $^{113}Cd^{2+}$ that exchanged rapidly with unbound $^{113}Cd^{2+}$ (the low-affinity, or L, resonance). The difference between the chemical shift of the H resonance and the chemical shift range of −90 to −125 ppm that has been reported for three other small calcium-binding proteins is postulated to be due to different coordination geometries for monocarboxylate and dicarboxylate ligands; Cd^{2+} binds to fragment 1 and factor X through the dicarboxylate side chains of γ-carboxyglutamate (Gla) residues. This allows contribution of only one oxygen per carboxyl group. At least one of the first few $^{113}Cd^{2+}$ ions bound to fragment 1 did not appear in the ^{113}Cd NMR spectrum until a total of five $^{113}Cd^{2+}$ had been added. This could be due to exchange broadening of initial $^{113}Cd^{2+}$ resonances due to sharing of ligands among several sites. Filling all sites would then restrict ligand exchange. Addition of Zn^{2+} displaced $^{113}Cd^{2+}$ from the H resonance sites. Factor X did not display the interactions among ion binding sites proposed for fragment 1.

The vitamin K dependent blood clotting proteins all contain the unusual amino acid γ-carboxyglutamate (Gla),[1] which is necessary for calcium binding and protein function [reviewed by Nemerson & Furie (1980) and Nelsestuen (1984a)]. The calcium binding sites have been studied most extensively in bovine prothrombin fragment 1, a thrombin cleavage product of prothrombin that contains all 10 Gla residues found in prothrombin and in factor X, the protein immediately preceding prothrombin in the blood coagulation cascade.

Calcium binding to both prothrombin and fragment 1 is cooperative (Henriksen & Jackson, 1975; Nelsestuen et al., 1981) with about six sites (Nelsestuen et al., 1981; Prendergast & Mann, 1977). Factor X has two high-affinity binding sites and several (up to 20) sites with lower affinity for calcium (Henriksen & Jackson, 1975; Jackson, 1980). Factor X also contains the recently discovered amino acid *β-erythro*-hydroxyaspartic acid (Fernlund & Stenflo, 1983; McMullen et al., 1983), which may be involved in the tight binding of one calcium (Sugo et al., 1984).

Cadmium-113 NMR has been very helpful in studies of other small (M_r 12000–18000) calcium binding proteins such as calmodulin (Andersson et al., 1983), troponin C (Forsen et al., 1979), and paravalbumin (Drakenberg et al., 1978). We have undertaken a ^{113}Cd NMR study of cadmium binding to bovine prothrombin fragment 1, factor X, and intermediate 1. The spectra showed distinct properties that may be characteristic of Gla-binding interactions.

Experimental Procedures

^{113}CdO was obtained from Merck and was converted into the perchlorate by the addition of $HClO_4$, followed by neutralization with NaOH. A small amount was converted to the chloride form with HCl for some studies. Prothrombin fragment 1 (Heldebrant & Mann, 1973), intermediate 1 (Heldebrant & Mann, 1973), factor X (Nelsestuen, 1984b), and Gla-domainless factor X (Sugo et al., 1984; Morita & Jackson, 1980) were prepared by published procedures. Prothrombin fragment 1 is generated by a very specific single cleavage of the prothrombin molecule, and methods for determining its purity and identifying pure, fully functional protein are well established (Nelsestuen, 1984b). It is unlikely, therefore, that protein impurities are responsible for some of the unexpected results. Protein concentrations were determined by using $E^{1\%}_{280nm}$ values of 10.1 for fragment 1 (Owen et al., 1974) and 16.4 for intermediate 1 (Butkowski et al., 1974) and molecular weights of 22500 for fragment 1 (Nelsestuen et al., 1981) and 50000 for intermediate 1 (Owen et al., 1974). Concentrations of factor X and Gla-domainless factor X were determined by dry weights with molecular weights of 55000 for factor X (Titani et al., 1975) and 50000 for Gla-domainless factor X. Fragment 1 activity was determined by measuring calcium-induced fluorescence quenching (Nelsestuen, 1976; Nelsestuen et al., 1976, 1981). All preparations showed at least 50% quenching before the studies and 44% quenching after the ^{113}Cd NMR spectra. Samples were prepared by dissolving the proteins, approximately 25 mg/mL (50 mg/mL for intermediate 1), in distilled deionized water, letting the protein itself act as a buffer. ^{113}Cd was added from a 1 M stock solution and did not cause any significant pH changes. Cadmium concentrations were determined at the University of Minnesota's Department of Soil Science Analytical Research Lab with an inductively coupled plasma (ICP) spectrometer.

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; fragment 1, bovine prothrombin fragment 1; intermediate 1, bovine prothrombin intermediate 1; Gla-domainless factor X, bovine factor X with amino acid residues 1–41 removed.

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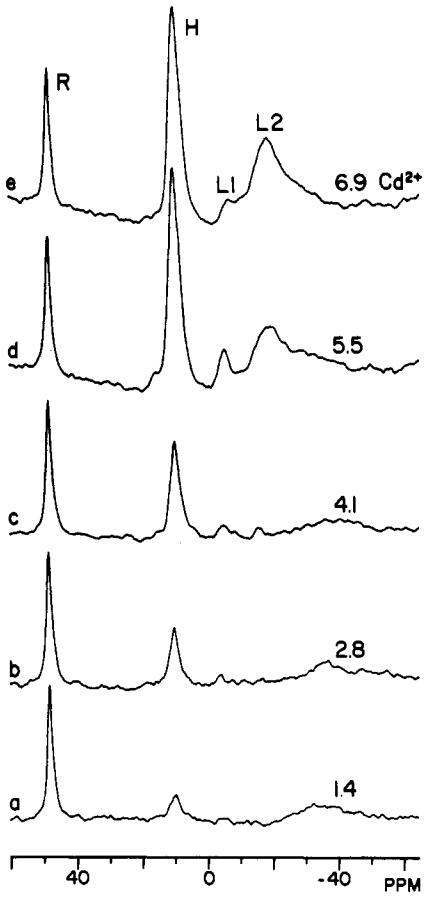

FIGURE 1: ^{113}Cd NMR spectra of 0.96 mM fragment 1 with increasing amounts of $^{113}Cd(ClO_4)_2$. Each spectrum contained 30 500–45 000 transients. The peak labeled R is from a reference capillary. The broad signal near −35 ppm is an artifact. $^{113}Cd^{2+}$:fragment 1 ratios are (a) 1.4, (b) 2.8, (c) 4.1, (d) 5.5, and (e) 6.9.

^{113}Cd NMR spectra were recorded on an 8.4-T Nicolet spectrometer at 80.08 MHz. Samples of approximately 5 mL were placed in a 20-mm solenoid probe without spinning. The sample was cooled to less than 4 °C, though the actual sample temperature could not be monitored during the acquisition. Acquisition times were usually 51.2 ms with a 90° pulse and a repetition time of 1 s, and the spectra were processed with 100-Hz line broadening. A coaxial reference capillary containing 1 M $CdSO_4$ and 1 M NaCl displayed a resonance at 48.0 ppm downfield from 0.1 M $Cd(ClO_4)_2$. Peak positions were measured relative to this capillary, and chemical shifts are reported relative to 0.1 M $Cd(ClO_4)_2$ at 0 ppm, downfield shifts being considered positive. Resonance intensities were measured by tracing the peaks on heavy paper and weighing the paper. The line width of the capillary reference peak was subtracted from the widths of other peaks in order to correct for magnetic field inhomogeneities.

In several spectra, a peak is labeled as an artifact. This broad signal was caused by a large spike in the free induction decay (FID), probably due to pulse feed-through, and appeared in the middle of the transformed spectra (only a portion of each spectrum is shown). The position of this signal relative to the other peaks changed as the spectrometer carrier frequency changed, always appearing in the middle of the spectra. In later spectra, the frequency was adjusted to remove this signal from the region of interest.

Results

Fragment 1. The ^{113}Cd NMR spectra of 0.96 mM fragment 1 with increasing amounts of $^{113}Cd(ClO_4)_2$ are shown in Figure

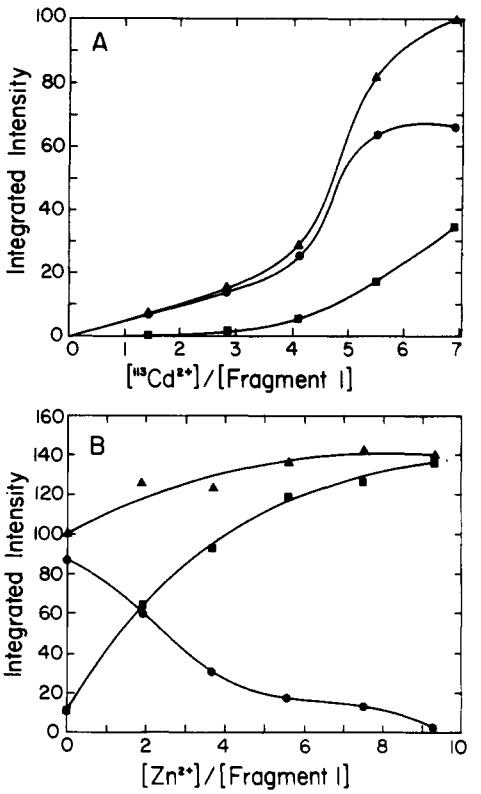

FIGURE 2: (A) Effect of increasing amounts of $^{113}Cd(ClO_4)_2$ on ^{113}Cd NMR resonance intensities with fragment 1, from the spectra in figure 1. (B) Effect of increasing amounts of $Zn(ClO_4)_2$ on ^{113}Cd NMR resonance intensities with fragment 1, from the spectra in Figure 3. (●) Resonance labeled H; (■) resonances labeled L1 and L2; (▲) total resonance intensity. The intensity is measured in arbitrary units.

1. A resonance (labeled H) first appeared at 10 ppm. As more $^{113}Cd^{2+}$ was added, the intensity of the first resonance increased, and two new resonances (L1 and L2) appeared at −5 and −18 ppm. Changes in the integrated resonance intensities are shown graphically in Figure 2A. The large and reproducible increase between 4.1 and 5.5 equiv of $^{113}Cd^{2+}$ is nearly twice as much as expected for 1.4 equiv. (The expected resonance intensity is obtained by dividing the total resonance intensity after addition of Zn^{2+} by the number of equivalents of $^{113}Cd^{2+}$ in the sample; see Figure 2.) Apparently, a previously unobserved $^{113}Cd^{2+}$ ion became visible at this point. No other resonances were detected in the range −160 to 340 ppm.

Line widths were approximately 150 Hz for the H resonance, 100 Hz for the L1 resonance, and 400–500 Hz for the L2 resonance. Relaxation times were measured on a sample of 0.89 mM fragment 1 with 6.75 equiv of $^{113}CdCl_2$. Transverse (T_2) relaxation times were 4 ms for the H resonance and 0.8 ms for the combined L1 + L2 resonance. Longitudinal (T_1) relaxation times were 0.5 s for the H resonance and 0.6 s for the combined L1 + L2 resonance. When the titration was repeated with $^{113}CdCl_2$ instead of $^{113}Cd(ClO_4)_2$ up to a final $^{113}Cd^{2+}$: fragment 1 ratio of 13.5:1, the initial resonance positions were the same, but as $^{113}CdCl_2$ was added beyond a 6:1 ratio, the L1 and L2 resonances merged and moved downfield, eventually becoming coincident with the (unchanged) H resonance.

When $Zn(ClO_4)_2$ was added to a solution of 0.81 mM fragment 1 and 5.9 equiv. of $^{113}Cd^{2+}$, the H resonance gradually disappeared while the L1 and L2 peaks merged and moved downfield (Figures 2B and 3). The combined areas of the H and L1 + L2 resonances also increased by 40% (Figure 2B). A similar result was obtained with 0.93 mM

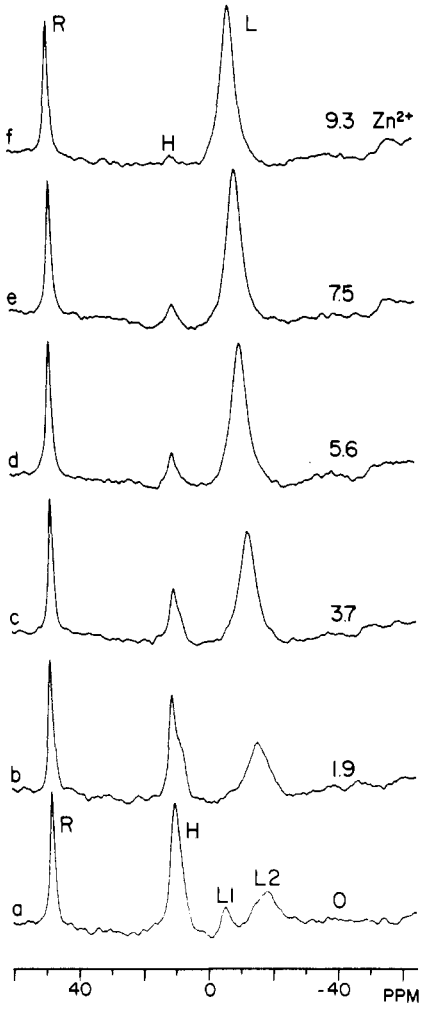

FIGURE 3: ^{113}Cd NMR spectra of 0.81 mM fragment 1 with 5.9 equiv of $^{113}Cd(ClO4)_2$ and increasing amounts of $Zn(ClO_4)_2$. Each spectrum contained 20 000 transients. The peak labeled R is from a reference capillary. Zn^{2+}:fragment 1 ratios are (a) 0, (b) 1.9, (c) 3.7, (d) 5.6, (e) 7.5, and (f) 9.3.

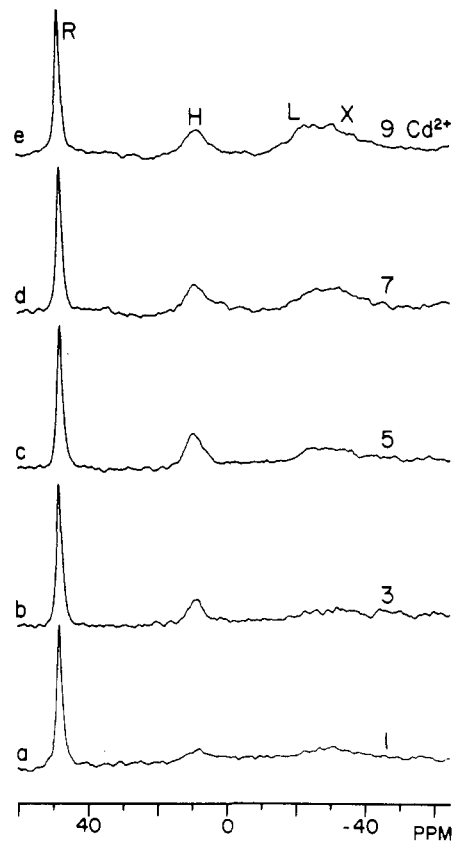

FIGURE 4: ^{113}Cd NMR spectra of 0.5 mM factor X with increasing amounts of $^{113}Cd(ClO_4)_2$. Each spectrum contained 20 000–50 000 transients. The peak labeled R is from a reference capillary. The X indicates that part of the broad signal near −30 ppm is an artifact. $^{113}Cd^{2+}$:factor X ratios are (a) 1, (b) 3, (c) 5, (d) 7, and (e) 9.

fragment 1 and 8.7 equiv of $^{113}Cd^{2+}$, but the increase in total areas was somewhat greater (100%), and the loss of area in the H resonance was more gradual (50% loss at 4.3 Zn/fragment 1 vs. 2.9 Zn/fragment 1).

Factor X. The ^{113}Cd NMR spectra of 0.5 mM factor X with increasing amounts of $^{113}Cd^{2+}$ are shown in Figure 4. The broad signal in the middle of each spectrum is due partly to an instrumental artifact (see Experimental Procedures) and partly to a ^{113}Cd resonance that increases in integrated intensity as more $^{113}Cd^{2+}$ is added. A resonance (labeled H) initially appeared at 9 ppm and increased in integrated intensity until 6 or 7 equiv of $^{113}Cd^{2+}$ had been added. A second resonance (labeled L) appeared near −25 ppm and moved downfield to −20 ppm as more $^{113}Cd^{2+}$ was added. When $Zn(ClO_4)_2$ was added to factor X with 11 equiv of $^{113}Cd^{2+}$, the H resonance disappeared quickly while the L resonance gradually increased in integrated intensity and moved downfield from −20 to −12 ppm. The final integrated intensity at 8 equiv of Zn was 50% greater than the combined H and L intensities before adding Zn.

Gla-Domainless Factor X. Mild treatment of factor X with chymotrypsin removes a small peptide (residues 1–41) that contains all the Gla residues (Sugo et al., 1984; Morita & Jackson, 1980). When $^{113}Cd^{2+}$ was added to a 0.4 mM solution of this Gla-domainless factor X, no resonance was visible up to 4.5 equiv of $^{113}Cd^{2+}$. At 9 equiv, a very broad peak (950 Hz) appeared near −11 ppm, and at 13.5 equiv the peak increased in intensity approximately 2-fold, moved downfield to 1 ppm, and became narrower (450 Hz). This resonance resembles the L resonance in fragment 1 and factor X. The addition of 2.4 equiv of $Zn(ClO_4)_2$ caused a further downfield shift to 5 ppm and a narrowing to 160 Hz but no measurable increase in total intensity.

Intermediate 1. When prothrombin is cleaved to produce fragment 1, the remaining peptide is called intermediate 1. No ^{113}Cd resonances were observed with 0.92 mM intermediate 1 and up to 3.6 equiv of $^{113}Cd^{2+}$ in the range −160 to 340 ppm.

Discussion

All the ^{113}Cd resonances observed in this study were in the chemical shift range expected for oxygen ligands (Armitage & Otvos, 1982). This result is consistent with the binding of cadmium at the same sites where calcium binds. The first resonance (labeled H) to appear near 10 ppm with fragment 1 and factor X (Figures 1 and 4) is assigned to $^{113}Cd^{2+}$ bound with high affinity to the protein, presumably through Gla side chains. The intensity of this H resonance increased with increasing $^{113}Cd^{2+}$ up to a certain point and then remained approximately constant (Figures 1, 2A, and 4). This resonance disappeared from the spectra of both proteins when Zn^{2+} was added to displace $^{113}Cd^{2+}$ (Figures 2B and 3).

As more $^{113}Cd^{2+}$ was added to fragment 1 and factor X, new resonances (labeled L1, L2, and L, and referred to as the L resonances in this discussion) appeared in the range −5 to −25 ppm (Figures 1 and 4) and are assigned to $^{113}Cd^{2+}$ bound with low affinity to the protein and rapidly exchanging with unbound $^{113}Cd^{2+}$. This assignment is based on the line width and

T_1 relaxation time, changes in the chemical shift when chloride or additional $^{113}Cd^{2+}$ are added, and the increased integrated intensity as excess $^{113}Cd^{2+}$ or zinc is added.

The sigmoidal shape of the integrated intensity plots (Figure 2) suggested that some ^{113}Cd nuclei were not observed continually in the spectra of fragment 1 and factor X. The sharp increase in intensity of the H signal for fragment 1 between 4.1 and 5.5 equiv of $^{113}Cd^{2+}$ (Figure 2A) suggested that some previously unobserved $^{113}Cd^{2+}$ became observable. It is possible that the signal from the first few $^{113}Cd^{2+}$ ions was broadened due to ligand exchange. Filling of all the Gla-containing sites may decrease the amount of ligand exchange and enhance the signal. Factor X showed a more linear increase in integrated intensity of the H signal. This interpretation requires that the high-affinity metal binding sites in fragment 1 are close enough to share some ligands.

The chemical shift of the H resonance in both proteins (10 ppm, Figures 1 and 4) is very different from the values of −90 to −125 ppm found in three other small calcium-binding proteins, calmodulin (Andersson et al., 1983), troponin c (Forsen et al., 1979), and parvalbumin (Drakenberg et al., 1978). The Gla residues of fragment 1 and factor X are essential to calcium binding and may be the basis for the unusual position of the H resonance.

In the recently published X-ray structure of the calcium–α-ethylmalonate complex (Zell et al., 1985), calcium is complexed to only one oxygen atom in each carboxylate group of α-ethylmalonate; calcium interaction with a single carboxylate group usually involves both oxygen atoms. Since ^{113}Cd chemical shifts are very sensitive to ligands (Armitage & Otvos, 1982), such a difference could account for the observed chemical shifts.

Gla-domainless factor X displayed no H resonance. The absence of this resonance, which was present in factor X, confirms the assignment of this resonance to high-affinity sites involving Gla side chains.

Registry No. L-Gla, 53861-57-7; ^{113}Cd, 14336-66-4; prothrombin, 9001-26-7; factor X, 9001-29-0.

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