Articles

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^{113}Cd and ^{13}C NMR of Cadmium(II) Transferrins

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The cadmium(II) derivatives of ovotransferrin and human serum transferrin have been investigated through ^{113}Cd and ^{13}C NMR spectroscopy. A sharp ^{113}Cd signal due to the bound Cd(II) ion is observed at 21.6 and 11.7 ppm for ovotransferrin and human serum transferrin, respectively. These chemical shift values are consistent with the involvement of only one histidine in each metal-binding set of the protein, as indicated by the recent X-ray structure of human lactoferrin. In the ^{13}C NMR spectra the protein-bound carbonate signal is found in both cases at 168.2 ppm, and it clearly splits into a doublet ($J \sim 20$ Hz) when the protein contains ^{113}Cd-enriched cadmium, thus giving further evidence of direct metal–carbonate binding. The addition of the nonsynergistic anion perchlorate to the ovotransferrin derivative resulted in the removal of the bound cadmium.

Introduction

Transferrins are globular proteins (M_r = 80000) acting as physiological iron carriers, involved in the biosynthesis of hemoglobin and other iron-containing proteins.[1] These double-site proteins show remarkable metal-binding properties, which have led to the characterization of several metal–transferrin derivatives, both of tripositive and bipositive metal ions. Many of the bound metals, used as spectroscopic probes in ESR [Cu(II), $V^{IV}O$],[2,3] EXAFS and resonance Raman [Fe(III)],[4] and multinuclear NMR investigations [Co(III), Tl(III), Al(III), Ga(III), Zn(II), Co(II)],[5-8] have shed some light on the structural features and the functional properties of the metal-binding sites: they were described as roughly equivalent sites, containing tyrosine and histidine ligands,[8,12] a water or hydroxide ion,[10,13-15] and carbonate[5,7] (as natural synergistic anion) arranged in a distorted octahedral geometry. Evidence has been obtained that the synergistic anion bridges the metal and a positively charged group of the protein.[5,7,16,17] The recent X-ray structure at 3.2-Å resolution of human lactoferrin,[18] which is likely to be close to that of human serum transferrin and ovotransferrin (particularly as far as the nature and spatial arrangement of the metal-binding residues are concerned[18]), confirms that two tyrosines bind the metal and indicates only one histidine and an aspartate as further ligands; furthermore, it confirms that carbonate bridges between the metal and protein, even if the resolution does not allow at present a statement of whether carbonate binds the metal in a monodentate or bidentate fashion and, consequently, if the sixth coordination position is occupied by a water molecule. Further information on the metal ion environment from solution measurements, which can be compared with the above structural findings, may be obtained by using the Cd(II) ion as an NMR probe. ^{113}Cd NMR is widely used in the investigation of protein binding sites due to the sensitivity of the ^{113}Cd chemical shift to both the nature of donor atoms and their coordination geometry.[19] Transferrins are known to bind the Cd(II) ion as tightly as Zn(II), Co(II), and Mn(II),[20] and such a binding was investigated through electronic spectroscopy.[21] Here a study is presented of the Cd(II) derivatives of both ovotransferrin (Otf) and human serum transferrin (Htf) performed through ^{113}Cd and ^{13}C NMR spectroscopy. In this case both the metal and anion are NMR sensitive, and complementary information on their synergistic binding can be obtained. Previous insertion of a magnetic nucleus, ^{205}Tl, gave evidence of direct carbonate binding to the metal ion.[6] ^{113}Cd, besides that, should also give information about the nature of the binding residues, due to the great variety of ^{113}Cd NMR data on both proteins and small complexes.[19]

Experimental Section

Ovotransferrin and human serum transferrin, both in the apo form, were purchased from Sigma and further purified according to the standard procedure.[16] Protein concentration was determined through UV absorption spectroscopy (ϵ_{280} = 91 200 M^{-1} cm^{-1} for Otf, and ϵ_{280} = 92 300 M^{-1} cm^{-1} for Htf). Solutions of isotopically enriched ^{113}Cd perchlorate were prepared by reacting 93.7 atom % ^{113}CdO (from Oak Ridge National Laboratory) with a stoichiometric amount of concentrated $HClO_4$ and then raising the pH to 4 with concentrated NaOH. The preparation of the bimetallic Cd(II)–transferrin derivatives was carried out according to the usual procedure,[4] in particular by adding stoichiometric amounts of cadmium perchlorate to millimolar protein solutions buffered at pH 8.2 with 5×10^{-2} M Tris–HCl in the presence of a 2-fold excess of bicarbonate. When ^{13}C-enriched bicarbonate was used, the apoprotein was first degassed at pH 4.5 by bubbling nitrogen through it in order to avoid carbon dioxide contamination from the air, and all the subsequent manipulations were performed under nitrogen atmosphere. The metal complex formation was checked through UV difference spectroscopy following the development of the tyrosinate bands at 250 and 297 nm upon metal addition.[20] The Fe_CCd_N monocadmium derivative (cadmium loaded in the N-terminal site) was prepared by reacting

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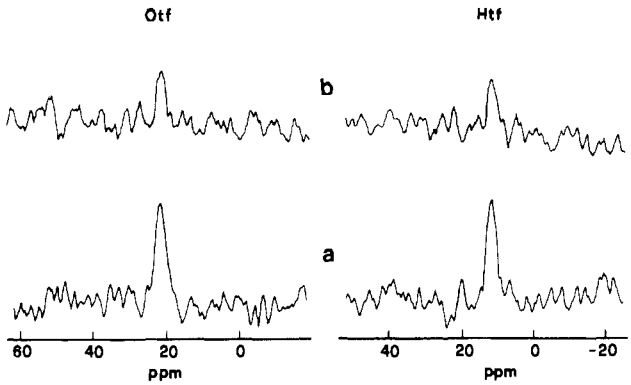

Figure 1. 44.4-MHz ^{113}Cd NMR spectra of (a) dicadmium-113 transferrins and (b) their corresponding $Fe_C{}^{113}Cd_N$ derivatives in 50% (v/v) D_2O and 0.05 M Tris–HCl, pH 8.2 (pH-meter reading uncorrected for isotope effect). Otf = ovotransferrin; Htf = human serum transferrin. Protein concentration = 2 mM; T = 23 °C.

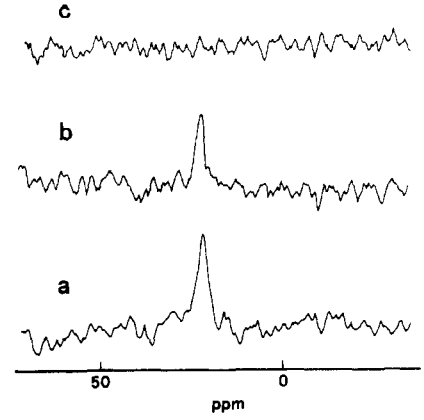

Figure 2. Effect of iron(III) addition on the ^{113}Cd NMR spectrum of $^{113}Cd_2$–Otf. Fe(III):Cd(II) ratio: (a) 0; (b) 0.5; (c) 1. The Htf derivative shows the same behavior.

apoprotein at pH 5.5 in the presence of an excess of bicarbonate with 1 equiv of iron(III) nitrate and then raising the pH to 8.2 with concentrated NaOH and adding 1 equiv of cadmium(II) perchlorate; iron(III) thus specifically reacts with the C-terminal site (that which maintains metal-binding capability at pH 5.5), while Cd(II) is subsequently forced to bind the N-terminal site. NMR measurements were performed with a Varian XL-200 spectrometer equipped with a tunable broad-band probe operating at 44.4 and 50.3 MHz for ^{113}Cd and ^{13}C, respectively. Experiments were performed at 23 °C by using 2 mM protein samples (2 mL in 10 mm o.d. tubes), and 50% (v/v) D_2O was used as field frequency lock. ^{113}Cd NMR acquisition parameters were as follows: flip angle, 30°; pulse delay, 1.5 s; spectral width, 16 kHz; collected number of scans, 30 000–50 000. The complete relaxation of the signals was checked by using longer delay times (up to 8 s). Spectra are externally referenced to 0.1 M aqueous (D_2O) $Cd(ClO_4)_2$ at 23 °C; quadrature detection was employed, and the reported spectra were obtained with a line broadening of 40 Hz. ^{13}C NMR spectra were obtained with a 45° flip angle, 3-s pulse delay, 4-kHz spectral width, and MLEV-16 proton decoupling; a line broadening of 4 Hz was applied. Typically, the reported spectra are the result of 15 000–17 000 scans.

Results

^{113}Cd NMR Spectra. A 2-equiv sample of isotopically enriched cadmium-113 perchlorate added to unbuffered apoprotein at pH 4.5 in the absence of bicarbonate gives a broad signal at 12.5 and 20 ppm for Otf and Htf, respectively, due to the Cd(II) ion not specifically interacting with the protein ("free" Cd(II) ion). When the pH is raised to 8.2 with Tris and concentrated NaOH and a 2-fold excess of bicarbonate is added, a relatively sharp signal ($\nu_{1/2} \sim 100$ Hz) appears at 21.6 ppm for Otf and 11.7 ppm for Htf (Figure 1a): its intensity in both cases approximately corresponds to 30% of that for the "free" Cd(II) resonance (pulse width and delay were carefully chosen in order to avoid any partial saturation of the signals). Dialysis of the sample against 5×10^{-2} M Tris buffer at pH 8.2 does not remove these signals, which, on the other hand, are reduced in intensity (with no changes in the line width) and completely disappear upon addition of 1 and 2 equiv of $Fe(NO_3)_3$, respectively (Figure 2): this behavior suggests their assignment to the Cd(II) ion specifically bound to the protein. No spectral changes were observed upon lowering the temperature to 4 °C. The spectra of both the Fe_CCd_N monocadmium derivatives at pH 8.2 show the same single signal observed for the Cd_2 derivatives but with a lower intensity (Figure 1b): the signal-to-noise ratio does not allow a precise evaluation of the intensity decrease, but the spectral features seem consistent with the lower metal occupancy of the N-site as compared to the C-site, as indicated by the development of the tyrosinate bands upon Cd(II) ion addition.[21]

The addition of a Cd(II) ion excess to both Cd_2 derivatives does not affect the protein-bound Cd(II) signal but results in the appearance of a broad signal at 85 ppm (not shown) whose intensity increases upon iron addition and which disappears upon dialysis against 5×10^{-2} M Tris buffer at pH 8.2; furthermore, it moves to lower field with increasing pH. An analogous signal was found for the Cd_6–alkaline phosphatase system in the presence of excess cadmium ion in the same buffer,[22] and both may be safely assigned to the excess cadmium interacting with Tris.[22] Addition of the chaotropic agent perchlorate to the Cd_2–Otf derivative causes a decrease of the intensity of the 21.6 ppm signal and no changes in the line width. The signal completely disappears for perchlorate concentration higher than 3 M (Figure 5); at the highest anion concentration, $CdCO_3$ precipitation was clearly detected.

^{13}C NMR Spectra. The ^{13}C NMR spectra of the Cd_2 derivatives of Otf and Htf containing natural-abundance Cd(II) ion and a 2-fold excess of ^{13}C-enriched bicarbonate at pH 8.2 show, besides the signal of free bicarbonate (161.4 ppm) and the broad protein carbonyl signal pattern, a resonance at 168.2 ppm (considerably broadened in the case of the human derivative), which may be safely attributed to slowly exchanging carbonate specifically bound to the two metal-binding sites[5,7] (Figure 3a,e). The ^{13}C NMR spectra of the same derivatives containing isotopically enriched ^{113}Cd (Figure 3b,f) show the same signal split into a doublet arising from the ^{113}Cd–^{13}C coupling between protein-bound cadmium and carbonate with ^2J = 22.3 and 20.0 Hz for Otf and Htf, respectively. The bound-carbonate signal of the human protein sharpens with increasing pH to 10, most likely as a consequence of a decreased site flexibility, possibly related to a change in the ionization state of a nearby residue and/or a change in the hydrogen-bonding network of the sites (Figure 3g,h). The ^{113}Cd NMR spectrum recorded at the same pH shows no detectable changes as compared to that at pH 8.2. For both cadmium derivatives, the addition of increasing amounts of $Fe(NO_3)_3$ lowers the intensity of the doublet until it completely disappears for a Cd(II):Fe(III) ratio of 1:1. The same doublet appears in the spectrum of the monocadmium derivative of both proteins (with cadmium specifically loaded in the N-site) at pH 8.2 (Figure 4): its intensity, compared with that observed for the (nominal) Cd_2 derivatives, supports the lower metal occupancy of the N-site as compared to the C-site.[21]

With higher bicarbonate concentration (8:1 excess) two signals at 164.4 and 165.5 ppm appear in the spectra of the Cd_2 derivatives at pH 8.2 (Figure 3c): the same signals were also previously observed for the $Co^{III}{}_2$ derivative of both proteins, and the latter was assigned to exchanging bicarbonate bound to the N-site.[5] Such a signal does not show any splitting when isotopically enriched cadmium is loaded to the protein (Figure 3d).

Addition of sodium perchlorate to the Otf derivative at pH 8.2 causes a decrease of the intensity of the metal-bound carbonate signal (Figure 5) which parallels that of the protein-bound Cd(II) signal in the ^{113}Cd NMR spectrum.

Discussion

The behavior of the signals at 21.6 and 11.7 ppm in the ^{113}Cd NMR spectrum of the Cd_2 derivative of Otf and Htf, respectively, closely parallels that of the metal-bound carbonate signal at 168 ppm in the ^{13}C NMR spectrum of the same derivatives. In

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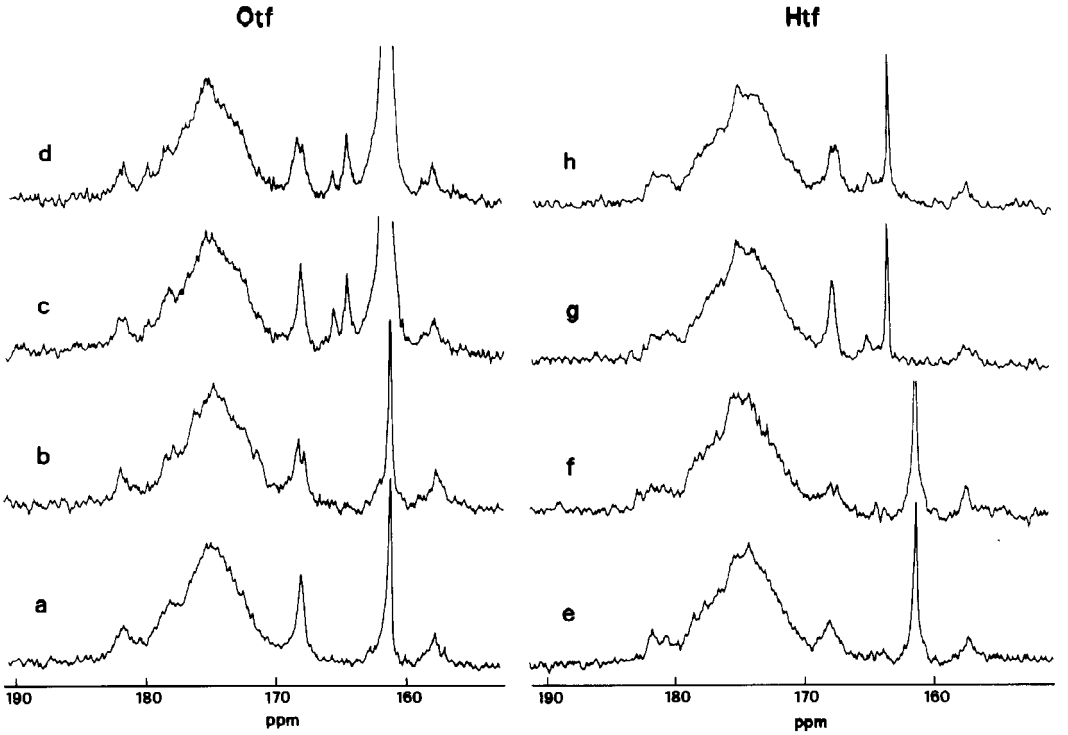

Figure 3. Proton-decoupled 50.3-MHz ^{13}C NMR spectra showing the carbonyl region of Cd_2–transferrin–bicarbonate complexes (Otf = ovotransferrin; Htf = human serum transferrin): (a, e) Cd_2 derivatives containing natural-abundance Cd(II) ion and a 2-fold excess of ^{13}C-enriched bicarbonate, pH 8.2; (b, f) the above derivatives obtained with isotopically enriched ^{113}Cd(II) ion; (c, d) as in Parts a and b, respectively, but in the presence of an 8:1 excess of ^{13}C-enriched bicarbonate; (g, h) as in parts e and f, respectively, but at pH 10. Protein concentration = 2 mM.

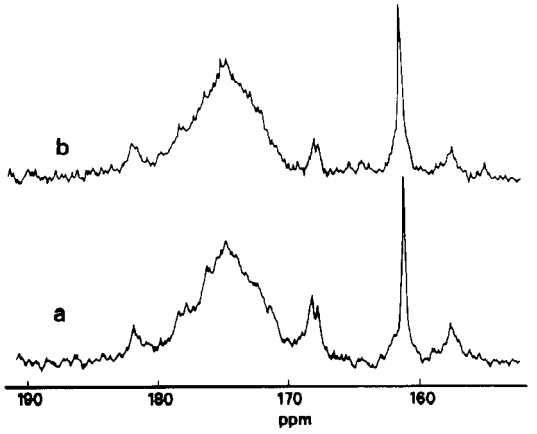

Figure 4. Proton-decoupled 50.3-MHz ^{13}C NMR spectra of the carbonyl region of (a) the $^{113}Cd_2$–Otf derivative and (b) the $Fe_C{}^{113}Cd_N$–Otf derivative, both in the presence of a 2-fold excess of ^{13}C-enriched bicarbonate, pH 8.2.

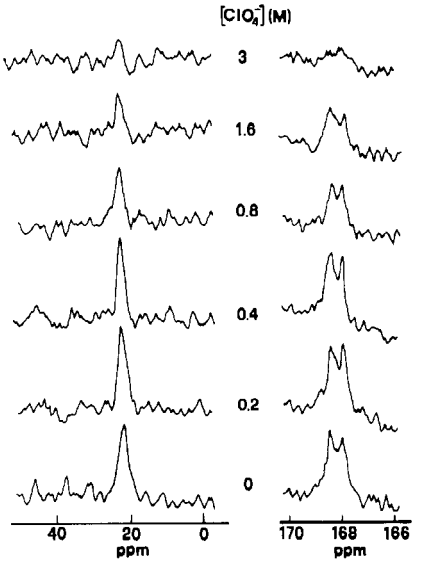

Figure 5. Effect of sodium perchlorate addition on the NMR spectral features of $^{113}Cd_2$–Otf: (left) ^{113}Cd NMR signal of the protein-bound Cd(II); (right) ^{13}C NMR signal of the protein-bound carbonate.

particular, they are still present after dialysis of the sample against buffer and disappear upon Fe(III) and perchlorate addition. Hence, due to the well-known synergistic nature of metal and carbonate binding, these signals may be assigned to protein-bound Cd(II). Their lower intensity as compared to that of the corresponding noninteracting Cd(II) ion at pH 4.5 is consistent with the presence of a multistate conformational equilibrium, most probably due to a fluctuation in the coordination geometry of the metal-binding sites: the observed signal may correspond to the most populated state slowly exchanging with at least two other states whose signals may be broadened beyond detection due to their low equilibrium concentration and their relative rates of interconversion of the same order of magnitude of their chemical shift difference.[23] The relatively weak binding of the Cd(II) ion to transferrins[21] may actually favor a certain flexibility of the metal chromophore; in addition, the presence of conformational equilibria in the binding sites has been previously proposed for several metal–transferrin derivatives.[14,15,24-29]

The ^{113}Cd chemical shift of cadmium complexes is sensitive to several ligand features, and in most cases its behavior in homologous compounds may be fairly well rationalized, also on a quantitative basis.[19] However, the same degree of confidence has not been reached yet for the relationship proposed for proteins.[19]

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The recent X-ray structure at 3.2-Å resolution of human iron(III) lactoferrin (which is likely to be very close to that of the iron derivatives of Otf and Htf, at least as far as the binding site arrangement is concerned) indicates two tyrosines, one aspartate, and one histidine as the metal ligands.[18] The so far obtained ^{113}Cd chemical shifts of protein binding sites where the Cd(II) ion is bound to oxygens and only one nitrogen ligand is around 30–50 ppm.[19] The 21.6 and 11.7 ppm values observed for Cd–Otf and Cd–Htf, respectively (relative to their dominant conformation), somewhat differ from the above values but are basically consistent with the presence of only one nitrogen ligand in the protein binding set if we consider the different (and not yet satisfactorily rationalized) contribution to the shielding of the many oxygen ligands. For the same reason, the different shielding of the protein-bound Cd(II) signal for Otf and Htf (far from being dramatic in the ^{113}Cd NMR chemical shift scale) has simply to be considered as indicative of a nonidentical cadmium-binding environment in the two proteins: any attempt to relate it to specific different structural features of the binding sites would be purely speculative. On the other hand, since in both cases a single signal of protein-bound Cd(II) is observed, the Cd(II) environments in the binding sites appear identical at the considered pH, at least within the resolution of the technique. The different binding properties of the two sites toward the Cd(II) ion[21] thus appear not to be directly related to factors concerning the first coordination sphere of the metal ion.

In the ^{13}C NMR spectra of some metal derivatives of Otf and Htf, the signal of the protein-bound carbonate lies in the region 165–169 ppm depending upon the nature and the charge of the metal ion.[5,7] This signal is found at 168.2 ppm for both the Cd(II) derivatives of Otf and Htf: the anion environments thus appear identical in these proteins and in each binding site. The splitting of such a signal upon isotopic enrichment of the Cd(II) ion, clearly attributable to ^{113}Cd–^{13}C magnetic coupling ($^2J \sim 20$ Hz), gives further evidence of direct metal–carbonate binding. The ^{113}Cd NMR spectra of the bimetallic derivatives containing ^{13}C-enriched bicarbonate do not show any splitting but are broader as compared to the corresponding ones with natural-abundance bicarbonate ($\nu_{1/2} \sim 130$ Hz vs ~ 100 Hz): obviously the line width is such as not to allow the observation of the coupling constant. Previous investigations of the ^{205}Tl derivatives of human serum transferrin[6] gave the same evidence of direct metal–carbonate binding; in that case the metal–carbon coupling constant was remarkably greater and slightly different for the two sites (290 and 265 Hz). For the present derivative the $^2J(^{113}Cd$–$^{13}C)$ coupling constants appear identical in each site, at least within the resolution available. The magnitude of the coupling is in good agreement with the $^2J(^{113}Cd$–$^{13}C)$ values observed for the carbonyl/carboxyl carbons present in the binding sites of cadmium-113 parvalbumin (6.9–24 Hz)[30] and for the carboxyl carbons in CdEDTA (13.2 Hz).[31] In addition, similar $^2J(^{113}Cd$–$^{13}C)$ values are observed in organocadmium compounds (19 Hz)[32] and polycadmium thiolate aggregates (9–16 Hz).[33] The Cd(II) ion is known to sequentially bind the protein with $\log K_1 \sim 6$ and $\log K_2 \sim 5$. The observation of a ^{113}Cd–^{13}C coupling constant as low as about 20 Hz sets the upper limit for the k_{off} rate for the Cd(II) ion (due to the synergistic nature of the binding) at about 40 s^{-1}; hence, by assuming that the metal on-rate is diffusion-limited[34] ($k_{on} = 10^9$ s^{-1}), one obtains a lower limit for the binding constant of Otf and Htf toward the Cd(II) ion of about 2×10^7. Since the metal on-rate used is, as a matter of fact, an upper limit,[35] the actual minimum value for the binding constant may be lower and therefore in line with the above spectroscopic values.

Nonsynergistic anions like perchlorate, thiocyanate, pyrophosphate, and chloride are known to decrease the stability of various metal–transferrin derivatives,[28b,36-39] most probably by binding positively charged residues near the binding site, which play a key role in the network of electrostatic interactions that stabilize the complex. In the present case, when increasing amounts of perchlorate are added, the intensities of both the NMR signals of the protein-bound Cd(II) and carbonate decrease (Figure 5), and a parallel $CdCO_3$ precipitation is observed. The cadmium ion thus appears to be removed from the protein, as previously observed for the Cu(II) ion.[39] In the present case, such a displacement takes places at higher anion concentrations, and this indicates a weaker binding of perchlorate to the cadmium derivative.

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Registry No. Cd, 7440-43-9; CO_3^{2-}, 3812-32-6; ClO_4^-, 14797-73-0.

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