Halogen Ion Probe Study of Bovine Mercaptalbumin

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Abstract: The halogen ion probe technique is applied to bovine mercaptalbumin using the ^{35}Cl resonance to explore the potential and sensitivity of the method. The chloride resonance is studied as a function of chloride concentration and pH using mercury to label the free SH group. The denaturation of the mercaptalbumin is followed as a function of urea concentration, and the behavior of the SH site is compared with that of the protein as a whole. Zinc and cadmium are shown to be useful metal exchange centers, and the competition between zinc, cadmium, and mercury for protein sites is observed.

The nuclear magnetic resonance of nuclei other than hydrogen provides a useful probe for investigating the structure or conformation of proteins and other macromolecules. In particular, the halogen ion probe technique introduced by Stengle and Baldeschwieler[1] has been recently applied to several systems of interest[2-6] and the results indicate that the method has great potential for the illucidation of certain kinds of structural and mechanistic problems. To date none of the experiments reported has offered a sound basis for comparison of this technique with other physical measurements such as fluorescence depolarization or electron spin resonance that may reflect structural changes in a macromolecule. Because bovine mercaptalbumin has been rather well characterized by a variety of physical techniques, this protein was studied over a range of conditions that should permit direct comparison with the results of other methods.

The halogen probe technique may be applied using the chloride, bromide, or iodide resonance;[7] since sodium and chloride ions are found at significant concentrations quite generally in biological environments, this work was done using the ^{35}Cl resonance. The ^{35}Cl line width is usually dominated by quadrupole relaxation so that in the limit of extreme narrowing the line width for a nucleus of spin $^3/_2$ is given by

$$\Delta\nu = \frac{2\pi}{5}(e^2qQ)^2\tau_c \qquad (1)$$

where $\Delta\nu$ is the full line width in hertz at half-height, e is the unit charge, q is the electric field gradient at the nucleus of quadrupole moment Q, τ_c is the correlation time for reorientation of the field gradient, and the asymmetry parameter has been neglected.[8]

In 1 M aqueous sodium chloride solutions, the chloride ion is found in an approximately symmetric environment producing a field gradient at the nucleus approaching zero and a line width of about 13 Hz. If the quadrupolar chlorine nucleus can be found at environmentally different sites in solution, the line width will depend on the relative concentration of each site, the values of $(e^2qQ)^2$ and τ_c associated with each site, as well as the frequency with which the ^{35}Cl nucleus samples the various sites. In the case where exchange of the chloride is fast with respect to $1/\pi\Delta\nu$ a single composite line is observed with the line width given by

$$\Delta\nu = (\Delta\nu_a)P_a + (\Delta\nu_b)P_b = \sum_{\text{sites}} \Delta\nu_i P_i \qquad (2)$$

where $\Delta\nu_a$ and $\Delta\nu_b$ are the contributions to the line width associated with sites a and b while P_a and P_b are the probabilities that the chlorine is at site a and b, respectively.[9] The probability that a ^{35}Cl nucleus may be found at any one site may be given directly in terms of concentrations and coordination numbers; for example, the probability, P_S, that a chlorine nucleus will be found directly in association with a site S on a protein molecule may be expressed as $P_S = N_S[E]/[Cl]_{tot}$ where N_S is the number of chloride ions per protein molecule that may associate at any instant with sites of type S, $[Cl]_{tot}$ is the total concentration of chloride, and [E] the concentration of protein. The $\Delta\nu_i$ in eq 2 are given in each case by eq 1 where the variables may be the field gradient and the correlation time. To summarize, a change in the contribution of a particular term in eq 2 to the total line width may arise from any of four sources: a change in concentration of sites, a change in the number of chloride nuclei associated with a particular site, a change in the electric field gradient at the chlorine nucleus at that site, or a change in the correlation time for that site.

When a 1 M sodium chloride solution is made 0.25% in bovine mercaptalbumin, the ^{35}Cl resonance is broadened to about 65 Hz. If mercury is added to make the Hg/BSA ratio equal to one, the resonance is broadened further to about 85 Hz. These changes may be explained by a rapid exchange of free chloride in the solution with chloride bound to the albumin

(1) T. R. Stengle and J. D. Baldeschwieler, *Proc. Natl. Acad. Sci. U. S.*, **55**, 1020 (1966).
(2) R. G. Bryant, *J. Am. Chem. Soc.*, **89**, 2496 (1967).
(3) R. P. Haugland, L. Stryer, T. R. Stengle, and J. D. Baldeschwieler, *Biochemistry*, **6**, 498 (1967).
(4) T. R. Stengle and J. D. Baldeschwieler, *J. Am. Chem. Soc.*, **89**, 3045 (1967).
(5) R. L. Ward and J. A. Happe, *Biochem. Biophys. Res. Commun.*, **28**, 785 (1967).
(6) A. G. Marshall, *Biochemistry*, **7**, 2450 (1968).
(7) Except for nuclear constants, the theoretical treatment for each nucleus is the same provided that the fast exchange limit obtains.
(8) A. Abragam, "The Principles of Nuclear Magnetism," The Clarendon Press, Oxford, 1961, p 314.
(9) T. J. Swift and R. E. Connick, *J. Chem. Phys.*, **37**, 307 (1962).

molecule itself and with the mercury atom which adds to the free sulfhydryl group of the mercaptalbumin molecule.[10] The dramatic changes in line width observed when mercaptalbumin is added to a chloride sample provide a direct means of probing the nature of the protein molecule itself. This paper reports studies of the ^{35}Cl line width of mercaptalbumin solutions as a function of the total chloride concentration, urea concentration, and pH. In addition, experiments with cadmium and zinc indicate that these metals are useful as metal ion exchange centers.

Experimental Section

^{35}Cl nmr spectra were taken on a Varian Associates V-4300 nmr spectrometer using a 4.3-MHz fixed frequency radiofrequency unit and a 12-in. electromagnet with standard accessories. The spectrometer system included a Princeton Applied Research JB-4 lock-in amplifier and an audio amplifier built in this laboratory to allow various modulation and detection options. Line widths were measured as the full line width at half-peak height of the absorption mode signal displayed as the first side band in the usual way.[11] Line-width values reported are the average of at least ten traces of the ^{35}Cl spectrum under each set of conditions, and the deviations shown are mean deviations. The spectra were calibrated by measuring the displacement of the audiofrequency side bands with the audiofrequency counted to ± 0.1 Hz with a Hewlett-Packard Model 521-C counter. The liquid samples were maintained at the temperature of the magnet gap of $27 \pm 2°$ in 14 or 15 mm o.d. Pyrex tubes in the Varian 15-mm probe insert.

Twice crystallized bovine mercaptalbumin was purchased from Mann Research Laboratories and used without further purification. All other chemicals were obtained as Baker Analyzed Reagents. The urea was recrystallized twice from 50% ethanol; other chemicals were used without further purification. The zinc and cadmium solutions were standardized by titration with EDTA using Eriochrome Black T as an indicator. Solutions of mercuric chloride were made by weight.

Crystalline mercaptalbumin was added to a series of sodium chloride solutions varying in concentration from 0.50 to 4.00 *M*. The pH was buffered at 4.75 with 0.05 *M* acetate and the final mercaptalbumin concentration was 0.25%. After recording the chlorine resonance, 1 equiv of mercury based on the mercaptalbumin concentration was added using a 10-μl Hamilton syringe and the solutions were remeasured.

The nmr metal titrations were performed by adding microliter quantities of stock metal ion solutions from a Hamilton 10-μl syringe to 0.125% mercaptalbumin solutions in 0.50 *M* sodium chloride buffered at the pH indicated with 0.1 or 0.05 *M* phosphate. Protein concentrations in these experiments were calibrated spectrophotometrically using a Cary 14 spectrophotometer.

Urea solutions were made by adding recrystallized urea to a solution of 0.25% mercaptalbumin in 1.00 *M* sodium chloride buffered in 0.1 *M* acetate at pH 4.75 to make an 8 *M* urea solution. The 8 *M* urea–mercaptalbumin solution was diluted volumetrically with the stock 0.25% mercaptalbum in solution to obtain intermediate concentrations of urea. After measuring each of these solutions, 1 equiv of mercury was added and the nmr measurements were repeated. Making solutions in this way introduces a volume change in obtaining the 8 *M* urea solution; however, this does not affect the probability term in the relaxation equation because the mercaptalbumin and chloride are diluted by exactly the same factor.

Mercaptalbumin solutions were made over a range of pH values by adding crystalline mercaptalbumin to a 1.00 *M* sodium chloride solution at a pH maintained with a 0.1 *M* acetate buffer. Mercury (1 equiv) was added after the first set of measurements as before. Measurements of pH were made directly in the sample tubes using a Beckman 39030 combination electrode in conjunction with a Beckman 76004 Expandomatic pH meter.

Results and Discussion

Figure 1 summarizes the ^{35}Cl line-width measure-

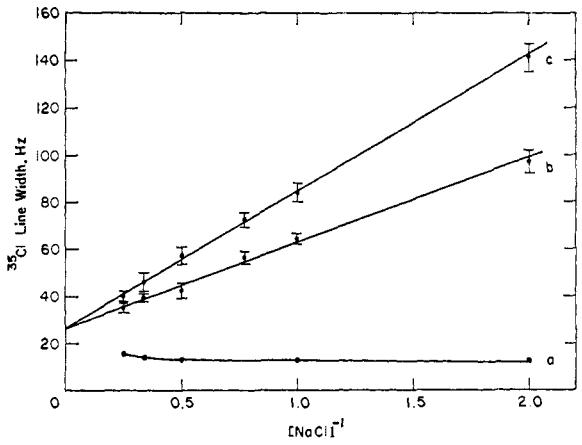

Figure 1. ^{35}Cl line width of mercaptalbumin solutions as a function of chloride concentration at pH 4.75. Curve a shows the sodium chloride line width in the absence of protein; curve b, 0.25% protein solutions containing 0.5 equiv of mercury per mol of protein; curve c, the same solutions containing 1 equiv of mercury per mol of protein.

ments made on solutions of constant mercaptalbumin as a function of total chloride concentration in 0.05 *M* acetate buffer at pH 4.75. These data demonstrate that the total line width is correctly described by eq 2, which may be rewritten in terms of coordination numbers and concentrations as

$$\Delta\nu_{tot} = \Delta\nu_{Cl_{free}} + \frac{1}{[Cl^-]} \times [N_{BSA}[BSA]\Delta\nu_{BSA} + N_{Hg}[BSAHgCl]\Delta\nu_{BSAHgCl}] \quad (2a)$$

where the total chloride concentration has been approximated as the molar chloride concentration. This equation clearly shows that measurements made with and without the metal site fully labeled with mercury should extrapolate to the same limiting line width. Because the viscosity and structure of the protein solutions are different from the sodium chloride solutions, it is not surprising that the sodium chloride solutions extrapolate to a line width slightly less than that of the protein solutions.

Within experimental error there is no evidence from these measurements that there is a change in the number of chloride ions associated with mercaptalbumin over this range of chloride concentrations. It should be pointed out that although the second term in eq 2a is a constant, it is not a well-known quantity because it is not likely that all the chloride binding sites offered by the mercaptalbumin molecule are identical.[12] In principle this is no limitation in the case where either all sites are saturated with chloride throughout an experiment, or where a separate probability and corresponding line-width parameter are known for each site.

In the presence of 0.5 *M* sodium chloride below pH 7 the tetrachloromercurate ion adds to an SH group and does not react with other sites in the molecule;[10] however at higher pH there is evidence for an increased number of mercury binding sites.[13] This behavior is

(10) I. M. Kolthoff, W. Stricks, and L. Morren, *Anal. Chem.*, **26**, 366 (1954).
(11) O. Haworth and R. E. Richards, *Progr. Nmr Spectry.* **1**, 1 (1966).
(12) G. Scatchard, J. S. Coleman, and A. L. Shen, *J. Am. Chem. Soc.*, **79**, 12 (1957).
(13) H. A. Saroff and H. J. Mark, *ibid.*, **75**, 1420 (1963).

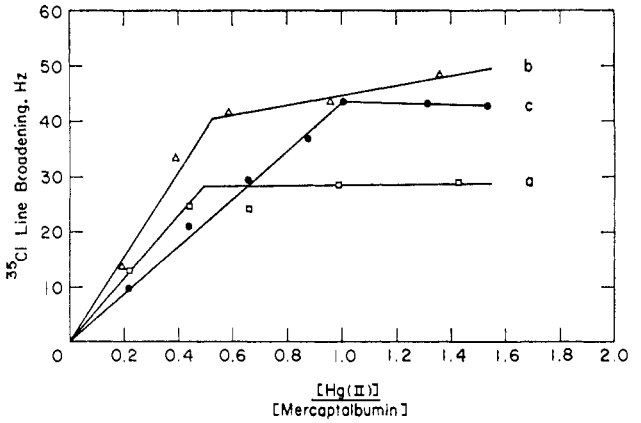

Figure 2. Titrations of bovine mercaptalbumin with mercury followed by ^{35}Cl line width in 0.5 *M* NaCl. Curve a, mercury titration at pH 5.0; curve b, mercury titration at pH 7.0; curve c, titration at pH 6.8 of mercaptalbumin sample treated with excess thioethanol and recovered.

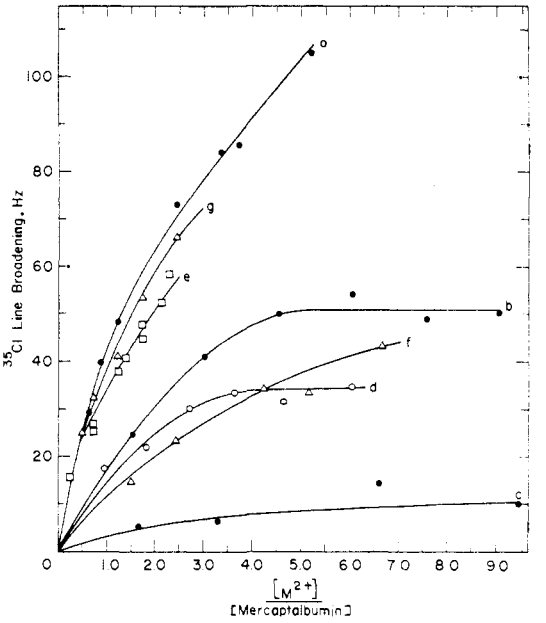

Figure 3. Mercaptalbumin–metal titrations at pH 8.0 in 0.5 *M* NaCl followed by ^{35}Cl line width: curve a, mercury(II) titration; curve b, cadmium(II) titration; curve c, zinc(II) titration. Curve d shows a cadmium titration of a mercaptalbumin solution to which 0.74 equiv of mercury had been added initially. Curve e, a mercury titration of mercaptalbumin solution to which 3.0 equiv of cadmium had been added intially. Curve f, a cadmium titration of a mercaptalbumin sample to which 6.6 equiv of zinc had been added intially. Curve g, a mercury titration of a mercaptalbumin sample to which 3.3 equiv of zinc had been added initially.

reflected in the mercury titrations of mercaptalbumin shown in Figure 2, curves a and b, where titrations at pH 5 and 7 show end points at 0.5 equiv of mercury. Titration with PCMB using the method of Boyer confirmed an SH titer of 0.5 per mol of mercaptalbumin.[14] Curve c, Figure 2, shows the mercury titration of a mercaptalbumin sample at pH 6 that had been treated with excess thioethanol and recovered. These titrations demonstrate that the mercaptalbumin initially contained 0.5 equiv of mercury as expected. It is interesting to note that the titrations carried out at pH 5 and 7 show different slopes even though the protein concentration of each is the same. This difference must arise from a change in $\Delta\nu_{BSAHgCl}$ with pH. Changes in this line-width parameter with pH may reflect a structural change of the protein as a whole, a change in the immediate environment of the SH moiety with pH, or both.

Cadmium and zinc also show a line broadening effect on the chloride resonance and may be potentially useful as metal ion labels.[15] However, the addition of zinc and cadmium ions to mercaptalbumin solutions in place of mercury produced effects on the order of experimental error at pH 5.

The metal ion titrations at pH 8 are summarized in Figure 3. The mercury titration at this pH shown in curve a lacks a well-defined end point and significant interaction with protein groups other than SH is clearly indicated. At this pH cadmium exhibits a well-defined titration curve, shown in curve 3b; the association is likely to be with imdiazole residues.[13] Although serum albumin is reported to contain 16 imidazole residues, the cadmium end point falls at approximately 4.5. There are at least two reasons for the difference: pH 8 may not be optimal for association of cadmium with the protein and this would shift the observed end point to lower cadmium values. The most important consideration is that residues must be accessible to chloride exchange in order to be observed by the chlorine resonance. Thus the largest end point possible at the pH most favorable for association would be only that number of cadmium binding sites that remained accessible for chloride exchange when the metal binds. However, the shape of the titration curve could indirectly indicate whether cadmium were binding to the protein at sites not accessible for chloride exchange. In contrast to cadmium and mercury, the broadening due to zinc is barely detectable in both pH ranges even though zinc is reported to compete effectively with cadmium for the protein sites.[13] It is possible that in the alkaline region OH^- competes with Cl^- at the zinc site thus reducing or eliminating the chloride coordination; it is also possible that the association of zinc with the protein is not maximal at either of these pH values. However, a titration with zinc at pH 6.2 did not differ from that at pH 5.

Metal ion additions followed by observing the halogen resonance permit detection of competition between two or more metals for binding sites offered by a protein molecule. As shown in Figure 3, curve d, the addition of 0.74 equiv of Hg before titration with cadmium at pH 8 shifts the cadmium curve to lower concentrations by an amount equal to the number of moles of mercury added. This implies that mercury protein equilibria are substantially stronger than the cadmium equilibria. The addition of 3.0 equiv of cadmium prior to the mercury titration shown in curve 3e produces a shift of the mercury titration curve only above a mercury to protein ratio of 0.5. The initial protein solution contained 0.5 equiv of mercury so that a break

(14) P. D. Boyer, *J. Am. Chem. Soc.*, 76, 4331 (1954).
(15) H. G. Hertz, *Z. Elektrochem.*, 65, 20 (1961).

at 0.5 equiv of mercury added corresponds to a mercury:protein ratio of 1. Thus cadmium is not effective in displacing the mercury–SH equilibrium, but these measurements suggest cadmium does compete with mercury for the other metal sites. Since curve 3e represents line-width increases above that of the starting solution which contained 3.0 equiv of cadmium, the difference between the mercury titrations with and without cadmium present contain two effects. The replacement of cadmium by mercury causes a decrease in the line-width contribution due to cadmium; however, the effect is counterbalanced by added line broadening caused by the mercury–protein complex. The observed curve is the sum of these effects. The difference between the mercury titrations in curves d and e is small, which implies that the mercury equilibria are dominant. The addition of 3.3 equiv of zinc prior to titration with mercury is even less effective in competing for the mercury sites as shown in curve g of Figure 3.

The addition of zinc prior to titration with cadmium at pH 8 alters the shape of the cadmium plot dramatically indicating direct competition between cadmium and zinc for binding sites. The line broadening plotted in Figure 3 as curve f represents the line-width increase beyond the line width of the starting solution which contained 6.6 equiv of zinc per mercaptalbumin molecule. The marked change in the cadmium titration curve indicates direct competition between zinc and cadmium for binding sites on the protein.

When the amounts of zinc and cadmium added are equal the line width is greater than is predicted by assuming that the equilibrium constants for zinc and cadmium binding to the protein are equal. Since the broadening is greater than that for zinc, this implies that cadmium competes somewhat more effectively for the protein sites than does zinc. This is consistent with the results of Gurd.[16] Since the chloride–cadmium equilibria are stronger than the chloride–zinc equilibria, this difference should become more pronounced in the absence of chloride. It is also possible, however, that at the higher metal concentrations of the competition experiment, more metal ions are bound at protein sites that are available for chloride exchange thus increasing the line width over that expected on the basis of the titrations with zinc and cadmium individually.

The small line broadening observed for zinc may be explained by considering the line broadening produced by the metal ion itself in aqueous sodium chloride. The ^{35}Cl line width of solutions containing metal ions discussed thus far is linear in metal ion concentration with a slope given by

$$\frac{\partial(\Delta\nu_{tot})}{\partial[M]} = \frac{N_m[\Delta\nu_m - \Delta\nu_{Cl}]}{[Cl^-]_{tot}} \simeq \frac{N_m\Delta\nu_m}{[Cl^-]}$$

The metal ion broadening is meaningfully analyzed in terms of $\Delta\nu_m$, the chlorine line width at the metal site; however, some ambiguity arises because there is often a distribution of species differing in the effective chloride coordination number. The slope defined above is thus the best measure of a metal ion's potential as a halogen ion exchange center.[17]

Table I summarizes the data for zinc, cadmium, and mercury, as well as several other metal ions of potential usefulness.[18]

Table I

Metal	Obsd slope in 0.5 *M* NaCl
Zn(II)	1.80×10^3
Cd(II)	1.98×10^4
Hg(II)	6.31×10^4
Cu(II)	2.03×10^5
Fe(II)	4.92×10^2
Fe(III)	2.24×10^3
In(III)	7.54×10^3

While zinc appears to be a useful probe at higher concentrations of metal and protein, mercury and cadmium offer more sensitivity permitting observations at metal and protein concentrations significantly more dilute. The other metal ions listed may be useful as halogen probe sites; however, in some cases the exchange rate may be most important in determining the line width.

The line-width measurements for the urea denaturation of mercaptalbumin at pH 4.75 are summarized in Figure 4. In this case the free chloride line width is a function of the urea concentration as shown in curve 4c. If urea does not interact directly with protein-bound chloride, then eq 2a may be modified to include the free chloride–urea interaction and the behavior of each term contributing to the total line width may be obtained. Curve b of Figure 4 represents the data recorded for samples of mercaptalbumin containing the initial 0.5 equiv of mercury per mol of protein while curve a samples contained 1.5 equiv of mercury per mole. The mercury titrations showed that the line broadening produced by the 0.5-equiv excess of mercury is negligible at these concentrations and pH so that curves a and b represent 0.5 and 1.0 mol of mercury bound to the SH site per mol of protein. Since the free chloride–urea interaction was measured, curve a may be decomposed into the contributions from each term of eq 2a. Curves 4c, 4d, and 4e represent the chloride–urea term, the protein–chloride term, and the protein-bound mercury term, respectively.

Curve 4d may reflect changes in the average number of chloride ions bound to the protein as well as the average correlation time or field gradient for the protein-bound chloride. In general, it is not possible to separate these effects because the product of these terms is observed; however, the contribution from this term decays to zero as the protein is denatured. This

(16) F. R. N. Gurd, in "Ion Transport Across Membranes," T. H. Clarke, Ed., Academic Press, New York, N. Y., 1954, p 259.

(17) The equilibrium constants for the chloride–metal ion systems have been reported and it is possible to calculate the concentration of each species present at any chloride concentration; however, this leaves the difficult problem of deciding what effect changing the coordination of the metal has on the quadrupole coupling constant at each associated chlorine nucleus.

(18) For some of these metal ions the exchange rate may be the most important factor in determining the line width.

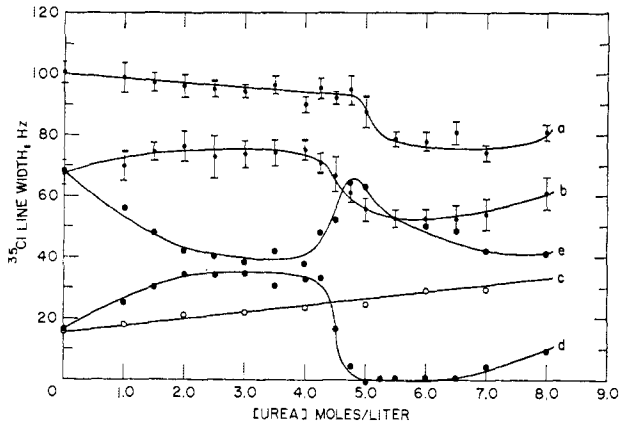

Figure 4. Urea denaturation of bovine mercaptalbumin followed by ^{35}Cl line width at pH 4.75 in 1.00 *M* NaCl: curve a, ^{35}Cl line width of mercaptalbumin solutions containing 1.5 equiv of mercury as a function of urea concentration; curve b, ^{35}Cl line width of mercaptalbumin solutions containing a total of 0.5 equiv of mercury as a function of urea concentrations; curve c, ^{35}Cl line width of sodium chloride–urea solutions at pH 4.75; curve d, the contribution of the chloride–mercaptalbumin interaction to the total line width shown in curve a; curve e, the contribution of the mercury–SH site to the total line width shown in curve a.

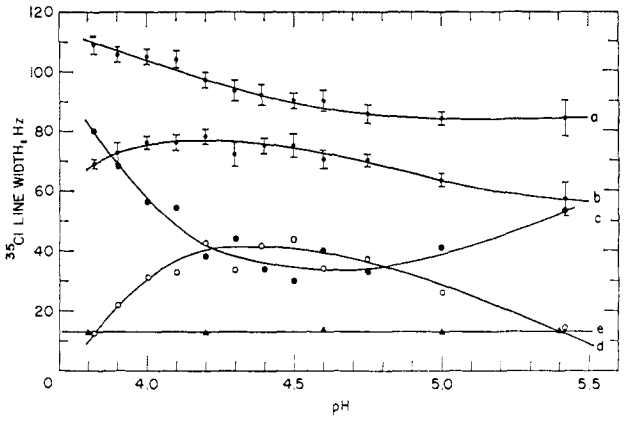

Figure 5. ^{35}Cl line width of bovine mercaptalbumin solutions at low pH in 1.0 *M* NaCl: curve a, ^{35}Cl line width of mercaptalbumin solutions containing total of 1.5 equiv of mercury; curve b, ^{35}Cl line width of mercaptalbumin solutions containing a total of 0.5 equiv of mercury; curve c, contribution of the mercury–SH site to the total line width shown in curve a; curve e, contribution of the mercaptalbumin–chloride interaction to the total line width shown in curve a; curve e, ^{35}Cl line width of buffer solutions.

strongly suggests that chloride binding is insignificant above urea concentrations of 5 *M*.

At the chloride concentrations of these experiments it is reasonable to assume that the chloride coordination number of the protein-bound mercury species is a constant. The changes in curve 4e then reflect changes only in the product $(e^2qQ)^2\tau_c$ where e^2 and Q are constants as defined earlier, q is the electric field gradient at the chlorine nucleus in the protein–HgCl species, and τ_c is the correlation time for reorientation of the mercury–chlorine bond with respect to the direction of the applied magnetic field. Because the field gradient arises from the covalent mercury–chlorine bond, q is large and not likely to be influenced by small changes in the conformation of the protein unless these changes involve a redistribution of electrons about the chlorine nucleus. Thus, changes in the line width at the mercury site are dominated by changes in τ_c. Because internal rotation of the residue containing the metal atom may in general be possible, the correlation time exhibited by the metal site may be shorter than the rotational correlation time of the protein as a whole.[19] This consideration makes detailed analysis of the SH site behavior impossible, but the irregular shape of curve 4e suggests that the SH site is located where structural changes are significant as the molecule is denatured. In this experiment it is possible for the excess mercury to add to newly exposed binding sites on the protein as it is denatured; however, curves 4c and 4a indicate an over-all decrease in the total line width and a decrease in the mercury contribution which is inconsistent with this possibility.

It should be noted that the line width decreases with urea denaturation in spite of the fact that the viscosity of similar solutions increases.[20] This point is important in the light of the usual hydrodynamic model for the correlation time where the line width is assumed to be proportional to the viscosity and inversely proportional to the temperature. This hydrodynamic model predicts that an increase in viscosity would cause an increase in line width. While this viscosity effect may explain the increase in the protein-bound chloride contribution prior to denaturation, the correlation time for the SH site suffers an over-all decrease on denaturation.

The acid denaturation measurements are summarized in Figure 5 where the data have been decomposed into line-width contributions as before. Curve 5d shows a smooth change in the second term of eq 2a that passes through a maximum in the region of pH 4.3. This may reflect changes in chloride coordination as well as the field gradient–correlation time product; however, Scatchard and coworkers[21] reported that chloride binding to serum albumin increases at lower pH. This indicates that the decrease in line-width contribution from chloride associating directly with the protein reflects a decrease in $(e^2qQ)^2\tau_c$. Fluorescence depolarization[22] and electrical birefringence[23] measurements on serum albumin solutions have shown that the rotational correlation time of the molecule as a whole decreases with decreasing pH, which is consistent with curve 5d.

In contrast with this behavior the mercury contribution shows that the SH site experiences an increase in the correlation time for reorientation below pH 4.3. This behavior parallels that of the intrinsic viscosity[24] and translational diffusion coefficient of serum albumin solutions at low pH; however, as stated above the measured rotational relaxation time decreases as the pH is lowered indicating more rapid rotational re-

(19) D. Wallach, *J. Chem. Phys.*, 47, 5258 (1967).
(20) H. K. Frensdorff, M. T. Watson, and W. Kauzmann, *J. Am. Chem. Soc.*, 75, 5167 (1953).
(21) G. Scatchard, J. S. Coleman, and A. L. Shen, *ibid.*, 79, 12 (1957).
(22) G. Weber, *Biochem. J.*, 51, 155 (1952).
(23) S. Krause and C. T. O'Konski, *J. Am. Chem. Soc.*, 81, 5082 (1959).
(24) J. T. Yang and J. F. Foster, *ibid.*, 76, 1588 (1954).

orientation time for the molecule as a whole. A reduction in τ_c is usually accompanied by less restricted motion of the nucleus observed and a decrease in line width. However, the decrease in rotational correlation time for the mercaptalbumin molecule is accompanied by an increase in line-width contribution from the mercury site which suggests that the motion of the SH site is slower than that observed at higher pH. A decrease in the reorientation time of the SH site could arise in at least two ways: steric interactions resulting from structural changes could decrease the amount of internal rotation between the mercury–chlorine bond and the polypeptide backbone as the pH is decreased, or the shape of the protein could change. In the second case, if the protein changed from an approximately spherical to an ellipsoidal form, the rotational motion of the ellipsoid would contain two components: rotation about the long axis which would be rapid, and rotation about the short axis which would be relatively slow. If such a geometric model is assumed, the increase in the correlation time of the SH site would result if the mercury site lies on or close to the long axis. Even though changes in geometry have been suggested to explain measurements on serum albumin in acid solutions,[25] the mercury site behavior by itself cannot be taken as evidence for the formation of an ellipsoidal structure at low pH because any structural change could produce steric hindrance of the SH motion and a resultant increase in the mercury contribution to the total line width.

An alternate explanation for the increase in the mercury-site contribution is that the 0.5 equiv excess of mercury present in these solutions could react with binding sites that become exposed as the protein changes structure at lower pH. This is unlikely because the urea denaturation experiment gave no evidence for increased mercury binding as the protein was denatured and the affinity of mercury for protein sites should decrease with pH.

Conclusion

These experiments indicate that the halogen ion probe technique offers a sensitive method for studying several important aspects of protein structure, and it compares well with other physical techniques directed toward this end. The metal ion exchange center, if appropriately chosen, may function to isolate the behavior of one particular residue in a protein, and the systematic study of the metal ion properties as well as those of related organometallic compounds should make labeling of specific residues a generally useful technique.

Since a requirement of the halogen ion probe technique is that the labeled residue must be accessible for chloride exchange to produce any line broadening, line-width measurements using a group specific label will determine whether a group is accessible for chloride exchange or buried in a hydrophobic region of the protein. A class of enzymes particularly amenable to this analysis are those containing intrinsic metal ions such as zinc. In carbonic anhydrase, for example, the zinc atom is accessible for chloride exchange and produces significant broadening of the ^{35}Cl line.[26] The competition experiments demonstrate that the technique may be used to study the metal ion–protein equilibria for the metal protein sites that are accessible to exchange.

There is no question that subjecting biological samples to salt concentrations as concentrated as 0.5 *M* sodium chloride may be no small distortion of a normal physiological environment. However, measurements have been made in 0.1 *M* sodium chloride solutions with signal to noise ratios that are quite workable.[27] The possibility of working this concentration range not only places the molecule or tissue in a more reasonable physiological environment, but it also increases the sensitivity of the line width to the concentration of protein by a factor of five over that in 0.5 *M* sodium chloride. It is thus possible to make measurements under chemically reasonable conditions at easily achievable concentrations of macromolecule. The question of the degree to which the metal ion perturbs the protein structure is almost impossible to assess. While the metal ions used are large, they are no larger than fluorescent or nitroxide spin labels, and although applications to enzymes with critical SH groups has obvious difficulties, the halogen probe technique appears to be as valid an approach as other labeling methods.

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(25) V. Bloomfield, *Biochemistry*, **5**, 684 (1966).

(26) The broadening due to exchange with metal site may be quantitatively eliminated by the addition of diamox, a known inhibitor of the enzyme: R. G. Bryant, unpublished results.

(27) R. G. Bryant, unpublished results.