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Evaluation of $[Co(gly)_3]^-$ as a $^{35}Cl^-$ NMR Shift Reagent for Cellular Studies

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We studied the efficacy of the tris-glycinatocobaltate(II) complex ($[Co(gly)_3]^-$) as a shift reagent (SR) for chloride by ^{35}Cl NMR spectroscopy and compared to that of $Co^{2+}_{(aq)}$. Due to the relatively low thermodynamic stability of $[Co(gly)_3]^-$, a 1:3 Co(II)/gly stoichiometric solution at physiological pH is approximately a 2:1 mixture of $[Co(gly)_2(H_2O)_2]$ and $[Co(gly)(H_2O)_4]^+$. This SR was found to be stable up to higher pH values than $Co^{2+}_{(aq)}$, better preventing $Co(OH)_2$ formation at alkaline pH. No significant differences in the $^{35}Cl^-$ NMR chemical shift induced by Co(II)/gly or $Co^{2+}_{(aq)}$ were observed in the presence of physiological concentrations of either Ca^{2+} or Mg^{2+}, or of either Na^+ or K^+. Although $Co^{2+}_{(aq)}$ was almost twice as effective as Co(II)/gly in shifting the $^{35}Cl^-$ NMR resonance at the same high ρ ([SR]/[Cl^-]) value and low ionic strength, $Co^{2+}_{(aq)}$ showed a significant decrease ($p < 0.05$) in the $^{35}Cl^-$ chemical shift at higher ionic strength. Line widths at half-height were significantly ($p < 0.05$) less for Co(II)/gly than for $Co^{2+}_{(aq)}$ at ρ values in the range 0.066–0.40. Intracellular chloride was clearly detectable by ^{35}Cl NMR spectroscopy in human skin fibroblast cells suspended in medium containing 40 mM Co(II)/gly SR. We determined that, although $Co^{2+}_{(aq)}$ provides a larger shift than Co(II)/gly at the same ρ value, there are significant advantages for using Co(II)/gly, such as pH stability, ionic strength independent chemical shifts, narrow $^{35}Cl^-$ NMR resonances, and reduced cellular toxicity, as a SR in biological systems.

Introduction

The study of chloride transport in cellular systems is of particular importance due to the associated chloride transport abnormalities present in cystic fibrosis[1] and Duchenne's muscular dystrophy patients.[2] Attenuation of chloride transport, through the inactivation of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, leads to a buildup of chloride inside the cell, resulting in pancreatic insufficiency, and mucosal buildup in the tracheae. Several techniques have been used to monitor chloride transport across cell membranes: whole-cell and partial-cell patch clamp, ion-selective electrodes, fluorescence, radioactive labeling, and more recently NMR spectroscopy by the use of either shift[3,4] or relaxation[5] reagents.

Aqueous lanthanide shift reagents (SRs), such as $Dy(TTHA)^{3-}$ and $HTm(DOTP)^{4-}$, have proven to be powerful tools to study cation transport using NMR spectroscopy.[6,7] These two SRs provide excellent means to study transport of cations; however, they do not work well for anion transport studies, presumably because their high negative charges repel anions. The resulting effect is little, or no, effective chemical shift of the anionic NMR resonance of interest. Because of the noninvasive nature of NMR spectroscopy, there are distinct advantages over the other techniques used to study anion transport, including the investigation of the number of chloride binding sites, of the thermodynamics of chloride interactions and of the kinetic properties of chloride transport.

A number of innovative $^{35}Cl^-$ NMR techniques have been employed using transition metal complexes, particularly

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$Mn^{2+}_{(aq)}$ as a relaxation reagent[5,8] for the extracellular chloride resonance, and $Co^{2+}_{(aq)}$ as a SR[3] to resolve the extracellular chloride resonance from that of intracellular chloride. Although both reagents have successfully resulted in the observation of intracellular chloride in vesicles and non-nucleated cell systems, they are toxic to cells because both $Mn^{2+}_{(aq)}$ and $Co^{2+}_{(aq)}$ slowly leak into cells.[4] Shachar-Hill and Shulman[3] reported the successful use of $Co^{2+}_{(aq)}$ as a SR in vesicles, but were unable to observe intracellular chloride in *Escherichia coli* cells. They interpreted this observation as being the result of plasma membrane binding the cytosolic chloride, thus broadening the resonance from the quadrupolar $^{35}Cl^-$ nucleus of intracellular chloride and making it unobservable.

We have reported the use of the Co(II)/gly SR to visualize the $^{35}Cl^-$ intracellular NMR resonance in red blood cell suspensions.[4] We designed this SR with the premise that the negative charge on the SR complex would cause slight repulsion from the membrane phospholipids and thus prevent entry of the SR into the cell.

The purpose of the present study was to compare the efficacy of the Co(II)/gly SR developed in our lab with the $Co^{2+}_{(aq)}$ SR for the application in biological systems by conducting ionic strength, concentration, pH, ionic competition, UV/vis, fluorescence, and magnetic susceptibility studies. Additionally, we provide the first report showing that intracellular chloride in nucleated cells is detectable using ^{35}Cl NMR spectroscopy and Co(II)/gly.

Experimental Section

$CoCl_2 \cdot 6H_2O$, glycine, $CaCl_2$, $MgCl_2$, $MgSO_4$, NaCl, NaH_2PO_4, KH_2PO_4, $NaHCO_3$, acetic acid, sulfuric acid, [4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid] (HEPES), and NaOH were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO) and used without further purification. 6-Methoxy-*N*-[3-sulfopropyl]-quinolinium (SPQ) was purchased from Molecular Probes (Eugene, OR). The human skin fibroblast (CCD-1001sk) cell line was purchased from American Tissue and Cell Culture (Manassas, VA).

Preparation of SR Solutions for pH Analysis. The nominal concentration of the Co(II)/gly SR or $Co^{2+}_{(aq)}$ was kept constant at 40 mM. All solutions contained HEPES, pH 7.4, 10 mM glucose, and NaCl. The pH was adjusted from pH 3.0 to pH 9.0 with NaOH or H_2SO_4. Ionic strength and [Cl^-] were adjusted to 0.15 and 0.10 M, respectively, with HEPES and NaCl.

Preparation of SR Solutions for Ionic Strength and Concentration Studies. The Co(II)/gly SR was prepared by dissolution of cobaltous chloride (or cobaltous nitrate for ionic competition studies; vide infra) in water with the addition of glycine in a molar ratio of 1:3 (cobalt:glycine). For comparison studies of $Co^{2+}_{(aq)}$ and Co(II)/gly, the ionic strength was adjusted to either 0.15 or 0.31 M with HEPES, pH 7.4. The final pH of each sample was adjusted to 7.4 with concentrated NaOH. Varying concentrations of SR and HEPES were added to a 10 mm NMR tube. NaCl was used to adjust the final concentration of Cl^- to 100 mM. The respective ratio ρ ([SR]/[Cl^-]) values were 0.033, 0.066, 0.13, 0.27, 0.33, and 0.40 for comparison of $Co^{2+}_{(aq)}$ and Co(II)/gly at the two ionic strength values studied.

Preparation of SR Solutions for Ionic Competition Studies. Solutions for cationic competition experiments contained 40 mM Co(II)/gly or $Co^{2+}_{(aq)}$, 10 mM glucose, 0.10 M [Cl^-], and 0.0−5.0 mM of either Ca^{2+} or Mg^{2+} for divalent cation studies or 0−0.10 M for K^+ or Na^+ monovalent cation studies, and varying amounts of HEPES, pH 7.4. Preparation of the SRs was from either $CoCl_2$ or $Co(NO_3)_2$ and glycine, as described above. Taking into account the concentration of $CoCl_2$ used, if any, adjustment of the concentration of chloride to 0.10 M in these solutions was achieved with NaCl, KCl, $CaCl_2$, $MgCl_2$, or tetramethylammonium chloride ($(CH_3)_4NCl$) depending on the particular competition experiments conducted. The pH was adjusted to 7.4 with tetramethylammonium hydroxide ($(CH_3)_4NOH$), and the ionic strength was kept constant at 0.31 M with HEPES, pH 7.4. We selected $(CH_3)_4NOH$, HEPES, and $(CH_3)_4NCl$ for adjusting pH, ionic strength, and [Cl^-] because the bulky counterion was unlikely to bind to the SRs and interfere in the competition experiments.

Preparation of SR Solutions for Fluorescence Spectroscopy. The effect of chloride on the fluorescence spectra of the SPQ dye was used to determine the amount of free chloride in SR solutions. All sample solutions, either with or without 0.04 M Co(II)/gly or 0.04 M $Co^{2+}_{(aq)}$ at pH values 7 or 8, contained 2 mM SPQ. Ionic strength was kept constant at 0.15 M, which was adjusted with 1 M HEPES (at either pH 7 or 8, respectively) and 0.5 M $NaNO_3$ solutions. Fluorescence spectra were recorded at 25 °C on a PTI QuantaMaster QM-1 fluorimeter. Emission spectra were acquired continuously at 443 nm, with the excitation wavelength set at 344 nm.[9] The SPQ fluorescence quenching effect by chloride is known to be purely collisional for chloride concentrations up to at least 0.16 M.[9a] We noticed that the SRs Co(II)/gly and $Co^{2+}_{(aq)}$ also quench SPQ through a collisional mechanism up to at least 0.5 M concentrations. Therefore, the modified Stern−Volmer equation to describe the relationship between the fluorescence intensity and the free concentrations of both quenchers is

$$F_o/F_{[Cl^-]+[SR]} = 1 + K_{sv}^{Cl}[Cl^-]_{free} + K_{sv}^{SR}[SR]_{free} \quad (1)$$

where F_o is the fluorescence intensity measured in the absence of both [Cl^-] and [SR], $F_{[Cl^-]+[SR]}$ is the fluorescence intensity in the presence of both [Cl^-] and [SR], and K_{sv}^{Cl} and K_{sv}^{SR} are the Stern−Volmer quenching constants for Cl^- and for SR, respectively. Here, we denote [Cl^-] and [SR] as the total concentrations of Cl^- and SR added to the solutions and $[Cl^-]_{free}$ and $[SR]_{free}$ as the free concentrations of Cl^- and SR in the solutions, respectively. K_{sv}^{Cl} and K_{sv}^{SR} were obtained through fitting of Stern−Volmer plots of fluorescence intensity data versus [Cl^-] in the absence of [SR] and versus [SR] in the absence of [Cl^-], respectively. In order to obtain $[Cl^-]_{free}$, the interaction between Cl^- and each SR needed to be considered,

$$Cl^- + SR \rightleftharpoons Cl^- \cdot SR \quad (2)$$

with association constants $K_a = [Cl^- \cdot SR]/[Cl^-][SR]$. Both $[Cl^-]_{free}$ and $[SR]_{free}$ can be expressed in terms of [Cl^-] and [SR]:

$$[Cl^-]_{free} = [Cl^-]/(1 + K_a[SR]_{free}) = (-1 - K_a[SR] + K_a[Cl^-] + ((1 + K_a[SR] - K_a[Cl^-])^2 + 4K_a[Cl^-])^{1/2})/(2K_a) \quad (3)$$

and similarly,

$$[SR]_{free} = [SR]/(1 + K_a[Cl^-]_{free}) = (-1 - K_a[Cl^-] + K_a[SR] + ((1 + K_a[Cl^-] - K_a[SR])^2 + 4K_a[SR])^{1/2})/(2K_a) \quad (4)$$

By substituting $[Cl^-]_{free}$ (eq 3) and $[SR]_{free}$ (eq 4) in eq 1, we

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obtained the function $F_o/F_{[Cl^-]+[SR]}$ in terms of [Cl^-] and [SR], and the parameters K_{sv}^{Cl}, K_{sv}^{SR}, and K_a. Through curve fitting of $F_o/F_{[Cl^-]+[SR]}$ vs [Cl^-], and assuming that [SR] was constant (0.04 M), the K_a values were obtained, and $[Cl^-]_{free}$ values were calculated using eq 3 for each SR.

Preparation of Human Fibroblast Cell Suspensions. Human skin fibroblast cells (CCD-1001sk) were grown until confluent, harvested, and washed once in a HEPES buffered saline solution (137 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, 20 mM glucose, 20 mM sucrose) and 40 mM Co(II)/gly. Cell viability was measured using the trypan blue exclusion test as described previously.[10]

UV/Vis Spectrophotometry. UV/vis spectra were determined on a Jasco-V550 UV/vis spectrophotometer. SR solutions were placed in disposable methacrylate cuvettes, and spectra were acquired using a wavelength scan between 285 and 700 nm, under the same conditions described above for the pH studies using NMR spectroscopy.

$^{35}Cl^-$, ^1H, and ^{13}C NMR Spectroscopy. $^{35}Cl^-$ NMR spectra of SR samples were acquired on either a Varian VXR-300 or a Varian VXR-400S NMR spectrometer at 25 °C. All solution spectra (without cells) were acquired with 512 transients, and a spectral width of 100000 Hz on a low-frequency 10 mm broadband probe tuned to ^{35}Cl (29.4 and 39.2 MHz for the Varian VXR-300 and Varian VXR-400S spectrometers, respectively). A 90° pulse width of 78 μs and a preacquisition delay of 40 μs were used for spectra obtained on the VXR-300, while a pulse width of 29 μs and a preacquisition delay of 0.4 s were used on spectra acquired on the VXR-400S. The total acquisition time for each SR sample was approximately 5 min. All samples were run with a reference tube placed within a sample tube, i.e., the inner tube contained 50 mM NaCl with 20% D_2O, and the outer tube contained 0.15 M NaCl and varying concentrations of SR. The D_2O in the inner tube was used to lock the frequency during the acquisition of the spectra.

$^{35}Cl^-$ NMR spectra of cell suspensions were acquired nonspinning on the VXR-300 equipped with a 10 mm broadband probe (29.4 MHz), with 8192 transients, a 90° pulse width of 78 μs, a preacquisition delay of 40 μs, a spectral width of 100000 Hz, and the variable-temperature unit set to 37 °C. Total acquisition time for cell samples was not more than 45 min.

^1H and ^{13}C NMR spectra of Co(II)/gly solutions in the absence and presence of Cl^- were acquired on the Varian VXR-400S NMR spectrometer at 25 °C, using a 5 mm broadband probe at 399.96 and 100.60 MHz, respectively. ^1H NMR spectra were typically run with 16 transients, a 33° pulse width, an acquisition time of 3.74 s, and a spectral width of 8000 Hz, while for the ^{13}C WALTZ-16 broadband decoupled spectra we used 7600 transients, a 66° pulse width, a spectral width of 66778 Hz, a preacquisition delay of 5.0 s, and an acquisition time of 0.96 s (12.7 h total acquisition). The ^1H and ^{13}C shifts were referenced internally to sodium 3-(trimethylsilyl)-1-propanesulfonate.

All ^{35}Cl NMR spectra were analyzed using Acorn Nuts (Acorn NMR, Livermore, CA). All spectra were integrated using the same vertical scale and spectral width. In the calculation of line width at half-height values of the $^{35}Cl^-$ resonances, the exponential multiplication, used for spectral smoothing, was subtracted from the experimental line widths.

Magnetic Susceptibility. Experiments were carried out using a modified version of Evans's method as previously described.[11,12]

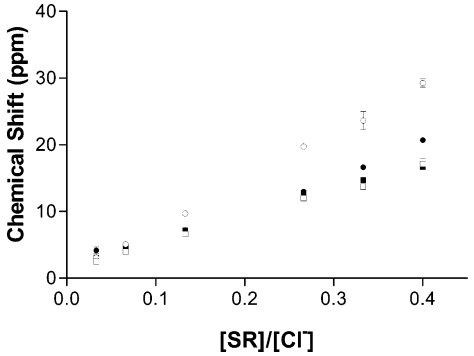

Figure 1. Plots of chemical shift (in ppm) vs ρ value for (○) $Co^{2+}_{(aq)}$, ionic strength (I) = 0.15 M; (●) $Co^{2+}_{(aq)}$, I = 0.31 M; (□) Co(II)/gly, I = 0.15 M; and (■) Co(II)/gly, I = 0.31 M. [Cl^-] was kept constant at 0.10 M in all experiments. For all points, n = 3.

Magnetic susceptibility measurements were carried out on a Varian VXR-400S NMR spectrometer at room temperature. A set of two coaxial NMR tubes was used. The inner tube was a capillary with 1.0 mm i.d., 1.2 mm o.d., and 90 mm length. The outer tube was a regular Wilmad NMR tube with 5 mm o.d. The outer tube was filled with 2% *tert*-butyl alcohol as an indicator in 99.9% D_2O. The inner tube was filled with the sample solution of 40 mM $Co^{2+}_{(aq)}$ or Co(II)/gly and 2% *tert*-butyl alcohol in 99.9% D_2O. The pH of the sample solution was adjusted to 7 or 8 with concentrated NaOH. At higher pH for $Co^{2+}_{(aq)}$, precipitation of $Co(OH)_2$ was observed. Therefore, Tris-Cl was used as a buffer for $Co^{2+}_{(aq)}$ at pH 8 to avoid such a precipitation. The solution level in the inner tube was lower than that in the outer tube to make the inner capillary stand straight up during fast spinning of the NMR tube. All solution spectra were acquired with 512 transients, a 28.4° pulse width, an acquisition time of 4.096 s, and a spectral width of 8000 Hz on a 5 mm broadband probe tuned to ^1H (399.96 MHz). The spinning frequency was set to 35 Hz. The number of unpaired electrons for $Co^{2+}_{(aq)}$ or Co(II)/gly was calculated from the chemical shift difference between the *tert*-butyl alcohol indicator in ^1H NMR resonance in the two compartments with or without $Co^{2+}_{(aq)}$ or Co(II)/gly.[12]

Statistical Analysis. Calculated average values are presented as mean ± SEM. Comparisons between two sets of experimental data were made using the paired Student's t-test, with $p < 0.05$ being considered significant.

Results

Ionic Strength Effects on $^{35}Cl^-$ NMR Chemical Shifts. For ρ values > 0.1, $Co^{2+}_{(aq)}$ produced a significant ($p < 0.05$) decrease in the $^{35}Cl^-$ paramagnetic induced shift at higher ionic strength (see Figure 1). For example, at ρ = 0.40, we observed a chemical shift difference of 29.3 ± 0.6 ppm (n = 3) at the ionic strength of 0.15 M vs 20.7 ± 0.1 ppm (n = 3) at the ionic strength of 0.31 M. The $^{35}Cl^-$ NMR shift induced by Co(II)/gly exhibited no significant dependence ($p < 0.36$) on ionic strength; at the same ρ value of 0.40, the chemical shift difference was 17.1 ± 0.9 ppm (n = 3) at the ionic strength of 0.15 M vs 16.2 ± 0.4 ppm (n = 3) at the ionic strength of 0.31 M.

Relationship between $^{35}Cl^-$ NMR Line Widths and Concentration of SR. The relationship between the line

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Table 1. Observed ^{35}Cl$^-$ NMR Chemical Shift for Cationic Competition with Either 40 mM [Co(gly)$_3$]$^-$ or 40 mM [Co$^{2+}_{(aq)}$][a]

[Ca^{2+}] or [Mg^{2+}] (mM)	Co(II)/gly		[Co$^{2+}_{(aq)}$]		[Na$^+$] or [K$^+$] (mM)	Co(II)/gly		[Co$^{2+}_{(aq)}$]	
	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}		Na$^+$	K$^+$	Na$^+$	K$^+$
5.0	23.8 ± 0.5	23.1 ± 0.4	37.6 ± 0.2	38.9 ± 0.2	100.0	23.7 ± 0.4	23.8 ± 0.3	36.2 ± 0.8	36.5 ± 0.4
4.0	23.6 ± 0.3	23.4 ± 0.4	39.0 ± 0.4	38.7 ± 0.4	80.0	23.2 ± 0.3	23.4 ± 0.2	36.0 ± 0.4	36.4 ± 0.3
3.0	22.8 ± 0.4	23.0 ± 0.4	38.3 ± 0.3	37.9 ± 0.1	60.0	23.0 ± 0.2	23.5 ± 0.3	36.4 ± 0.3	36.9 ± 0.1
2.0	22.5 ± 0.3	22.8 ± 0.6	38.1 ± 0.4	39.1 ± 0.2	40.0	22.7 ± 0.1	22.3 ± 0.3	37.3 ± 0.2	38.1 ± 0.5
1.0	23.6 ± 0.3	24.0 ± 0.4	39.2 ± 0.3	38.3 ± 0.3	20.0	24.5 ± 0.3	23.4 ± 0.4	38.2 ± 0.3	38.1 ± 0.4
0.0	23.8 ± 0.4	23.8 ± 0.4	38.2 ± 0.5	38.2 ± 0.5	0.0	23.8 ± 0.4	23.8 ± 0.4	38.2 ± 0.5	38.2 ± 0.5
p values	0.50		0.36			0.87		0.39	

[a] All entries in the table, except for cation concentrations and *p* values, are ^{35}Cl$^-$ NMR chemical shifts (in ppm). [Cl$^-$] and the ionic strength were adjusted at 0.10 and 0.31 M, respectively. All data reported as mean ± SEM, $n = 4$.

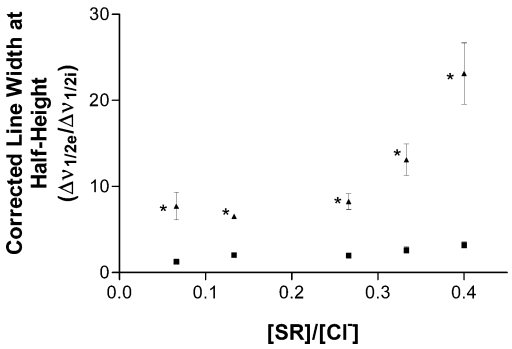

Figure 2. Corrected line width at half-height vs ρ value for (▲) Co$^{2+}_{(aq)}$ and (■) Co(II)/gly at ionic strength 0.15 M. [Cl$^-$] was held constant at 0.10 M. * statistically significant, $p < 0.05$, $n = 3$. Error bars smaller than points for Co(II)/gly, and at $\rho = 0.13$ for Co$^{2+}_{(aq)}$. Corrected line widths at half-height were calculated by (external resonance line width at half-height)/(internal resonance line width at half-height) to correct for magnetic field inhomogeneity and shimming between samples.

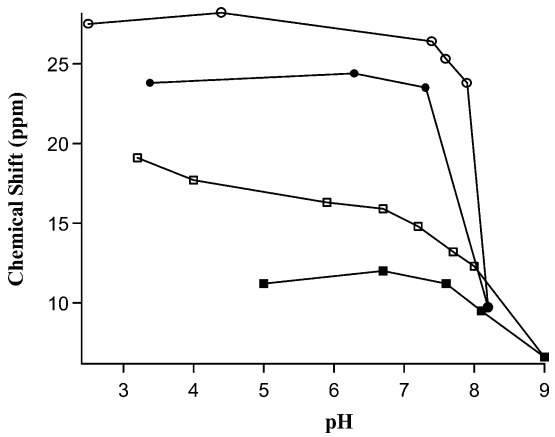

Figure 3. Plots of chemical shift (in ppm) vs pH value for (○) Co$^{2+}_{(aq)}$ forward titration; (●) Co$^{2+}_{(aq)}$ reverse titration; (□) Co(II)/gly forward titration; and (■) Co(II)/gly reverse titration. Note that only a partial restoration in chemical shift is evident for the reverse titrations of Co$^{2+}_{(aq)}$ or Co(II)/gly.

width at half-height of the ^{35}Cl$^-$ NMR resonance and varying SR concentrations at the ionic strength of 0.15 M are shown in Figure 2. The corrected line width values at half-height are significantly larger for Co$^{2+}_{(aq)}$ than for Co(II)/gly ($n = 3$, $p < 0.05$) for all ρ values investigated.

Cationic Competition Studies. Data from cationic competition studies with Co(II)/gly or Co$^{2+}_{(aq)}$ are shown in Table 1. No significant differences in the ^{35}Cl$^-$ shift induced by Co(II)/gly or Co$^{2+}_{(aq)}$ with or without Ca^{2+} or Mg^{2+} and with or without K$^+$ or Na$^+$ were observed.

pH Dependence of ^{35}Cl$^-$ NMR Chemical Shifts. At the physiological ionic strength of 0.15 M, the ^{35}Cl$^-$ NMR chemical shift values in the presence of either Co$^{2+}_{(aq)}$ or Co(II)/gly were compared at varying pH values (Figure 3). A significant decrease in chemical shift was observed at higher pH (pH > 8.0) for both Co$^{2+}_{(aq)}$ and Co(II)/gly. As the pH increased from pH 3 to 7, the chemical shift remained almost constant until the physiological pH was reached, but it decreased as the pH approached values above 8. It was also observed that the color of the Co$^{2+}_{(aq)}$ solution changed from light pink to a blue precipitate at higher pH, whereas the color of [Co(gly)$_3$]$^-$ remained light pink and no precipitate was visible at higher pH. Upon addition of acid to decrease the pH, the ^{35}Cl$^-$ NMR chemical shift in the presence of Co(II)/gly or Co$^{2+}_{(aq)}$ was only partially restored, but the color of the system was fully restored for Co$^{2+}_{(aq)}$.

^{1}H and ^{13}C NMR Shifts of Co(II)/gly Solutions in the Absence and Presence of excess Cl$^-$. At pH 7.4, the ^{1}H chemical shift (3.62 ppm) of the C_α methylene protons of a Co(II)/gly aqueous solution at a nominal concentration of 40 mM displays a very small, positive shift of 0.07 ppm relative to 120 mM gly (3.55 ppm). This small paramagnetic shift does not change significantly in the presence of 100 mM NaCl ($\rho = 2.5$) or in the presence of 267.5 mM HEPES, pH 7.4 ($I = 0.31$ M). At the same pH value, the ^{13}C paramagnetic shifts of a Co(II)/gly solution at a nominal concentration of 200 mM [Co(gly)$_3$]$^-$ relative to 120 mM gly are very large and positive: 130.9 ppm for C_α and 121.2 ppm for C_o. These shifts, however, only experienced a very small but significant variation in the presence of 600 mM NaCl ($\rho = 3.0$).

UV/Vis and Magnetic Susceptibility Measurements. In order to study the coordination chemistry occurring at higher pH, the pH titrations were followed by UV/vis spectrophotometry as well as with magnetic susceptibility measurements. The UV/vis titration spectra of Co(II)/gly and Co$^{2+}_{(aq)}$ are shown in Figure 4A and Figure 4B, respectively. The insets in Figure 4 display the increases in the absorbance of the bands with increasing pH for the two Co(II) complexes. The pH effect was partially reversible upon acidification. There was no significant change ($p < 0.45$, $n = 5$) in the magnetic susceptibility of Co(II)/gly at pH 7 or 8, yielding values of 3.33 ± 0.06 and 3.25 ± 0.07 unpaired electrons for pH 7 and 8, respectively. For Co$^{2+}_{(aq)}$, a significant difference ($p < 0.05$, $n = 5$) between pH 7 and pH 8 was observed, yielding values of 3.22 ± 0.04 and 2.98 ± 0.03 unpaired electrons, respectively.

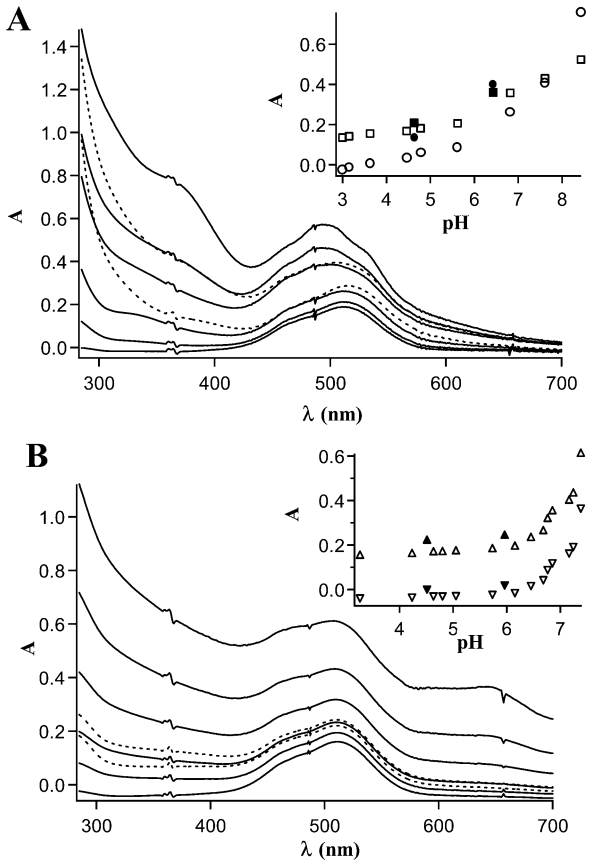

Figure 4. UV/visible spectra of (A) Co(II)/gly and (B) $Co^{2+}_{(aq)}$ at various pH values. Titrations from low to high pH are indicated by full spectral lines, whereas titrations from high pH back to low pH are shown with broken lines. Insets show the variations in absorbance of the bands at 369 nm (○) and 477 nm (□) for Co(II)/gly, and at 509 nm (△) and 610 nm (▽) for $Co^{2+}_{(aq)}$. Reverse titration absorbance values are indicated by filled symbols in each figure inset.

Interaction of Cl^- with the Cobalt Complexes. The data plots of SPQ fluorescence intensity ratios F_o/F vs $[Cl^-]$ and the Stern−Volmer constants K_{sv}^{Cl} obtained at pH 7 and 8 are shown in Figure 5 and Table 2. There was no significant difference ($p < 0.15$) between the K_{sv} values in the absence of SRs at pH 7 and 8 ($92 \pm 2\ M^{-1}$, $n = 3$, and $87 \pm 3\ M^{-1}$, $n = 3$, respectively). These values are somewhat smaller than the reported K_{sv}^{Cl} value of 118 M^{-1} in water at 23 °C,[9a] possibly due to the weak quenching effect of 0.5 M nitrate present in our solutions. K_{sv}^{SR} constants were obtained from the appropriate Stern−Volmer plots in the absence of Cl^- (data not shown), yielding values of $59 \pm 1\ M^{-1}$ and $45 \pm 1\ M^{-1}$ for $Co^{2+}_{(aq)}$ and $56 \pm 1\ mM^{-1}$ and $44 \pm 1\ M^{-1}$ for Co(II)/gly at pH 7 and 8, respectively. There was no significant difference between the K_{sv}^{SR} values for $Co^{2+}_{(aq)}$ and Co(II)/gly at pH 7 ($p < 0.18$, $n = 3$) and at pH 8 ($p < 0.36$, $n = 3$). However, for the values between pH 7 and pH 8 a significant difference was observed ($p < 0.05$, $n = 3$). The data plots of $F_o/F_{[Cl^-]+[SR]}$ vs $[Cl^-]$ in the presence of 0.04 M SR (either $Co^{2+}_{(aq)}$ or Co(II)/gly) are also shown in Figure 5. The K_a values obtained through curve fitting of eq 1 are $3.00 \pm 0.15\ M^{-1}$ and $3.23 \pm 0.10\ M^{-1}$ for $Co^{2+}_{(aq)}$ and $1.81 \pm 0.03\ M^{-1}$ and $3.92 \pm 0.28\ M^{-1}$ for Co(II)/gly at pH 7 and 8, respectively. The $[Cl^-]_{free}$ values in the presence of SR, calculated as described in the Experimental Section,

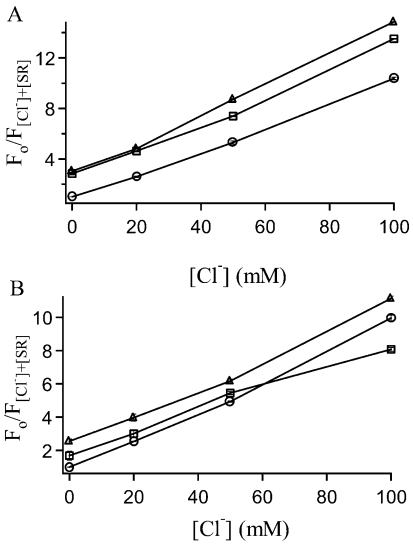

Figure 5. Normalized fluorescence intensities of Cl^-−SPQ complex at pH 7 (A) and at pH 8 (B), in the absence of SR (○), in the presence of 40 mM $Co^{2+}_{(aq)}$ (△), and in the presence of 40 mM Co(II)/gly (□).

Table 2. $\Delta[Cl^-]_{free}$ (nM) Values Obtained from SPQ Fluorescence Quenching by Cl^- in the Presence of SR (0.04 M) at Increasing Total $[Cl^-]$[a]

pH = 7			pH = 8		
$[Cl^-]$	$Co^{2+}_{(aq)}$	Co(II)/gly	$[Cl^-]$	$Co^{2+}_{(aq)}$	Co(II)/gly
0	0	0	0	0	0
20	2.41 ± 0.12	1.45 ± 0.02	20	2.58 ± 0.08	3.14 ± 0.22
50	6.01 ± 0.30	3.63 ± 0.06	50	6.45 ± 0.21	7.84 ± 0.55
100	12.0 ± 0.60	7.26 ± 0.12	100	12.9 ± 0.40	15.7 ± 1.10

[a] The entries in the table, $\Delta[Cl^-]_{free}$ (in nM), are the differences between the $[Cl^-]_{free}$ values in the presence and absence of SR (the latter values being equal to the total $[Cl^-]$ (in mM) used). The K_{sv}^{Cl} values in the absence of SR at pH 7 and pH 8 were $92 \pm 2\ M^{-1}$ and $87 \pm 3\ M^{-1}$, respectively.

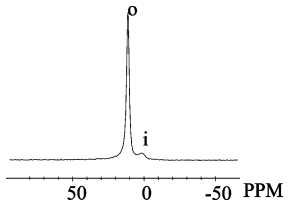

Figure 6. ^{35}Cl NMR spectrum of a human skin fibroblast suspension: (o) the extracellular $^{35}Cl^-$ resonance; (i) the intracellular resonance.

are not significantly different from the total $[Cl^-]$ values ($p < 0.15, 0.15, 0.15, 0.15$, $n = 4$, for solutions containing either $Co^{2+}_{(aq)}$ or Co(II)/gly at pH 7 or 8, respectively).

NMR Studies on Cellular Systems. A representative $^{35}Cl^-$ NMR spectrum from our studies on cellular systems is shown in Figure 6. Intracellular chloride was clearly detectable using a nominal concentration of 40 mM Co(II)/gly in a fibroblast cell suspension. Cell viability was measured to be 81% at the conclusion of the experiment, indicating good cell viability during the course of spectrum acquisition.

Discussion

The ability for a SR to be effective in NMR studies of cell compartmentation of anionic species, such as $^{35}Cl^-$,

depends upon a number of factors.[13] In the absence of SR, the ^{35}Cl$^-$ signals in the two compartments coincide. The presence of SR in the extracellular medium should, however, be effective in shifting the extracellular signal from the intracellular one at low, nontoxic [SR] concentrations and without causing too much broadening, and the SR should be thermodynamically stable in the extracellular medium and not leak into the cell.

Fast exchange conditions prevail for the ^{35}Cl NMR spectrum between the free and SR-bound Cl$^-$ ions. As the interaction between the SR and Cl$^-$ is weak, the shift observed for the extracellular resonance relative to the unshifted intracellular one is given by $\Delta\delta_{obs} = \Delta\delta_t f_b$, where $\Delta\delta_t$ is the fully bound shift and f_b is the SR-bound Cl$^-$ fraction. While $\Delta\delta_t$ depends on the shift mechanisms present, which will be discussed later, f_b depends on ρ and n, the number of binding sites of Cl$^-$ to the SR, as well as the respective association constants of Cl$^-$ to the SR, K_n. If ρ decreases, e.g., because [Cl$^-$] in the solution increases relative to [SR], f_b and the observed shift will decrease, due to the decreased number of anions being in close proximity to the SR compared to bulk Cl$^-$. Conversely, as [SR] in the solution is increased relative to [Cl$^-$], the observed shift will increase until it reaches a plateau at ρ values when all the SR binding sites are saturated. We did not reach such a plateau using the ρ values reported under the current experimental conditions, indicating that the SR$-$Cl$^-$ interaction is too weak to be measured by this technique in both cases (Figure 1). The present experiments were limited by our wish not to change the [Cl$^-$] and the ionic strength too much, keeping them whenever possible at values relevant to physiological conditions.

The thermodynamic stability of the SR complex is a determining factor in the kind of species present in solution. The concentration stability constants (defined as $\beta_{pqr} = [M_pL_qH_r]/[M]^p[L]^q[H]^r$) reported for the Co^{2+}/glycinate system at 25 °C and an ionic strength of 0.1 are log $\beta_{110} = 4.64 \pm 0.01$ ([Co(gly)(H$_2$O)$_4$]$^+$ species), log $\beta_{120} = 8.46 \pm 0.04$ ([Co(gly)$_2$(H$_2$O)$_2$] species), and log $\beta_{130} = 10.81 \pm 0.1$ ([Co(gly)$_3$]$^-$ species).[14] Because of these low values, the conditional overall stability constant of tris-glycinatocobaltate(II) at pH 7.4 is even lower. Figure 7 shows the distribution diagram vs pH of the soluble species calculated for the Co(II)/glycinate system at total concentrations of Co(II) and glycinate of 40 mM and 120 mM, respectively, using the above constants, as well as the formation constant log $\beta_{11-1} = -5.42$ for the hydrolysis product [Co(gly)(H$_2$O)$_3$(OH)]$^+$, the glycinate protonation constants of 9.78 and 2.35, and the ion product of water, p$K_w = 13.755$.[14] This species distribution was calculated using the HySS program within the HYPERQUAD package.[15] Although this diagram does not take into account the formation of the hydroxide Co(OH)$_{2(s)}$ at high pH (vide infra), it clearly predicts that at pH 7.4 the [Co(Gly)$_3$]$^-$ species is virtually nonexistent (3%), and the active SR species for ^{35}Cl$^-$ are the 1:2 [Co(Gly)$_2$(H$_2$O)$_2$] (57%) and 1:1 [Co(Gly)(H$_2$O)$_4$]$^+$ (37%) species. The presence of 10% excess glycinate is predicted to only increase to 4% the speciation of [Co(Gly)$_3$]$^-$, which increases to 21% at a 10-fold excess of ligand. Indeed, we observed that preparation of [Co(gly)$_3$]$^-$ using more than a 3-fold excess of glycine over Co^{2+} did not result in a further increase in the ^{35}Cl$^-$ shift.

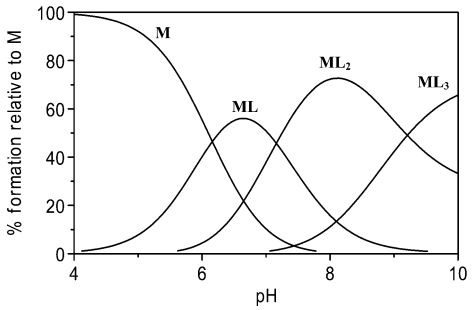

Figure 7. Species distribution as a function of pH calculated for a total Co(II) concentration of 40 mM and a total glycinate (gly = L) concentration of 120 mM using the thermodynamic data from the literature,[14] but not taking into account the formation of Co(OH)$_{2(pp)}$. M, ML, ML$_2$, and ML$_3$ represent the species [Co(H$_2$O)$_6$]$^{2+}$, [Co(gly)(H$_2$O)$_4$]$^+$, [Co(gly)$_2$(H$_2$O)$_2$], and [Co(gly)$_3$]$^-$, respectively.

At the same ρ values, the chemical shift induced by the Co(II)/gly SR was almost twice as small as that of Co$^{2+}_{(aq)}$ (Figure 1), presumably either because the different magnitude and sign of the charges in the complex ions present ([Co(gly)$_2$(H$_2$O)$_2$] and [Co(gly)(H$_2$O)$_4$]$^+$) result in the interaction between SR and Cl$^-$ not being as strong as that between Co$^{2+}_{(aq)}$ and Cl$^-$ (indicated by a smaller f_b value), or the $\Delta\delta_t$ values being smaller for [Co(gly)$_3$]$^-$ than for Co$^{2+}_{(aq)}$, or a combination of the two (vide infra).

The concentration of chloride in extracellular fluids is reported to be between 0.03 and 0.14 M.[16] Since the effectiveness of the SR is dependent largely upon [Cl$^-$], the choice of the [SR] to [Cl$^-$] ratio (ρ) to be used is of tantamount importance. As the [SR] increases, it may become more toxic to the cells, but as the [Cl$^-$] increases more SR is required to provide an adequate resolution of the ^{35}Cl$^-$ chemical shift. Therefore, the amount of SR required should be high enough to resolve the intra- and extracellular chloride resonances in the NMR spectrum, but not too high to cause cell toxicity and death.

Increasing the ionic strength of the Co(II)/gly SR solution had no significant effect on the induced ^{35}Cl$^-$ shift (Figure 1), indicating that the outer-sphere ion pair interaction with chloride is negligible even for the positively charged Co(II) complex present. A decrease in the ^{35}Cl$^-$ shift induced by the Co$^{2+}_{(aq)}$ SR was expected with an increase in ionic strength, due to the attenuation of ion$-$ion attractive interactions at higher ionic strength. This effect was indeed observed (Figure 1), in agreement with the decreased stability

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constant reported for the monochloro Co(II) complex at 25 °C when the ionic strength increases ($\log K = 0.0$ at $I = 0.5$ M and $\log K = -0.2$ at $I = 3.0$ M).[14] Therefore, ionic strength plays a more important role for the $Co^{2+}_{(aq)}$ SR in affecting the $^{35}Cl^-$ induced shift than ion competition. By contrast, neither ionic strength (Figure 1) nor ion competition (Table 1) significantly affected the $^{35}Cl^-$ shift induced by Co(II)/gly.

As described above, the $^{35}Cl^-$ shift induced by Co(II)/gly was not as large as that of $Co^{2+}_{(aq)}$ at the same ρ values (Figure 1), indicating that $\Delta\delta_t$ is larger for the latter SR. There are a number of factors contributing to the overall chemical shift, as given by the following expression: $\Delta\delta_t = \Delta\delta_d + \Delta\delta_c + \Delta\delta_{pc}$, where $\Delta\delta_d$ is the diamagnetic complex formation shift, $\Delta\delta_c$ is the contact shift, and $\Delta\delta_{pc}$ is the pseudo-contact or dipolar shift.[13,17,18] The diamagnetic complex formation shift, usually small, is the result of electrostatic interactions of metal ion induced ligand conformational changes.[17,18] Substitution of Co^{2+} in $[Co(gly)_3]^-$ for Mg^{2+} (40 mM Mg^{2+} at a ρ value of 0.40, in the absence of $Co^{2+}_{(aq)}$ or Co(II)/gly), its isomorphous diamagnetic analogue,[19] gave no significant ($p < 0.54$, $n = 4$) $^{35}Cl^-$ chemical shift (data not shown), indicating that the complex formation shift is not a significant contributor.

The paramagnetic shift induced by the Co(II) shift reagents on a given nucleus, in the simpler case of axial magnetic symmetry for the SR−Cl^- complex, is given by[18]

$$\Delta\delta_c + \Delta\delta_{pc} = -(2\pi A/h\gamma_I B_o)\langle S_z\rangle + DG \qquad (5)$$

The Fermi-contact contribution to the shift ($\Delta\delta_c$), originating from the additional magnetic field at the observed nucleus I caused by the electron magnetic moment of the Co(II) ion, depends on the hyperfine coupling constant A of the nucleus with a gyromagnetic ratio γ_I and on the spin expectation value $\langle S_z\rangle$ of the Co(II) ion (h is Planck's constant and B_o the external magnetic field). A is proportional to the total unpaired spin density at the nucleus, which is a sum of the contributions from each molecular orbital containing an unpaired electron, and is transmitted to the nucleus through its s orbital contribution to that molecular orbital. Spin density can be positive or negative, but because $\langle S_z\rangle$ is negative for Co(II), a positive spin density implies $A > 0$ and a positive (downfield) hyperfine shift. Unpaired electrons in Co^{2+} may result in positive contributions to spin density through direct unpaired spin delocalization into the ligand nucleus, while negative contributions to spin density may result from spin polarization mechanisms. The pseudo-contact contribution ($\Delta\delta_{pc}$), deriving from the Co(II)-centered magnetic moment, depends on the magnetic anisotropy constant D, which is characteristic of the Co(II) SR complex and is proportional to ($g_{||}^2 - g_\perp^2$) (where $g_{||}$ and $g_\perp$ are the principal g values parallel and perpendicular to the main symmetry axis of the SR complex), and the geometric factor G of the nucleus ($G = (3\cos^2\theta - 1)/r^3$) describing the position of the nucleus relative to the Co(II) ion through the Co(II)−I nucleus distance r and the angle θ between r and the main symmetry axis of the SR complex.[18] The pseudo-contact shifts have been estimated for the ^1H and ^{13}C nuclei of pyridine bis-coordinated to the tetragonal high-spin Co(II) bis-acetylacetonate complex using single-crystal magnetic susceptibility data.[20] While these were all negative, indicating that all the pyridine nuclei had G values of the same sign, the contact shift often changed sign between adjacent nuclei, reflecting a polarization of the unpaired electron spin density through the ligand bonds, which resulted in sign changes in the respective spin density and A values.

The larger positive $^{35}Cl^-$ shift induced by the $Co^{2+}_{(aq)}$ SR relative to Co(II)/gly results from a combination of the Fermi contact and the dipolar contributions. The interactions of Cl^- with both SRs are very weak, as predicted from the very low stability constant reported for the monochloro Co(II) complex,[14] and as inferred from the absence in decreases of free chloride concentrations in solutions containing either SR, as sensed by the indirect method based on SPQ fluorescence quenching by Cl^- (Table 2). The weak inner-sphere complexes formed by Cl^- ions with $Co^{2+}_{(aq)}$ lead to replacement of at least one of the H_2O ligands surrounding the paramagnetic center. The presence of a direct Co(II)−Cl^- bond leads to a large positive contact contribution to the $^{35}Cl^-$ shift induced by $Co^{2+}_{(aq)}$, because of direct σ and π spin delocalization of the unpaired electrons (e_g and t_{2g}, respectively) of Co^{2+} onto the ^{35}Cl nucleus, as observed before for the ^{17}O shifts of water in the aqua complexes.[18b] This large positive contribution is dominant relative to the dipolar contribution, which is likely to be negative,[20] and dominated by the orbital contribution and the distortion from the regular octahedral symmetry to the magnetic anisotropy constant D of the inner-sphere $[Co(H_2O)_5Cl]^-$ complex. The outer-sphere complexes are expected to have no significant contact and dipolar shifts.

The $^{35}Cl^-$ shifts induced by the Co(II)/gly SR result from the interaction of Cl^- with the $[Co(gly)_2(H_2O)_2]$ and $[Co(gly)(H_2O)_4]^+$ complexes present in solution. The outer-sphere contribution to this interaction is negligible, as shown by the absence of ionic strength effects on the observed shifts, and, if present, would give a negligible contribution to the $^{35}Cl^-$ shifts, because of the negligible Cl^--bound fraction and the very small shifts induced. The very small positive ^1H shift observed for the $C_\alpha H_2$ protons of the glycinate ligands in the complexes present in the SR solution complexes, which results from canceling of the opposing effects of the two spin polarization mechanisms from the Co(II) center through the carboxylate and nitrogen paths, clearly indicates that any contact interaction due to polarization of the unpaired electron spin density of Co(II), through the three bidentate glycine ligands, into Cl^-, is negligible. The dipolar

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shift should also average out. Thus, the large observed $^{35}Cl^-$ shifts induced by the SR are due to formation of inner-sphere complexes, such as $[Co(gly)_2(H_2O)Cl]^-$ and $[Co(gly)(H_2O)_3Cl]$, with Co(II)−Cl^- bonds allowing direct σ and π spin delocalization mechanisms, as discussed above for $[Co(H_2O)_5Cl]^-$. The efficacy of these mechanisms is reflected in the very large positive ^{13}C shifts observed for the carboxylate C_o and C_α carbons, with a sizable negative dipolar shift contribution for C_o, which has a much larger G value than C_α. Inner-sphere Cl^- complexation is also demonstrated by the extremely small effect of a 3-fold excess Cl^- ($\rho = 3$) on the ^{13}C shifts of the complexes present in the Co(II)/gly system, which are in fast exchange on the NMR time scale.

One advantage of the Co(II)/gly SR over $Co^{2+}_{(aq)}$ is that the line width at half-height of the SR-exposed $^{35}Cl^-$ resonance was much narrower (Figure 2). This should result from a smaller paramagnetic relaxation effect of Co(II) in the former SR, reflecting a lengthening of its electron spin relaxation time,[18b] as the percentage of inner-sphere complexes present should be comparable. This narrow resonance has a number of advantages in biological systems, particularly because lines tend to be broadened due to magnetic susceptibility effects in high-density cell suspensions undergoing nonisotropic tumbling.

The effect of pH on the $^{35}Cl^-$ chemical shift induced by $Co^{2+}_{(aq)}$ (Figure 3) showed an abrupt decrease at pH $\geq$ 8, which is attributed to the formation of insoluble $Co(OH)_2$ at high pH from the $Co^{2+}_{(aq)}$ solutions,[21] whose low solubility product (K_{sp} value of 1.3×10^{-15} M^3) indicates a relatively insoluble product in H_2O.[22] A decrease in pH results in a reversal of the above process, with a concomitant increase in the $^{35}Cl^-$ chemical shift as would be expected from the ionization of $Co(OH)_2$ into $Co^{2+}_{(aq)}$. From $K_{sp} = [Co^{2+}][OH^-]^2$, we calculated that, at pH 8, $[Co^{2+}]_{eq} = 1.3$ mM, which is much smaller than the starting concentration, 40 mM. Therefore, most $Co^{2+}_{(aq)}$ (about 38.7 mM) was converted into insoluble precipitate $Co(OH)_2$, and the effective $[Co^{2+}]$ decreased dramatically. At a pH value of 7, it can also be calculated that $Co^{2+}_{(aq)}$ is soluble up to 0.13 M.

With the Co(II)/gly system, the decrease of the induced $^{35}Cl^-$ shift with increasing pH is more gradual, starting at pH 7 and leading to negligible shifts only at pH 9 (Figure 3). As a result of the complexation of Co^{2+} by the glycinate ligand, the free $[Co^{2+}]$ is now much lower in those solutions. Its calculated value in a solution containing 40 mM total Co(II) and 120 mM total glycinate, using the speciation method described above, is substantially lower than its solubility obtained from the K_{sp} value of $Co(OH)_2$. The respective values of free $[Co^{2+}]$ for Co(II)/gly and $Co^{2+}_{(aq)}$ are 0.21 mM vs 1.3 mM at pH 8.0 and 1.77×10^{-5} M vs 5.2×10^{-5} M at pH 8.7 indicating that no hydroxide precipitation is predicted, as observed. Thus, while for $Co^{2+}_{(aq)}$ at pH > 8 a significant amount of $Co(OH)_{2(s)}$ was formed, and the actual $[Co^{2+}_{(aq)}]$ in the solution decreased dramatically resulting in a decrease of the $^{35}Cl^-$ chemical shift, the observed decrease of $^{35}Cl^-$ shift with the Co(II)/gly SR at alkaline pH does not result from $Co(OH)_{2(s)}$ formation but rather in part from a combination of the increased solution concentration of the shiftless $[Co(gly)_3]^-$ at higher pH (Figure 7), as well as to some other complex being generated, other than the $[Co(gly)(H_2O)_3(OH)]^+$ hydroxo complex.

The UV/vis spectra observed for both SRs at different pH values (Figure 4) are consistent with d^7 complexes, and at higher pH the observed absorbance changes could be due to a shift from high-spin $t_2^5e^2$ to low-spin $t_2^6e^1$ spin systems. The shift in spin configuration at high pH could be the result of coordination of the OH^- ligand to Co^{2+} or to the formation of other Co^{2+} complexes. With more OH^- present, the $Co^{2+}_{(aq)}$ system favors the formation of $Co(OH)_{2(s)}$ resulting in a decrease in chemical shift because of lower availability of the soluble $Co^{2+}_{(aq)}$ complex. However, the data from magnetic susceptibility studies of the Co(II)/gly system do not support the formation at higher pH of a complex with reduced spin multiplicity since no significant difference in magnetic susceptibility was observed. For $Co^{2+}_{(aq)}$ at pH 7, the complex is entirely in the high-spin configuration. At pH 8, however, approximately 1% of the complex is in the low-spin configuration.

If Cl^- ions were covalently bound to or strongly interacting with the SR and therefore not available for collision with SPQ, the concentration of free Cl^- detected by fluorescence would be significantly lower than the total $[Cl^-]$ in the absence and presence of SR. The absence of a significant difference of $[Cl^-]_{free}$ from the total $[Cl^-]$ in the SPQ fluorescence intensity in SR-containing solutions (Figure 5 and Table 2) reflects a weak interaction between Cl^- and the SR. The significant decrease of K_{sv}^{SR} values for the two SRs between pH 7 and 8 could be due to the formation of $Co(OH)_2$ at higher pH, resulting in smaller concentrations of $Co^{2+}_{(aq)}$ or Co(II)/gly complexes available to interact with SPQ.

Human fibroblast cell lines have been used extensively to study chloride transport[23−26] and have clinical relevance due to the associated abnormalities expressed in the CFTR gene product.[27] The limitations of the $^{35}Cl^-$ NMR technique are that the signal viewed by NMR spectroscopy takes an average of over 45 min (Figure 6) and the overall viability of the cell system is threatened without the use of perfusion. The theoretical $^{35}Cl^-$ NMR visibility of chloride being exchanged in a cellular system is low, namely, 60%.[28,29] Given the visibility limitations and that $^{35}Cl^-$ is a relatively insensitive NMR nucleus, longer experimental times are

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necessary, and this precludes the use of this technique to monitor short-lived transport phenomena. We have begun to conduct experiments where the cell line is constantly perfused with oxygenated medium in order to alleviate concerns with viability.

Although the $[Co(gly)_3]^-$ complex is not stable at physiological pH in the experimental stiochiometric conditions used, the $[Co(gly)_2(H_2O)_2]$ and $[Co(gly)(H_2O)_4]^+$ complexes present in the 1:3 Co(II)/gly SR formulation provide several advantages as a $^{35}Cl^-$ NMR SR over $Co^{2+}_{(aq)}$ for application to biological systems. First, at a pH of 8 or greater, and even near pH 7.4, $Co^{2+}_{(aq)}$ begins to precipitate out of solution. This precipitate is not observed with the Co(II)/gly SR up to pH 9. Second, the narrower $^{35}Cl^-$ line width at half-height as seen with Co(II)/gly is crucial for resolution of intracellular chloride in cellular systems. Finally, since there is no ionic strength effect or interference from the physiologically relevant cations Na^+, K^+, Ca^{2+}, and Mg^{2+} on the $^{35}Cl^-$ chemical shift when using Co(II)/gly, it should provide better versatility in biological applications.

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