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# Study of a Sulfonyl Derivative of $\alpha$-Chymotrypsin by Chlorine Nuclear Magnetic Resonance*

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ABSTRACT: The width of the $^{35}Cl$ nuclear magnetic resonance line provides a measure of the rotational motion of the inhibitor, *p*-mercuribenzenesulfonyl fluoride (RHgCl), attached to the active site of $\alpha$-chymotrypsin in aqueous NaCl solution. The inhibitor appears to bind to the active serine at the catalytic site. If a single methionine residue on the enzyme is first alkylated with benzyl bromide, subsequent binding of RHgCl is still specific for the active site, but the observed $^{35}Cl$ resonance line is broadened relative to the inhibited native enzyme.

This indicates a more restricted rotational motion of the inhibitor attached to the *S*-benzylmethionine-chymotrypsin. In 8 M urea, however, the line width for RHgCl attached to *S*-benzylmethionine-chymotrypsin is the same as for RHgCl attached to native chymotrypsin. Apparently a well-defined secondary and tertiary structure at the active region is necessary for the alkyl group to affect motion of the inhibitor at the active site.

Attempts to correlate structure and function of enzymes in solution have drawn heavily from studies of chemical binding specificity and catalytic activity toward substrates. An enzyme commonly chosen for study is $\alpha$-chymotrypsin since the binding and catalytic constants for a wide spectrum of substrates are known (Bender *et al.*, 1964). A common technique has been to examine binding or catalytic constants before and after some chemical modification of part of the enzyme. However any conclusions about steric relations between various residues at the active site and the various parts of a substrate require the testing of a substantial number of substrates, and the result is still often ambiguous.

It has recently been shown that relaxation and exchange of quadrupolar nuclei at suitable sites can provide a general method for nuclear magnetic resonance study of proteins in solution (Stengle and Baldeschwieler, 1966, 1967). In particular it is possible to obtain direct information about the accessibility of a particular region of a macromolecule to a solvent and also the degree of motional freedom of a label attached to a site on the macromolecule. In the present work, this technique is used to probe the active site of $\alpha$-chymotrypsin by observing the effect of alkylating a particular methionine residue.

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## Theory

For a nucleus of spin greater than one-half (*e.g.*, $^{35}Cl$, $^{79}Br$, $^{81}Br$, $^{23}Na$, or $^{127}I$) the interaction of the nuclear electric quadrupole moment $Q$ with the fluctuating electric field gradient $q$ at the nucleus can provide a simple and dominant relaxation mechanism. In the extreme narrowing approximation ($\omega\tau_0 \ll 1$), the contribution to the nuclear resonance line width from quadrupole relaxation is

$$\Delta\nu = K(e^2qQ)^2\tau_c \qquad (1)$$

where $\Delta\nu$ is the full line width at half-height in cycles per second, $(e^2qQ)$ is the quadrupole coupling constant in cycles per second, $\tau_c$ is the correlation time for molecular rotation in seconds, and $K$ equals $2\pi/5$ for a nucleus of spin three-halves if the asymmetry parameter is neglected (Abragam, 1961). A large range of line widths is possible depending on the values of these quantities. For example, for a chloride ion in dilute aqueous solution, the solvation of the ion is essentially symmetric, and the electric field gradient at the nucleus is nearly zero. This results in a line width of 15–20 cps for the $^{35}Cl$ signal in aqueous solutions of NaCl. However when the chlorine atom is involved in covalent binding, the value of $(e^2qQ)$ is quite large; the line width for $CCl_4$ is 14.5 kcps. Even greater line widths are expected for molecules larger than $CCl_4$ with longer $\tau_c$.

If a quadrupolar nucleus can be located at different kinds of sites in solution, the resulting line shape depends on the relative concentrations of the various sites, the values of $(e^2qQ)$ and $\tau_c$ at each site, and the rate of

exchange of chlorine among the various sites. If there are two possible sites, and the exchange of chlorine between them is sufficiently rapid, the spectrum will be a single composite signal of line width

$$\Delta\nu = \Delta\nu_a P_a + \Delta\nu_b P_b \qquad (2)$$

where $\Delta\nu_a$ and $\Delta\nu_b$ are the line widths at sites a and b, and $P_a$ and $P_b$ are the probabilities that the nucleus is at sites a and b. It is apparent from eq 2 that if $\Delta\nu_b$ is very large, a very small value of $P_b$ may produce an observable effect on the line width $\Delta\nu$. Hence the chloride ion can be used as a probe for interesting sites in low concentrations, and the exchange process functions as a chemical amplifier. For small values of $P_b$

$$P_a \cong 1 \text{ and } \Delta\nu - \Delta\nu_a \cong \Delta\nu_b P_b \qquad (3)$$

The conditions for the binding and exchange process are reasonably restrictive. The chloride ion must be able to enter the first coordination sphere of the site and form a sufficiently strong bond to give a large value of $q$, the electric field gradient at the nucleus. Furthermore, the ion must remain bound for a time long compared with $\tau_c$, while exchange with ions in the bulk solvent must occur in a time short compared with $1/\pi\Delta\nu_b$. The binding of chloride to mercuric ion appears to satisfy these requirements (Stengle and Baldeschwieler, 1966).

It is possible to attach to the active site of an enzyme a label containing exchangeable chloride, and thus use the chloride ion as a probe to examine this site. The experimental measurements are conveniently carried out as a titration, where the concentrations of chloride and enzyme are constant, and increments of the label, RHgCl, are added. As the label molecules become attached to the active site, the $^{35}Cl$ nuclear magnetic resonance line width increases, due to the relatively longer effective correlation time for $^{35}Cl$ bound *via* the label to the enzyme. The slope of the titration curve is proportional to the effective correlation time for $^{35}Cl$ bound to enzyme; thus a steep slope indicates that rotational motion of the attached label is markedly restricted. It is expected that $(e^2qQ)$ for the chloride in RHgCl will be essentially the same for all R.

The correlation times observed by this technique are frequently one or two effective orders of magnitude shorter than the time of rotation of the protein molecule as a whole (Stengle and Baldeschwieler, 1966). In eq 1, $\tau_c$ refers to the correlation time for the change in angle between the electric field gradient at the chlorine nucleus, $q$, and the external magnetic field, $H_0$. This angle is affected by motions within the protein–mercury complex as well as by rotations of the entire molecule. Hence the effective correlation times will be a composite of these two factors. Thus $\tau_c$ provides a measure of the rigidity of the structure at the labeled site, as well as the rotational correlation time of the entire protein, and should be quite sensitive to any conformational changes of the molecule or restriction of the motion of the labeling group.

## Experimental Section

Three-times-crystallized $\alpha$-chymotrypsin and diisopropylphosphoryl-chymotrypsin (DIP[1]-chymotrypsin) were obtained from Worthington Biochemical Corp. All enzyme samples contained 0.2% enzyme, 2.0 M NaCl, and 0.5 M phosphate buffer at pH 7. The effect on the activity of $\alpha$-chymotrypsin by such a concentration of NaCl is negligible (Warren and Cheatum, 1966).

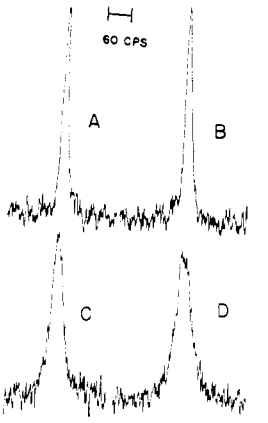


FIGURE 1: Chloride ion nuclear magnetic resonance for: (A) 2.0 M NaCl–0.05 M phosphate (pH 7.0), (B) contents of A plus 0.2% $\alpha$-chymotrypsin, (C) contents of B plus 1.0 equiv of *p*-mercuribenzenesulfonyl fluoride (RHgCl), and (D) contents of B treated first with 20 equiv of benzyl bromide, then with 1.0 equiv of RHgCl.

*p*-Mercuribenzenesulfonyl chloride was prepared in several steps from reagent grade sulfanilic acid by the method of Kraut (Sigler *et al.*, 1965). Then *p*-mercuribenzenesulfonyl chloride (0.0015 mole, 0.617 g) was dissolved in 12 ml of dimethoxyethane in a 50-ml round-bottom flask. While this solution was stirred on a magnetic stirrer, 15 ml of 1 M aqueous potassium fluoride (0.015 mole of KF) was added to it. The reacting mixture was stirred at room temperature for 24 hr. The dimethoxyethane was removed by rotary evaporation and the residue collected by filtration, washed with water, and allowed to dry. The product was obtained in high yield (J. Kraut, personal communication of unpublished synthesis). A stock solution consisted of $4.0 \times 10^{-3}$ M RHgCl in acetonitrile.

The results of an elemental analysis by the Microanalytical Laboratory, Stanford University, were as follows: C, 17.78; H, 0.95; Cl, 9.20; F, 5.0; Hg, 50.8; S, 7.72. The calculated composition of *p*-mercuribenzenesulfonyl fluoride is: C, 18.24; H, 1.02; Cl, 8.97; F, 4.81; Hg, 50.76; S, 8.11.

Alkylation of a methionine residue of the enzyme was achieved by addition of 0.01 ml of a 10% (by volume) solution of benzyl bromide in ethanol to 5.0 ml of a solution containing 0.2% $\alpha$-chymotrypsin, 2.0 M NaCl, and 0.05 M phosphate (pH 7). Although this is a 20-fold excess of benzyl bromide, it has been shown that only one methionine is affected (Schramm and Lawson, 1963).

Titrations of native *S*-benzylmethionine, and DIP-chymotrypsins with *p*-mercuribenzenesulfonyl fluoride

[1] Abbreviations used that are not listed in *Biochemistry 5* 1445 (1966), are: DIP, diisopropylphosphoryl.

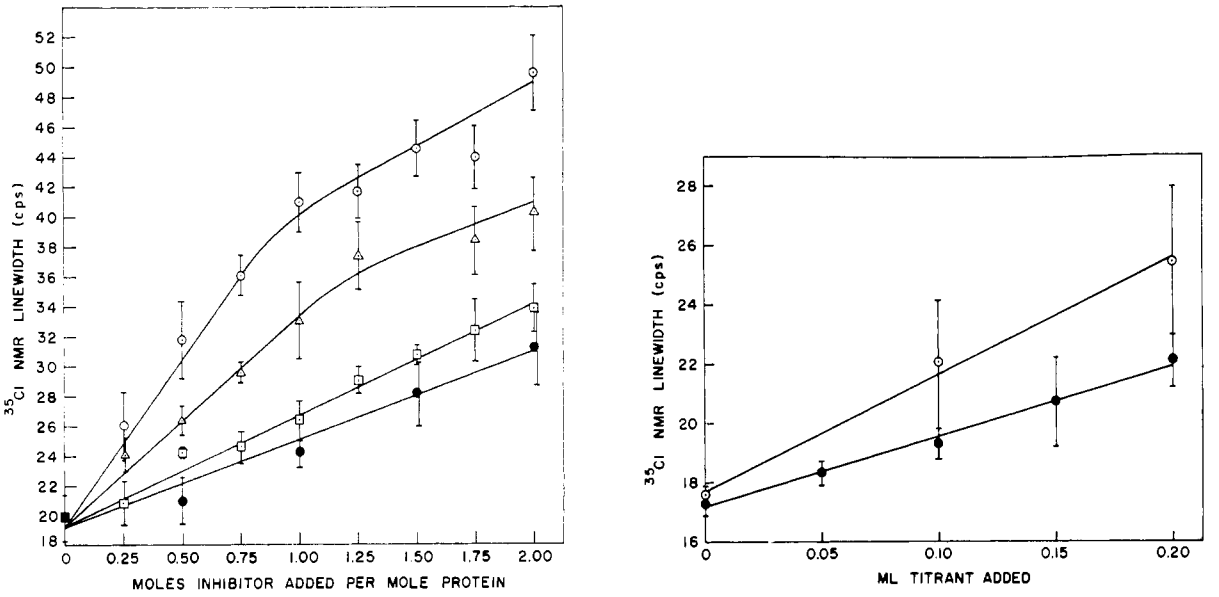


FIGURE 2: Titration experiments. (a, left) Of enzyme with the inhibitor, RHgCl, in increments of 25 $\mu$l of inhibitor dissolved in acetonitrile; each solution contains in addition 2.0 M NaCl and 0.05 M phosphate (pH 7.0). (●) 0.2% DIP-chymotrypsin ($\alpha$-chymotrypsin which had previously been treated with diisopropyl fluorophosphate). (⊡) 0.2% DIP-chymotrypsin plus 20 equiv of benzyl bromide (added as 10 $\mu$l of a 10% solution in ethanol). (△) 0.2% $\alpha$-chymotrypsin. (⊙) 0.2% $\alpha$-chymotrypsin plus 20 equiv of benzyl bromide. (b, right) Of a solution of 2.0 M NaCl–0.05 M phosphate (pH 7.0) with: (●) pure acetonitrile and (⊙) $4 \times 10^{-3}$ M RHgCl (dissolved in acetonitrile).

(RHgCl) were carried out by addition of 0.025-ml aliquots of $4.0 \times 10^{-3}$ M RHgCl stock solution to 5.0 ml of a solution containing 0.2% of the respective enzyme and the usual 2.0 M NaCl and 0.05 M phosphate. Incubation of each aliquot for a minimum of 1 hr proved sufficient for binding, as evidenced by the titration curves in Figure 2a. The enzyme solutions were therefore as high as 4% in acetonitrile; such a concentration of acetonitrile does not affect enzyme activity (D. E. Koshland, Jr., personal communication).

The $^{35}$Cl nuclear magnetic resonance spectra were obtained at 4.33 Mc, using a Varian HR-60 nuclear magnetic resonance spectrometer. The nuclear magnetic resonance signal was modulated at 300 cps and detected from a side band using a lock-in amplifier to eliminate base-line drift. Line widths were calibrated from side bands of a saturated aqueous NaCl sample. All experiments were conducted at room temperature, and each measured line width is the average of at least ten spectra. Representative spectra are shown in Figure 1.

## Results and Discussion

Titration curves for the addition of RHgCl to solutions of $\alpha$-chymotrypsin, DIP-chymotrypsin, and *S*-benzylmethionine-chymotrypsin are shown in Figure 2a. The $\alpha$-chymotrypsin curve shows a break after the addition of approximately 1 equiv of RHgCl, and the DIP-chymotrypsin curve (bottom trace) shows no such break. Since $\alpha$-and DIP-chymotrypsin differ primarily in the presence or absence of an available serine binding site, it seems clear that the site at which RHgCl is bound in the native enzyme is indeed the active-site serine residue (amino acid 95 on the *c* chain). This is consistent with previous X-ray and chemical observations (Sigler *et al.*, 1965). If one subtracts away the contribution from the DIP-chymotrypsin titration, then the titration curve for $\alpha$-chymotrypsin levels off after the end point. The nonzero slope for the DIP-chymotrypsin titration is explained by the controls in Figure 2b. The top trace shows the $^{35}$Cl nuclear magnetic resonance line width of a 2.0 M NaCl solution with no enzyme present as a function of RHgCl added. The slope of that line is due partly to the presence of free RHgCl and partly to the effect of the added acetonitrile. The lower trace of Figure 2b shows the marked effect of acetonitrile alone. Presumably the addition of a small amount of acetonitrile disrupts the symmetrical environment at the solvated aqueous chloride ion and thereby increases the nuclear magnetic resonance line width.

Also shown in Figure 2a is the curve obtained from addition of RHgCl to *S*-benzylmethionine-chymotrypsin. (The appropriate DIP-chymotrypsin control is also shown). It is known that *S*-benzylmethionine-chymotrypsin has markedly reduced catalytic activity compared to the native enzyme, but the ability of either molecule to react with diisopropyl fluorophosphate is the same in both cases (Schramm and Lawson, 1963). It is clear that the slope of this curve is decidedly greater than for the native enzyme and that there is an end point at about 1 equiv of RHgCl. Thus the binding seems unaffected (stoichiometrically) by the presence of excess benzyl bromide, and the greater slope indicates that the motion of the inhibitor attached to the active-site serine residue is rotationally restricted compared to the native enzyme. The restriction could arise either from the proximity of the methionine group to serine 195 on the same primary amino acid chain or from an interaction mainly dependent on the secondary and tertiary structure at the active site. The addition of urea to a concentration of 8

M causes the protein to unfold (Martin, 1964) and should eliminate any effect due to higher order structure. The results of such experiments are shown with appropriate controls in Table I. Upon addition of 8 M urea, the line width for RHgCl attached to *S*-benzylmethionine-chymotrypsin is the same as for RHgCl attached to native chymotrypsin, within experimental uncertainty. Thus in order for the alkyl group on the methionine to affect motion of the inhibitor at the active site, a well-defined secondary and tertiary structure at the active site is necessary.

In view of the fact that sulfonyl inhibitors may bind in more than one way at the active site (Sigler *et al.*, 1965), it is possible that the effect of alkylating a methionine is to favor one of these configurations compared with the native enzyme; in any case the result is a perturbation of the higher order structure at the active site.

The broader resonance for the *S*-benzylmethionine-chymotrypsin relative to the native enzyme might also be explained by postulating an increase in $P_b$ in eq 2 either (a) by having the labeled site exposed to the solution for a larger percentage of the time or (b) by an increase in the number of chlorides which may coordinate to a given inhibitor. If condition a applied, one would expect that an unfolding of the enzyme at the labeled site (as by addition of 8 M urea) would more fully expose the labeled region and thus broaden the resonance by increasing $P_b$. An addition of 8 M urea does broaden the resonance for labeled $\alpha$-chymotrypsin (see Table I and the end point of the titration of Figure 2a). However the resonance for DIP-chymotrypsin is also broadened by about 50%; this is thought to arise from a bulk viscosity change, since addition of sucrose to a solution of 2.0 M NaCl (with or without enzyme present) produces a line broadening which is linear in sucrose concentration, at least up to 1 M sucrose (A. G. Marshall, unpublished results). Thus if the urea results are adjusted for this bulk viscosity effect (*i.e.*, by multiplying all line widths in urea solution by the factor $^{25}/_{36}$), the broadening due to the $P_b\Delta\nu_b$ term is actually *smaller* than that observed when no urea was present. As to the possibility of additional chlorides, some preliminary solubility and nuclear magnetic resonance measurements (R. G. Bryant, personal communication) indicate that in aqueous NaCl solution compounds of the type RHgCl exhibit only one exchangeable $Cl^-$, even when an excess of $Cl^-$ is present.

TABLE I[a]

| Sample | $^{35}$Cl Nuclear Magnetic Resonance Line Width (cps) |
|---|---|
| 0.2% DIP-chymotrypsin | 36.6 ± 1.1 |
| 0.2% DIP-chymotrypsin + 20 equiv of benzyl bromide | 35.7 ± 0.7 |
| 0.2% $\alpha$-chymotrypsin | 49.3 ± 1.4 |
| 0.2% $\alpha$-chymotrypsin + 20 equiv of benzyl bromide. | 46.8 ± 1.4 |

[a] Each solution contains in addition: 2.0 M NaCl, 0.05 M phosphate (pH 7.0), 1 equiv of RHgCl, and 8 M urea.

## Conclusion

These results illustrate the utility of the halide ion probe technique for elucidating the steric relations between the active site of an enzyme and various parts of an inhibitor. This general approach can be extended by similar studies with a series of structurally similar inhibitors with mercury atoms substituted at different places. It should then be possible to map the steric restrictions for the enzyme–inhibitor complex. Such experiments would have direct bearing on a currently popular theory (Bender *et al.*, 1964) that the efficiency of catalysis for a given substrate is directly related to the rigidity with which it is bound at the catalytic site. Since the $^{35}$Cl nuclear magnetic resonance line widths are a function of protein configuration, it should be possible to study the effect *at the active site* of various disruptive agents such as heat, urea, guanidine, pH, etc. Most other physical measurements (optical rotatory dispersion, viscosity, etc.) measure only gross conformational changes (Weiner and Koshland, 1965) and are difficult to correlate with changes at a single region of the enzyme. Since heavy atoms are usually involved for these halide ion nuclear magnetic resonance experiments (Stengle and Baldeschwieler, 1966) it should be interesting to relate the nuclear magnetic resonance studies with X-ray crystallographic studies of proteins with the same heavy atom substituents.

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