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Chemical Sciences Division

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Chloride Binding Effects on Lysozyme Solubility

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Abstract

Lysozyme salting-out phase equilibria were examined in ammonium-sulfate solutions in the presence of low concentrations of either of two anions (chloride or trichloroacetate) that associate with lysozyme. Addition of either anion enhances lysozyme partitioning to the dense phase. Trichloroacetate increases partitioning more than chloride. At pH > 4, 0.1 m chloride enhances lysozyme partitioning in concentrated ammonium-sulfate solutions; however, at lower pH, chloride decreases lysozyme partitioning. The pH-dependent enhancement of partitioning by chloride is attributed to competition between chloride and sulfate binding. Ion binding may increase the effective protein diameter and thereby increase protein partitioning to the dense phase. ^{35}Cl NMR spectroscopy in the presence of $Au(CN)_2^-$ (which competes for chloride binding sites) provide a quantitative measure of chloride ions bound to lysozyme. These ion-binding and salting-out experiments suggest that, at constant ionic strength, pH-dependent ion binding to proteins may be responsible for the pH dependence of protein salting-out phase equilibria.

Introduction

Protein-ion interactions are known to play an important role in determining protein-solution phase behavior. Czok and Bücher (*1*) showed that addition of small amounts of anions that bind tightly to proteins can drastically reduce the solubility of protein in aqueous ammonium-sulfate solutions. Anions play a more crucial role in influencing protein solubility than cations, presumably because of water's ability to attract negative charge (*2*) . In general, those anions which have the highest molal surface tension increment (e.g., sulfate > thiocyanate) are the best for salting-out. The Hofmeister series also correlates with an ion's ability to structure the neighboring water molecules; e.g., sulfate is a better water "structure maker" than thiocyanate. Several studies, most notably those of Melander and Horváth (*3*) and Arakawa and Timasheff (*4-6*) , have described the effect of various salts on the salting-out behavior of proteins. Arakawa and Timasheff were able to predict qualitatively the observed effect of the Hofmeister series on protein salting-out behavior.

However, as discussed by Rothstein (*7*), there are numerous exceptions to the Hofmeister series for protein salting-out behavior. Experimental results with bovine α-chymotrypsin (*8, 9*) and with hen egg-white lysozyme (*10*) indicate that the general trend is not always observed. Riès-Kautt and coworkers demonstrated that basic proteins, such as lysozyme, are more easily crystallized by salts that are low in the Hofmeister series, such as thiocyanate, than better salting-out salts, such as sulfate (*11-13*) . Riès-Kautt and coworkers suggest that the stronger protein-binding properties of the ions lower in the Hofmeister series may be responsible for decreasing lysozyme's solubility, although the mechanism is not known. For example, only very small quantities of TCA[1] are necessary to precipitate most of a protein from solution (*7, 14*) . Czok and Bücher (*1*) demonstrated that the addition of a small amount (less than 50mM) of TCA greatly enhances protein crystallization from ammonium-sulfate solution. Protein titrations, ion-binding studies, and light-scattering experiments have shown that TCA binds strongly to proteins

(*15-18*) . The mechanism of TCA's effect on protein precipitation is not well understood but it may involve partial protein denaturation (*19*) and increased protein-surface hydrophobicity (*20*) .

An ion's effect on protein phase behavior can be considered as the sum of two separate short range effects (*5, 21*): 1) alteration of water structure in the vicinity of the ion, reflected in the molal surface tension increment and 2) specific interaction or binding of the ion with the protein. This work describes the effects of specific ion-binding interaction on protein salting-out phase behavior.

Numerous researchers have studied the interaction of proteins with ions using a variety of techniques. Osmotic-pressure experiments measure the protein-ion osmotic second virial coefficient which quantifies the attraction or repulsion between a protein and an ion (*22*) . Proton titrations in the presence of salt ions can be used to calculate the number of anions and cations associated with the protein. Scatchard and coworkers used this method to study the binding of several ions to human serum albumin (*16*) . Their results indicate that the lower an ion is in the Hofmeister series (i.e., the closer to thiocyanate), the stronger the ion binds to human serum albumin; ion binding followed a reverse-Hofmeister-series order. Arakawa and Timasheff's extensive densitometry investigations also indicate the reverse Hofmeister series trend for ion binding to proteins (*4-6*) . Fraaije and Lyklema (*23*) developed an analysis to determine anion and cation-binding numbers from protein-titration data. Their analysis, unlike Scatchard's, included electroneutrality constraints and indicated that a significant number of ions binds in the diffuse layer as well as at the protein surface. A recently developed experimental technique, electrospray-ionization mass spectroscopy, has been used to examine complexes of sulfuric acid (*12*) and heavy metals (*24*) with lysozyme.

Ion-binding investigations have also used ion-selective-membrane electrodes to examine the decrease in bulk-solution ion concentration on addition of protein to the solution. In a study of a diverse group of proteins over a range of pH, Carr used ion-selective-membrane electrodes to

investigate anion binding to proteins (*15*). Carr showed significant increases in chloride binding to proteins at low pH where proteins are more positively charged. In agreement with the titration and densitometry experiments, Carr's study also showed that the binding of anions follows the reverse Hofmeister series. In further agreement, Scatchard et al. (*25, 26*) showed that thiocyanate binds more strongly than chloride to human serum albumin. Binding of both chloride and thiocyanate ions increased with rising salt concentration. Similarly, more chloride ions bind to α-chymotrypsin than sulfate ions, except at very low pH where sulfate and chloride bind in similar numbers (*27*) .

Cl, Br and I NMR have been used to investigate ion binding to proteins; detailed reviews of the technique are available (*28*). The NMR technique is based on the decrease in the relaxation rate of a quadrupolar halide ion when it is moved from bulk solution into the presence of the protein. This decrease is not attributable to viscosity changes on addition of the protein to solution (*29*). Direct measurements of relaxation times have been made for Cl^- in the presence of proteins (*30, 31*). The half-height linewidth broadening of Fourier-transformed halide NMR spectra is measured in the presence of protein. This broadening is observable due to the rapid exchange of ions between protein-bound and free-solution states while the bound species itself is difficult to detect (*30*) . For example, on addition of 9.0 g/L lysozyme to a NaBr solution, the ^{81}Br spectra were broadened four-fold by the presence of protein (*29*). Linewidth broadening is typically reported as the excess line broadening, $\Delta\nu_{ex}$, defined as

$$\Delta\nu_{ex} = \Delta\nu_{obs} - \Delta\nu_0$$

where $\Delta\nu_{obs}$ is the experimentally-measured spectral line broadening (Hz) in the presence of the protein and $\Delta\nu_0$ is the line broadening of the reference salt-solution in the absence of protein.

The number of halide ions strongly bound to the protein can be determined by titrating with an agent that competitively binds to the same sites as the halide ion. A plot of $\Delta\nu_{ex}$ against the

ratio of moles of the agent per mole of protein gives a curve with two distinct slopes. The intersection of the two limiting tangents indicates the number of competing molecules necessary to remove the tightly bound ions, yielding the number of ions strongly bound to the protein. The analysis follows the two-site binding model of Scatchard (*32*), although it does not yield equilibrium binding constants for the ions. Ion-binding equilibrium constants for both weak and strong halide binding sites may be measured by varying concentrations of the halide ion (*33*) .

Although sodium dodecyl sulfate (SDS) is most commonly used to compete for protein-bound halides, one molecule of SDS may potentially block multiple ion-binding sites because ions are likely to bind most strongly near hydrophobic surface areas of proteins (*33, 34*). Therefore, use of SDS may result in underestimation of chloride binding. $Au(CN)_2^-$ (dicyanoaurate) competes with chlorides bound to human serum albumin nearly as effectively as SDS (*35*) but without the undesirable steric hindrance.

We report the effects of the addition of small quantities of anions that bind to proteins (chloride and TCA) on the salting-out phase equilibria of lysozyme in ammonium-sulfate solutions. We also report the pH-dependence of chloride binding to lysozyme measured by a quantitative NMR technique based on linewidth broadening of chloride spectra caused by ions associating with proteins.

Experimental Procedures (Materials and Methods)

Hen-egg-white lysozyme (L-6876) and $KAu(CN)_2$ (potassium dicyanoaurate) were purchased from Sigma, St. Louis, MO. A.C.S.-grade ammonium sulfate, sodium chloride and trichloroacetic acid were used. Deuterium oxide was purchased from Aldrich, Milwaukee, WI. Distilled water was de-ionized and filtered (0.20 μm) by a NANOpure system. Lysozyme concentrations were determined by absorbance at 280 nm, where the extinction coefficient is 2.635 L/(g cm) (*36*). All experiments were at 25±0.1°C.

Salting-out phase-equilibrium experiments are described elsewhere (*37*). Low concentrations of either trichloroacetic acid (TCA acid) or ammonium chloride were added to the protein and ammonium-sulfate solutions. Initial protein concentrations before phase separation were between 20-40 mg lysozyme/g water. For lysozyme, these initial concentrations do not affect phase equilibria (*10*).

For the NMR experiments, 6.5 g/L (0.45 mM) lysozyme solutions were prepared. All solutions contained 5% (v/v) D_2O. 0.1 M NaCl was optimal for the experiments: lower concentrations gave weak NMR signals, while at higher concentrations the solution-chloride signal overwhelmed the bound-chloride signal making detection difficult. Nitrogen was bubbled through the stock salt solutions and all samples were stored under nitrogen to minimize the presence of paramagnetic oxygen which can induce signal broadening (*38*).

^{35}Cl NMR experiments were performed on a Bruker AMX-300 at 29.41 MHz. A •/2 pulse of 26 μsec was used. A sweep width of 5000 Hz was used with 1K or 2K digitization. Acquisition times were longer than $5T_1$ (T_1 = longitudinal relaxation time). Price et al. (*31*) have reported $T_1 = 27.3$ μsec for chloride in aqueous solution. Spectrometer temperature was maintained at 25°C, although ion-binding results have been shown to be independent of small changes in temperature (*31*). An average of 4000-5000 scans were taken for each 0.1 M NaCl sample yielding a signal-to-noise ratio of 20 or better. Half-height linewidths were calculated from Lorentzian fits to the spectra.

Results

Protein Salting-Out Phase Equilibria with Two Salts

Lysozyme salting-out phase equilibria were measured as a function of ammonium-sulfate ionic strength in the presence of low concentrations of either TCA or chloride. Ammonium hydroxide and sulfuric acid were used to attain pH 5. Lysozyme partition coefficients are

presented in Figures 1 and 2 in the presence of TCA and chloride, respectively. The protein partition coefficient, K, is defined as the ratio of dense-phase (precipitate) to supernatant-phase protein concentration resulting from the salting-out phase separation. Phase-equilibrium results for lysozyme salted out from ammonium-sulfate solutions (*37, 39*) are also given in each plot for comparison. Dense-phase protein concentrations were not significantly affected by addition of either TCA or chloride; thus, the supernatant-phase protein-concentration behavior is reflected inversely in the partition coefficient.

In Figure 1, a slight enhancement of the partition coefficient was observed in ammonium-sulfate solutions with 0.001 m TCA (0-30% increase in K). In the presence of 0.05 m TCA, however, a large enhancement in partitioning was observed: a five-fold increase on average. The higher TCA concentration used in this study corresponds to 0.8% (w/v) TCA, well below the threshold where protein denaturation has been observed (about 10% (w/v) (*19*)).

Figure 2 shows that the addition of 0.1 m ammonium chloride increases partitioning to the dense phase by about a factor of two. This increase is significantly greater than would be expected by the small increase in ionic strength due to the chloride ion. In additional experiments (not shown), lysozyme dissolved in ammonium-sulfate solutions phase separated upon addition of small amounts of ammonium chloride.

Effects of low concentrations of chloride on lysozyme salting-out behavior were investigated as a function of pH. Protein partition coefficients are presented in Figure 3 as a function of pH at three ammonium-sulfate ionic strengths. At pH • 5, addition of 0.1 m ammonium chloride enhances lysozyme partitioning as indicated by Figure 2. However, at a pH between 4 and 5, the effect of chloride changes from enhancing salting-out separations at higher pH to reducing salting out at pH • 4.

Chloride Binding

^{35}Cl NMR was used to investigate chloride binding to lysozyme as a function of pH using varying concentrations of the chloride-binding inhibitor $Au(CN)_2^-$. Solutions were prepared by dissolving approximately 6.5 g/L lysozyme in 0.1 M NaCl. Line broadening ($\Delta\nu_{ex}$) at pH 3, 4, and 7 is shown in Figures 4, 5, and 6, respectively. The abscissa (the ratio of moles $Au(CN)_2^-$ to moles lysozyme) corresponds to the number of chlorides strongly bound to a lysozyme molecule. The intersection of the two linear regions determines the number of tightly bound Cl^- ions. Error bars represent the standard deviation of at least two experiments with the same sample and reflect NMR-instrument-related variations. The estimated error in the graphical determination of the chloride binding number is ±1 chloride per lysozyme molecule. Protein-free chloride-solution spectra are unaffected by pH (*34*). $\Delta\nu_0$ was 9.0-9.5 Hz for 0.10 M NaCl.

Figure 4 shows a significant broadening of the chlorine spectra at pH 3 due to lysozyme. Approximately 4 chlorides (3.8 as shown; a second trial gave 3.9) are bound strongly to lysozyme at pH 3. Figure 5 shows less linewidth broadening at pH 4; a more subtle slope change and correspondingly lower reproducibility are observed. About 3 chlorides (3.1 as shown; a second trial gave 2.3) are strongly bound to lysozyme at pH 4. Figure 6 shows that, at pH 7, the two linear regions are absent; we conclude that there are no strongly-bound chloride ions to lysozyme at pH 7, although there are weak chloride-lysozyme interactions that broaden the chlorine spectra relative to the bulk-chlorine spectra. Because lysozyme binds fewer chlorides per molecule than human serum albumin, chlorine linewidth broadening is smaller for lysozyme than for human serum albumin (*35*) .

Discussion

While the TCA-enhanced separations are subject to the interpretation that partial protein denaturation (*19, 40*) or perhaps increased surface hydrophobicity (*20*) enhanced the separation,

the chloride-enhanced separations are not. Chloride enhancement of salting-out separations is probably due to ion binding because chloride is neither a denaturant nor a hydrophobic anion.

Ion binding may affect protein salting-out phase behavior through an increase in the effective protein diameter. Force-plate measurements have observed interactions between colloidal surfaces attributable to ions bound to these charged surfaces (*41*) . These force-plate measurements also indicate that the bound ions appear to retain at least a fraction of their bulk-solution hydration layer. Table 1 gives ionic radii (R) and the increase in radius due to hydration (ΔR) for chloride and sulfate (*42*) . As a maximum, if a monolayer of sulfate ions is bound to a protein, the effective radius of a spherical protein may increase as much as 5Å. A more modest increase in effective protein diameter would be expected when a few sulfate ions are bound to a protein.

Protein diameter (protein excluded volume) has been shown strongly to influence phase separation behavior. Theoretical studies (*43, 44*) have demonstrated that globular protein solubility in a solution containing a precipitating agent (salt or polymer) depends strongly on the protein diameter, and only a few Å increase in protein diameter may double the calculated partition coefficients. Experimentally, the larger the protein, the lower the solubility (*7*). Therefore, increases in effective protein diameter caused by ion binding may increase protein partitioning to the dense phase.

For the lysozyme salting-out behavior in mixtures of chloride and sulfate, Figure 3 suggests one possible explanation. At high pH, addition of chloride increases the total number of ions bound to lysozyme, thus increasing the effective protein diameter and enhancing partitioning. At low pH, the stronger binding chloride ion displaces the larger sulfate from the protein surface leading to a smaller effective protein diameter and a decrease in partitioning relative to the sulfate solution. This possible explanation is supported by experimental studies of chloride and sulfate binding to α-chymotrypsin. Friedberg and Bose (*27*) have shown that at high pH, more

chloride ions bind to the protein, but at pH < 4, sulfate ions bind in similar or greater numbers. Competitive binding studies of chloride and sulfate for human serum albumin show that bound chloride ions are not displaced by an excess of sulfate ions (*31*).

A summary of the chloride binding results is given in Table 2. For comparison, chloride binding determined using an ion-selective-membrane electrode (*15*) are also provided, along with the net charge on lysozyme (*45*). Agreement of the NMR results with Carr's ion-selective-membrane results is excellent for pH 4 and 7, but at pH 3, the NMR binding number is lower than that reported by Carr. This difference is not unexpected as the ion-selective-membrane electrode measures depletion of bulk chloride but, unlike the NMR linewidth-broadening method, does not distinguish between strong and weak binding. As discussed elsewhere (*23*), measurements of such nonspecific ion binding are likely to measure ions weakly associated with proteins (perhaps in the counterion double layer), especially when the protein is highly charged. Since sulfate was observed not to affect line broadening of chloride in experiments with human serum albumin (*31*), the number of chlorides bound to lysozyme is unlikely to be affected by the high sulfate concentrations in our salting-out experiments, suggesting that even only a few tightly bound anions can significantly enhance protein salting out.

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Tables

Table 1 Ionic Radii[a]

Ion	R	ΔR
Cl^-	1.81	0.43
SO_4^{-2}	2.40	0.38

[a]Ionic radius, *R*, and radial hydration increment, Δ*R*, for chloride and sulfate (*46*) Radii are in Å.

Table 2 Chlorides Bound to Lysozyme[b]

pH	$z_{lysozyme}$	NMR	Carr
3	+16	4	6.7
4	+13	3	3
7	+7	0	0

[b]Number of chlorides bound per lysozyme molecule as measured by NMR experiments and by ion-selective-membrane electrodes (*15*). Lysozyme net charge, $z_{lysozyme}$, taken from Tanford and Wagner (*49*).

Figure Legends

Figure 1 Lysozyme partition coefficients for TCA-enhanced phase separation of lysozyme in ammonium-sulfate solutions at pH 5.

Figure 2 Lysozyme partition coefficients for chloride-enhanced phase separation of lysozyme in ammonium-sulfate solutions at pH 5. Each value measured twice for the 0.1 m NaCl experiment.

Figure 3 Lysozyme partition coefficients for chloride-enhanced phase separation of lysozyme in ammonium-sulfate solutions as a function of pH. Ionic strengths are based on ammonium sulfate only. Filled symbols (dashed lines) are for ammonium-sulfate solutions and open symbols (solid lines) are for solutions of ammonium sulfate plus 0.1 m ammonium chloride.

Figure 4 Excess linewidth broadening ($\Delta\nu_{ex}$) of ^{35}Cl NMR spectra for lysozyme in 0.1 M NaCl at pH 3 plotted against the ratio of moles of chloride-binding inhibitor to moles lysozyme. Lines drawn to determine the number of chlorides strongly bound per lysozyme molecule.

Figure 5 Excess linewidth broadening ($\Delta\nu_{ex}$) of ^{35}Cl NMR spectra for lysozyme in 0.1 M NaCl at pH 4 plotted against the ratio of moles of chloride-binding inhibitor to moles lysozyme. Lines drawn to determine the number of chlorides strongly bound per lysozyme molecule.

Figure 6 Excess linewidth broadening ($\Delta\nu_{ex}$) of ^{35}Cl NMR spectra for lysozyme in 0.1 M NaCl at pH 7 plotted against the ratio of moles of chloride-binding inhibitor to moles lysozyme.

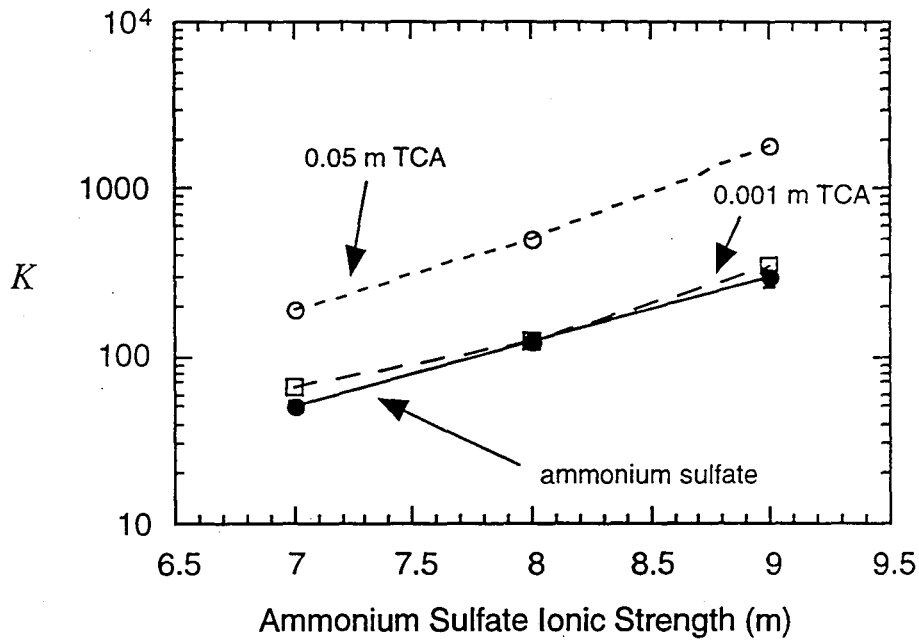
10^4
1000
100
10
K
0.05 m TCA
0.001 m TCA
ammonium sulfate
6.5
7
7.5
8
8.5
9
9.5
Ammonium Sulfate Ionic Strength (m)

Figure 1

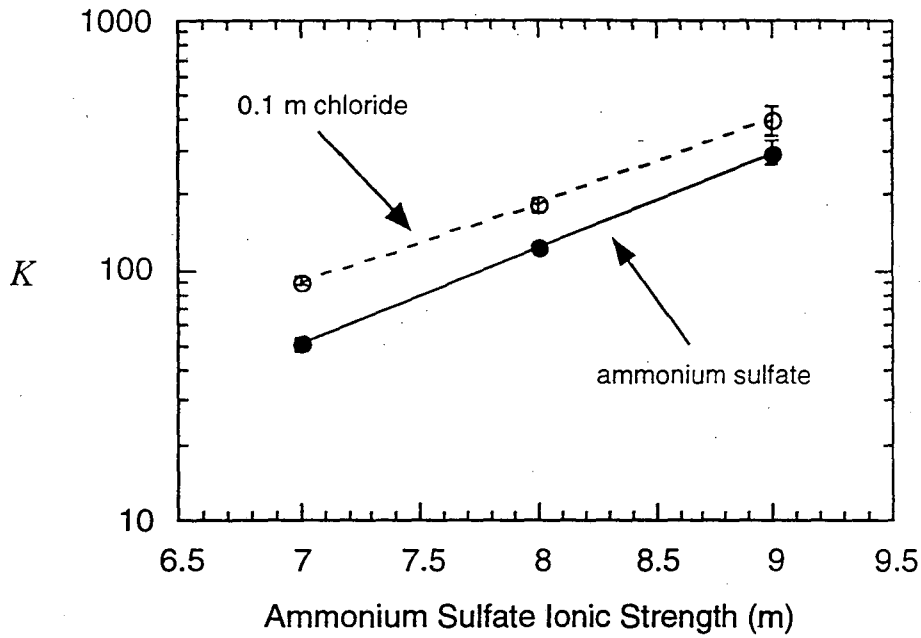
1000
100
10
K
0.1 m chloride
ammonium sulfate
6.5
7
7.5
8
8.5
9
9.5
Ammonium Sulfate Ionic Strength (m)

Figure 2

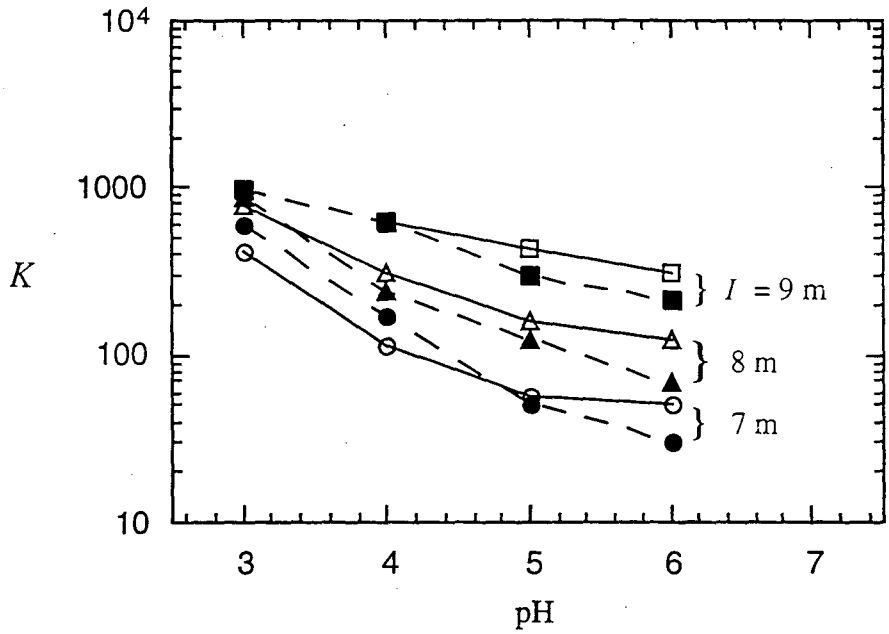

Figure 3

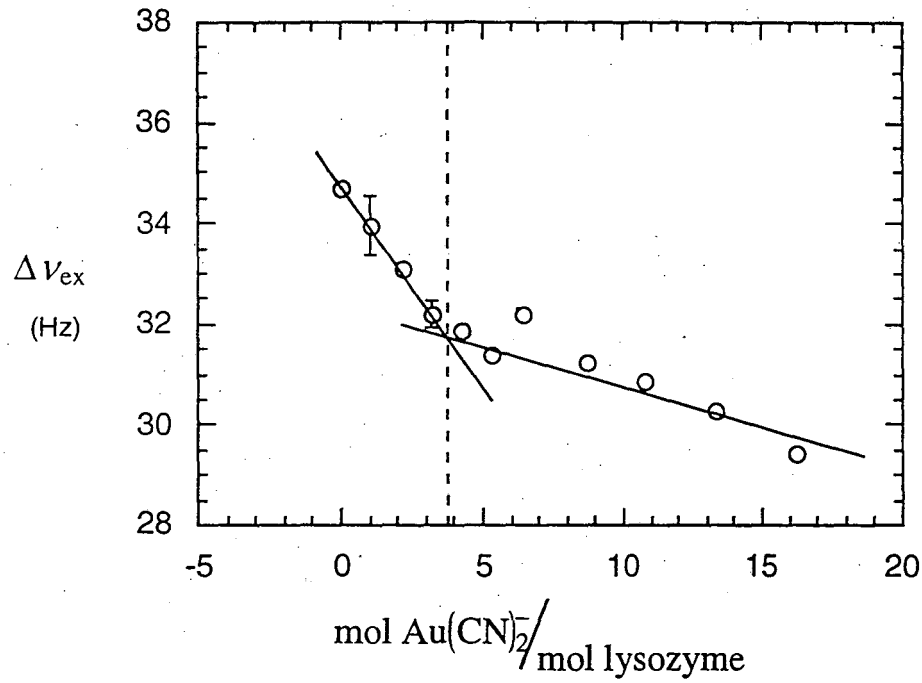
38
36
34
32
30
28
$\Delta \nu_{ex}$
(Hz)
-5
0
5
10
15
20
mol $Au(CN)_2^-$/mol lysozyme

Figure 4

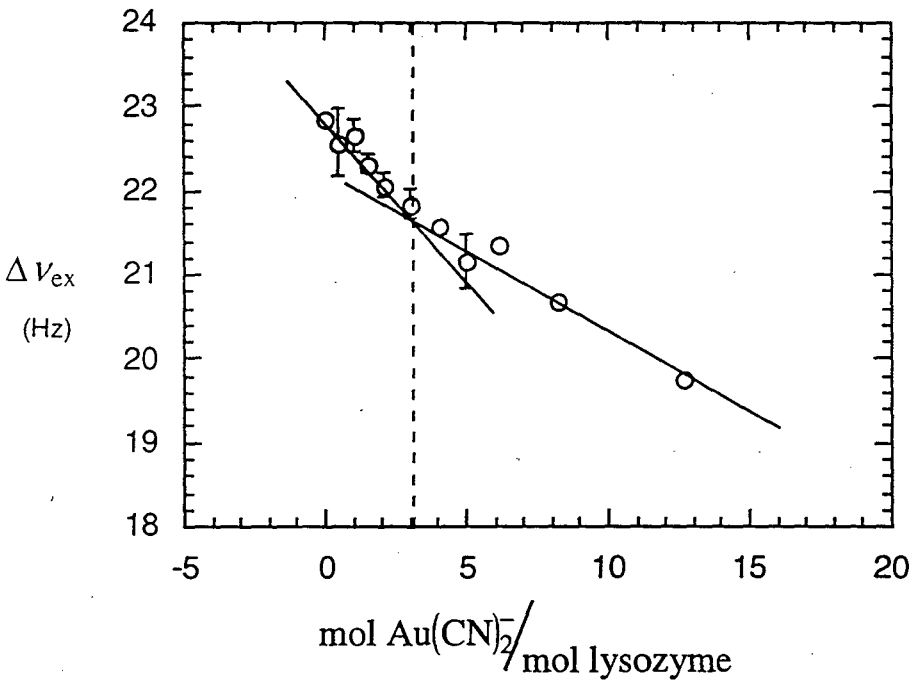

Figure 5

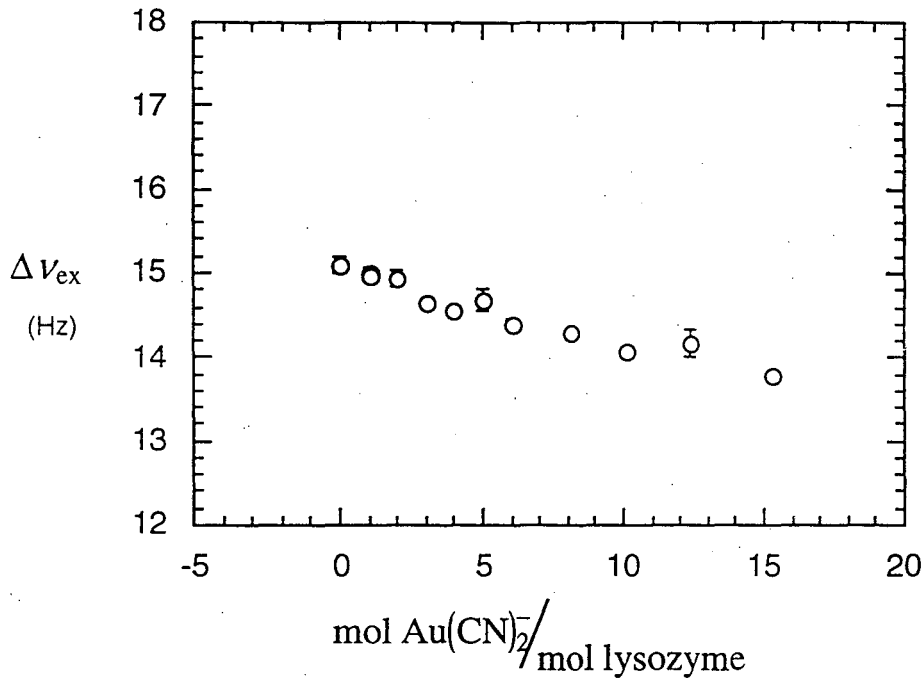

Figure 6

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AAY632
LBL Libraries