n-butyllithium TMEDA complex at 0°, was treated with 5.2 ml of benzene and stirred at room temperature for 3 hr. Subsequently, the reaction mixture was treated with 3.5 g (0.033 mol) of benzaldehyde and worked up as described above to give 3.3 g (67%) of α-(*N*,*N*-dimethylaminomethyl)benzyl alcohol, bp 50–52° (0.03 mm), 0.8 g (12%) of $(CH_3)_2NCH_2CH_2N(CH_3)CH_2CH(OH)$-$C_6H_5$, bp ~100° (0.03 mm), 10.2 g of tetrabutyltin, and ~0.4 g (8%) of benzhydrol.

Reaction of Triphenylphosphine Oxide with 1′. Treatment of a suspension of 5.56 g (0.02 mol) of triphenylphosphine oxide in 50 ml of anhydrous ether with a suspension of 0.02 mol of **1′** in 20 ml of hexane at 27°, followed by stirring for 2 hr, gave a deep red-brown solution. The reaction mixture was then quenched with 20 ml of water and worked up in the usual manner to afford 3.3 g (64%) of (*N*,*N*-dimethylaminomethyl)diphenylphosphine oxide, mp 180–182° (lit.[29] mp 185–187°) and 6.0 g (87%) of tetrabutyltin, bp 91–94° (0.1 mm). Subsequent recrystallization of the phosphine oxide from benzene–hexane afforded 3.15 g of compound, mp 181–182°. ^{1}H nmr spectral analysis of the phosphine oxide in $CDCl_3$ showed aromatic protons (10, m) at τ 2.5, methylene protons (2, d, *J* = Hz) at 6.7, and methyl protons (6, s) at 7.6.

Preparation of *N*-(2,2-Diphenylethylidine)-*N*,*N*-dimethylamine and Diphenylacetaldehyde. A solution of 3.62 g (0.02 mol) of benzophenone in 15 ml of anhydrous ether was added to 0.02 mol of [α-lithio-α-(*N*,*N*-dimethylamino)methyl]diphenylphosphine oxide (prepared as described above). The reaction mixture rapidly lost color and was mildly exothermic. After 1.5 hr stirring at 27°, the reaction mixture was hydrolyzed with 40 ml of a 10% sulfuric acid solution. The reaction mixture was filtered and extracted with ether, and the organic layer washed to neutrality. Removal of the solvent *in vacuo* afforded 12.8 g of a two-phase oil. Distillation gave 1.75 g (44%) of diphenylacetaldehyde, bp 91–100° (0.05 mm), and 6.0 g (87%) of tetrabutyltin. The aldehyde was identified by comparison of its ^{1}H nmr spectrum and glpc data with that of an authentic sample.

In a separate experiment, the enamine intermediate was isolated and characterized in the following way. Prior to the hydrolysis step, the reaction mixture was taken up in ether, washed quickly with water (three 40-ml portions), dried, and solvent removed *in vacuo* to afford a two-phase oil. Glpc analysis showed that the mixture contained tetrabutyltin, unreacted benzophenone, and the enamine. The enamine was collected by glpc and characterized by ^{1}H nmr spectral analysis (in $CDCl_3$) with aromatic protons (10, d, *J* = 14 Hz) at 2.9, vinyl proton (1, s) at 3.7, and methyl protons (6, s) at 7.5. A mass spectral analysis of the compound was consistent with the structure assignment.

Stability of 1′ in Mixed Hexane–Tetrahydrofuran Solvent. A solution of 0.03 mol of **1′** in 30 ml of hexane and 5 ml of THF was allowed to stand for 18 hr at room temperature and then treated with 3.5 g (0.033 mol) of benzaldehyde. The usual work-up gave 2.5 g (50%) of α-(*N*,*N*-dimethylaminomethyl)benzyl alcohol, bp 52–56° (0.1 mm). A higher boiling fraction was also obtained, but not characterized. No attempt was made to isolate the neutral product(s) of the reaction.

Acknowledgment. The author is grateful to Dr. C. D. Broaddus for helpful discussions and to Messrs. K. Yates and D. Toepker for technical assistance.

(29) A. Aquiar, K. C. Hansen, and J. T. Maque, *J. Org. Chem.*, **32**, 2383 (1967).

Proton Magnetic Resonance Study of the Indole N*H* Resonances of Lysozyme. Assignment, Deuterium Exchange Kinetics, and Inhibitor Binding

J. D. Glickson,[1a] W. D. Phillips,*[1a] and J. A. Rupley[1a]

Contribution No. 1717 from the Central Research Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898, and the University of Arizona, Department of Chemistry, Tucson, Arizona 85721.
Received October 1, 1970

Abstract: Resonances of the indole N*H* protons of the six component tryptophan residues of hen egg white lysozyme are coincident in the denatured form of the protein. Five of six indole N*H* protons are resolved in the 220-MHz pmr spectrum of the protein in the native conformation. Chemical modification, deuterium exchange kinetics, and inhibitor perturbation studies, in combination with published X-ray results, permit identification of these resonances with specific tryptophan residues.

Concomitant with improvements in instrumention, a number of laboratories have undertaken over the past few years studies by proton magnetic resonance (pmr) spectroscopy of the structures of proteins in solution. As was to be expected, because of the large number of structurally and environmentally nonequivalent protons, the pmr spectra of proteins are exceedingly complex and by no means competely resolved even at the highest resonance field presently available on commercial spectrometers.

In spite of this complexity, it is clear that the maximal and probably unique contributions of pmr spectroscopy to the elucidation of structural and dynamic processes of proteins in solution will depend on the extent to which resolved resonances can be uniquely identified with specific protons of the protein. Over the past few years there has been a good deal of progress in this direction. Resonances of the C-2 protons of the four histidine residues of bovine ribonuclease have been resolved and assigned.[2a] Resolved resonances in the high-field portion of the pmr spectrum of HEW lysozyme have been attributed to methyl protons of, principally, valine, leucine, and isoleucine residues perturbed by ring-current field effects.[2b,3,4] Resonances

(1) (a) E. I. du Pont de Nemours and Co.; (b) University of Arizona.

(2) (a) D. H. Meadows, O. Jardetzky, R. M. Epand, H. H. Ruterjans, and H. A. Scheraga, *Proc. Nat. Acad. Sci. U. S.*, **60**, 766 (1968); (b) C. C. McDonald and W. D. Phillips, *J. Amer. Chem. Soc.*, **89**, 6332 (1967).

Table I. Exposure of Tryptophan Side Chains in Lysozyme[a]

Residue	Exposure of ring: Free protein	Exposure of ring: Bound protein +NAG	Exposure of ring: Bound protein $+NAG_2$	Exposure of ring: Bound protein $+NAG_3$	Exposure of ring nitrogen: Free protein	Exposure of ring nitrogen: Bound protein +NAG	Exposure of ring nitrogen: Bound protein $+NAG_2$	Exposure of ring nitrogen: Bound protein $+NAG_3$
28	0.0				0.0 (+)			
62	0.60	0.45	0.35	0.25	0.55 (−)	0.0 (+)	0.0 (+)	0.0 (+)
63	0.20	0.10	0.05	0.05	0.35 (−)	0.0 (+)	0.0 (+)	0.0 (+)
108	0.10	0.0	0.0	0.0	0.15 (+)	0.0 (+)	0.0 (+)	0.0 (+)
111	0.10				0.0 (+)			
123	0.25				0.45 (−)			

[a] Exposure calculated as the surface area accessible to water in the protein or protein saccharide complex, relative to that in small peptides (these estimates were made in collaboration with D. C. Phillips). The symbol + indicates the ring nitrogen participates in a hydrogen bond.

displaced far to low field or to high field of their normal positions by contact interactions in the paramagnetic forms of proteins such as the heme proteins[5,6] and the ferredoxins[7] have yielded valuable structural and magnetic information.

The greatest success, then, in identifying resonances of proteins with specific protons has been for protons whose resonances are displaced either by structural or environmental factors from the bulk of largely unresolved resonances of the central portion of pmr spectra of proteins. Not to be ignored, however, are the extremely promising initial results of studies of proteins whose pmr spectra have been greatly simplified by partial deuterium substitution of side-chain and backbone protons.[8,9] Certainly isotope replacement, including ^{13}C enrichment, will be pursued, in spite of inherent expense and technical difficulties, because of the wealth of detailed information potentially available in the nmr spectra of proteins so prepared.[10,11] Nevertheless, continued efforts to resolve and assign resonances of proteins containing natural isotopic abundances are essential.

In this vein, we have continued pmr studies of hen egg white (HEW) lysozyme. The six tryptophan residues at sequence positions 28, 62, 63, 108, 111, and 123 of HEW lysozyme are distributed throughout the three-dimensional structure of this enzyme.[12,13] Of particular significance are tryptophans 62, 63, and 108, which are located in the active-site cleft of lysozyme. X-ray studies[14–16] indicate that the indole N*H*'s of Trp-62 and Trp-63 form hydrogen bonds with oxygen atoms of reducing end residues of the β anomers of the competitive inhibitors *N*-acetylglucosamine (NAG) and β-1,4-*N*,*N*'-diacetyldiglucosamine ($(NAG)_2$). Trp-108 lies close to the active site of the enzyme, and just below the nonproductive inhibitor binding site. (Some inhibitors like $(NAG)_2$ are actually weak substrates, which bind predominantly in the region above the active site. In this region true competitive inhibitors like NAG are also bound.) In the free enzyme the extent of exposure of the indole side chains to solvent increases in the following order (Table I): Trp-108 (completely buried with regard to solvent access), Trp-111 and Trp-108, Trp-123, Trp-63, Trp-62 (which shows 0.6 the exposure of indole in a small peptide).[14–16] Exposures of the indole nitrogens parallel those of the rings. The nitrogens of tryptophans 28, 108, and 111 hydrogen bond with amide carbonyls; the nitrogens of the other three indoles can bond to solvent. Binding of NAG and larger saccharides completely covers the nitrogens of residues 62, 63, and 108, and involves those of 62 and 63 in hydrogen bonds. Tryptophan resonances of lysozyme, therefore, should reflect perturbations of the active site such as by inhibitor binding, and monitor the environment on the surface and in the interior of the protein.

A number of potentially useful studies could be pursued if it were possible to assign specific resonances in the pmr spectrum of lysozyme to protons of the six tryptophan residues. The complexity of resonance absorption from the C*H* protons of the indole moiety of tryptophan coupled with overlap by other C*H* resonances makes such assignments exceedingly difficult in the aromatic region of resonance absorption.[17] In H_2O, rather than the more customary solvent D_2O, five indole N*H* proton resonances are cleanly resolved in the extreme low-field region of the pmr spectrum of native HEW lysozyme. We have employed inhibitor binding, deuterium exchange kinetics, and chemical modification to identify each of these resonances with a unique tryptophan residue of HEW lysozyme. A preliminary report of these results appeared.[18]

Experimental Section

Materials and Methods. Crystalline salt-free lysozyme was purchased from Worthington. Specific oxidations of tryptophans 62 and 108 were done as previously described, using NBS[19] and

(3) H. Sternlicht and D. Wilson, *Biochemistry*, **6**, 2881 (1967).
(4) C. C. McDonald and W. D. Phillips in "Biological Macromolecules," Vol. 4, S. N. Timasheff and G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1970.
(5) K. Wüthrich, R. G. Shulman, and J. Peisach, *Proc. Nat. Acad. Sci. U. S.*, **60**, 373 (1968); R. G. Shulman, K. Wüthrich, T. Yamane, E. Antonini, and M. Brunori, *ibid.*, **63**, 623 (1969); R. G. Shulman, S. Ogawa, K. Wüthrich, T. Yamane, J. Peisach, and W. E. Blumberg, *Science*, **165**, 251 (1969).
(6) R. J. Kurland, D. G. Davis, and C. Ho, *J. Amer. Chem. Soc.*, **90**, 2700 (1968).
(7) M. Poe, W. D. Phillips, C. C. McDonald, and W. Lovenberg, *Proc. Nat. Acad. Sci. U. S.*, **65** (4), 797 (1970).
(8) H. L. Crespi and J. J. Katz, *Nature (London)*, **224**, 560 (1969).
(9) I. Putter, J. L. Markley, and O. Jardetzky, *Proc. Nat. Acad. Sci. U. S.*, **65**, 395 (1970).
(10) W. Horsely and H. Sternlicht, *J. Amer. Chem. Soc.*, **90**, 3738 (1968); W. Horsley, H. Sternlicht, and J. S. Cohen, *Biochem. Biophys. Res. Commun.*, 37 (1), 47 (1969); W. Horsley, H. Sternlicht and J. S. Cohen, *J. Amer. Chem. Soc.*, **92**, 680 (1970).
(11) W. A. Gibbons, J. Sogn, A. Stern, L. C. Craig, and L. F. Johnson, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **29** (2), 1233 (1969).
(12) P. Jolles, J. Juarigini-Adell, and J. Jolles, *C. R. Acad. Sci.*, **258**, 3926 (1964).
(13) R. Canfield and A. K. Liu, *J. Biol. Chem.*, **240**, 1997 (1965).
(14) D. C. Phillips, *Sci. Amer.*, **216**, 78 (Nov 1966).
(15) C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. Roy. Soc., Ser. B*, **167** (1009), 378 (1967).
(16) D. C. Phillips, *Proc. Nat. Acad. Sci. U. S.*, **57**, 484 (1967).
(17) D. H. Meadows, J. L. Markley, J. S. Cohen, and O. Jardetzky, *ibid.*, **58**, 1307 (1967).
(18) J. D. Glickson, C. C. McDonald, and W. D. Phillips, *Biochem. Biophys. Res. Commun.*, **35** (4), 492 (1969).
(19) K. Hayashi, T. Imoto, G. Funatsu, and M. Funatsu, *J. Biochem. (Tokyo)*, **58**, 227 (1965).

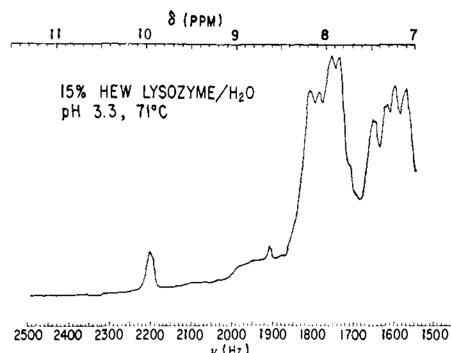


Figure 1. The low-field region of resonance absorption of thermally denatured HEW lysozyme. Solvent H_2O, 220 MHz, internally referenced to DSS.

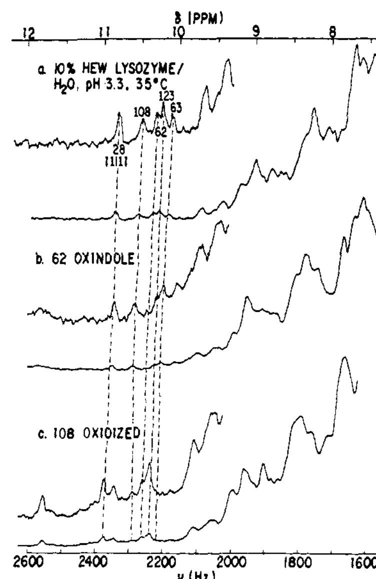


Figure 2. The low-field region of resonance absorption of native HEW lysozyme and two tryptophan-modified derivatives: (a) 10% (w/v) native lysozyme, (b) 10% (w/v) 62-oxindole lysozyme, and (c) 10% (w/v) 108-oxidized lysozyme (solvent H_2O, pH 3.3, 35°, internally referenced to DSS, 220 MHz).

iodine,[20,21] respectively. $(NAG)_2$ was prepared and determined using charcoal–Celite columns.[22]

Pmr Spectra. Spectra were recorded on a Varian Associates HRSC-IX 220-MHz pmr spectrometer equipped with a V-4540 variable-temperature accessary. The temperature was determined (to ±1°) from the separation of the resonances of a sample of ethylene glycol. Samples were contained in Royal Imperial 527-PP nmr tubes (Wilmad Glass Co., Buene, N. J.) fitted with a Teflon plug to prevent vortexing. Chemical shifts were measured relative to the methyl resonance of sodium 2,2-dimethylsilapentane-5-sulfonate (DSS) by the side-band technique employing a Hewlett-Packard 3734A electronic counter. Signal-to-noise was improved with the aid of a Varian Associates C-1024 computer of average transients (CAT).

Deuterium Exchange Kinetics. Protein was added to a solution of *N*-acetylglucosamine (NAG) in D_2O (ice bath, pD 3.5–4.5). Final pD (pH meter reading plus 0.40) adjustments were made with NaOD and/or DCl (no buffer) at room temperature. Concentrations of lysozyme were determined from the absorbance at 280 mμ ($E_{1\,cm}^{1\%}$ = 26.35).[23] Accumulation of a spectrum (60 CAT passes, recur mode) required 25 min. Peak areas (A) were measured with a planimeter. Least-squares analysis of plots of log A *vs.* $\bar{t}$ (mean of the time at which spectral accumulation was initiated and terminated) yielded rate constants and their standard deviations (Appendix I).

Results

Indole N*H* Proton Resonances. The pmr spectra of proteins ordinarily are obtained in D_2O to reduce interference from the intense water resonance that is present when H_2O is employed as solvent. In D_2O, protons such as N*H*, O*H*, S*H*, and some C*H* ordinarily are replaced by deuterium and therefore are nonobservable in pmr spectroscopy. H_2O can, however, be employed as a solvent for pmr studies of proteins, and at pH's < 8, the rate of exchange between protons of water and N*H* protons of amide and indole groups of proteins is sufficiently slow that separate resonances can be resolved over the 10–80° temperature range.

The low-field region of resonance absorption of HEW lysozyme dissolved in H_2O under conditions of thermal denaturation is shown in Figure 1. Resonance absorption in the 1470–1700-Hz region in Figure 1 includes contributions from aromatic C*H* protons,[2,4,18] arginine guanidino, and primary amide N*H* protons of asparagine and glutamine residues.[24] The 1700–2140-Hz region contains resonances of peptide N*H* protons.[24] The sharp resonance at 1915 Hz arises from the C-2 proton of the single histidine residue of lysozyme, His-15. In D_2O as solvent, deuterium replacement eliminates the 1700–2194-Hz region of resonance absorption and modifies the 1500–1700-Hz region. The C-2 proton of His-15 can also be replaced by deuterium on prolonged exposure to D_2O at elevated temperatures.[2a]

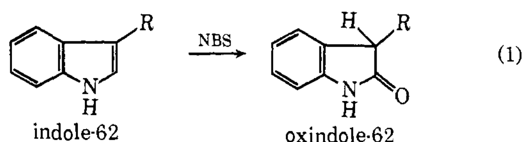
The 2194-Hz resonance of Figure 1, whose intensity is that of six protons, also is observed only in H_2O. This resonance is close to the resonance originating from the indole N*H* proton of free tryptophan at the same pH and temperature (2211 Hz), but is at considerably lower field than the N*H* resonances of model compounds of amides, amines, and guanidines.[24] The 2194-Hz resonance is absent in pmr spectra of thermally denatured bovine ribonuclease dissolved in H_2O at 71°; tryptophan is not present in this protein. Based on these observations, the 2194-Hz resonance is assigned to the indole N*H* protons of the six tryptophan residues of thermally denatured HEW lysozyme.

Upon thermal renaturation, profound changes are observed in the N*H* region of resonance absorption of lysozyme (Figure 2a). The peptide N*H* protons of native lysozyme exhibit a complex and largely unresolved series of peaks extending to about 2100 Hz. The resonance positions and exchange characteristics of amide N*H* protons as derived from pmr studies will be discussed elsewhere.[24] Here we are principally concerned with the observation that upon thermal denaturation the 2194-Hz resonance that was assigned to the indole N*H* protons of all six tryptophan residues is replaced by five resolved resonances, each of intensity equivalent to one proton, over the absorption range 2350–2150 Hz. Consistent with the assignment of these low-field resonances to indole N*H* protons, five similar resonances were observed in the spectrum of native human lysozyme (which contains five tryp-

(20) F. J. Hartdegan and J. A. Rupley, *J. Amer. Chem. Soc.*, **89**, 1743 (1967).
(21) F. J. Hartdegan, Ph.D. Thesis, University of Arizona, Tucson, Ariz., 1967.
(22) J. A. Rupley, *Biochim. Biophys. Acta*, **83**, 245 (1964).
(23) A. J. Sophianopoulos, C. K. Rhodes, D. N. Holcomb, and K. E. Van Holde, *J. Biol. Chem.*, 237, 1107 (1962).
(24) J. D. Glickson, C. C. McDonald, and W. D. Phillips, in preparation.

tophan residues),[18] and no such resonances were observed in native bovine ribonuclease (which lacks tryptophan). In contrast to the denatured form of lysozyme where indole N*H* proton resonances indicate all tryptophan side chains to be in similar shielding environments (probably dominated by solvation), five of the six nonequivalent environments of the tryptophan residues in the native enzyme are clearly reflected in resolvable resonances of the indole N*H* protons. The sixth, as yet unlocated, resonance probably has been shifted into the complex region of resonance absorption of the amide N*H* protons. We now proceed to identify the five resolved indole N*H* proton resonances of HEW lysozyme with specific tryptophan residues. Anticipating final assignment, we have labeled the resonances by their respective sequence positions (Figure 2a). In the subsequent discussion the six indole N*H* protons are designated H_{28}, H_{62}, H_{63}, H_{108}, H_{111}, and H_{123}.

Chemical Modification. Treatment of lysozyme with 1 equiv of *N*-bromosuccinimide (NBS) selectively converts the indole group of Trp-62 to an oxindole (eq 1).[19]



(1)

The pmr spectrum of native lysozyme so modified by NBS treatment (Figure 2b) is little perturbed from the spectrum of the unmodified protein. The high-field region of resonance absorption of NBS-modified lysozyme (−100 to 1000 Hz, not shown here) appears identical with that of the unmodified protein. Absence of an effect in this region of the spectrum indicates that the conformation of lysozyme was not significantly perturbed by this particular chemical modification. ORD studies support this conclusion.[25]

The tryptophan indole N*H* region of resonance absorption of lysozyme is, however, selectively affected upon conversion of Trp-62 to its oxindole derivative. The indole N*H* peak at 2266 Hz in the spectrum of the unmodified enzyme (Figure 2a) disappears (Figure 2b), and a new resonance at 2180 Hz appears (Figure 2b). The 2266-Hz resonances of lysozyme thus are provisionally identified with indole N*H* protons of Trp-62.

The N*H* resonance of oxindole (R = H in eq 1) appears about 60 Hz to high field from the N*H* resonance of indole in dimethyl-d_6 sulfoxide (DMSO-d_6) (Table II). In H_2O at 80° the corresponding oxindole–indole N*H* shift is 65 Hz (Table II). A similar comparison was not feasible at lower temperatures in H_2O because of the low solubility of indole. The N*H* protons of these model compounds probably are predominantly hydrogen bonded to the solvent in DMSO-d_6 and H_2O, as are the N*H* protons of the indole or oxindole moieties of tryptophan residues such as Trp-62 that are on the surface of lysozyme. Assignment of the 2180-Hz resonance of NBS-oxidized lysozyme (Figure 2b) to the oxindole N*H* proton of Trp-62 is indicated by the 86-Hz oxindole–indole N*H* shift, a value roughly consistent with the shifts observed in model compounds. Inspection of the crystal structure shows that Trp-62 has the most exposed indole of lysozyme. Thus the normal shifts observed for it and the oxindole are expected, as is the absence of a structural change following NBS oxidation.

Table II. Chemical Shifts of N*H* Resonances of Indole and Oxindole

Solvent	Temp, °C	pH	Chemical shift[a]: Indole, Hz	Chemical shift[a]: Oxindole, Hz
H_2O	35	3.3	*b*	2291
	80	3.3	2450	2385
DMSO-d_6	23		2450	2292

[a] Relative to internal DSS at 220 MHz. [b] Not sufficiently soluble to permit determination of chemical shift.

Lysozyme specifically oxidized at Trp-108 has been isolated from the mixture of products resulting from treating this enzyme with 0.5 equiv of iodine.[26,27] Blake and Beddell[28] have extended their X-ray study of the oxidation product to high resolution and have shown it to be the oxindolyl ester of Glu-35. This product forms with essentially no change in protein conformation (a one-third rotation of the carboxyl of Glu-35, with no displacement of the indole ring or other residues). Thus, detailed comparisons of properties of the modified and native proteins are justified. Solution studies of the ester[29] are in accord with the crystallography. In particular, substrate binding is unaffected by iodine reaction. These active-site cleft perturbations are reflected in the indole N*H* resonances of the Trp-108-oxidized enzyme (Figure 2c) in which resonances originating from H_{63} and H_{108} have been shifted from their positions in the unmodified enzyme (Figure 2a), and two new resonances appear at 2362 and 2700 Hz (Figure 2c). Assignment of these new resonances to specific protons is not yet possible. Oxidation of Trp-108 will be discussed in more detail elsewhere.[29] From the perturbations of resonances of H_{63} and H_{108} in the spectrum of Trp-108-oxidized lysozyme, we can only conclude that one of these resonances probably originates from the indole N*H* of Trp-108, but that detailed assignment is not possible at this time.

Inhibitor Binding. Thomas[30] and Raftery and coworkers[31] have employed perturbations of the pmr spectra of *N*-acetylglucosamine inhibitors to study the binding of these molecules to lysozyme. Meadows, *et al.*,[2] assigned histidine C-2 proton resonances to specific active-site residues of ribonuclease partially on the basis of effects of bound inhibitors on their chemical shifts. Here we utilize perturbations of the resonances of resolved indole N*H* protons of lysozyme by inhibitors to identify these resonances with specific tryptophan residues and to evaluate binding constants.

The binding constant *K* for a 1:1 complex between a small molecule S and a protein E is given by eq 2 where

(25) T. Takahashi, K. Hamaguchi, K. Hayashi, T. Imoto, and M. Funatsu, *J. Biochem.* (*Tokyo*), **58** (4), 385 (1965).

(26) F. J. Hartdegan and J. A. Rupley, *Biochim. Biophys. Acta*, **92**, 625 (1964).

(27) C. C. F. Blake, *Proc. Roy. Soc., Ser. B*, **167** (1009), 435 (1967).

(28) Private communication.

(29) T. Imoto and J. A. Rupley, in preparation.

(30) E. W. Thomas, *Biochem. Biophys. Res. Commun.*, **24** (5), 611 (1966).

(31) M. A. Raftery, F. W. Dahlquist, S. M. Parsons, and R. G. Wolcott, *Proc. Nat. Acad. Sci. U. S.*, **62**, 44 (1969); F. W. Dahlquist and M. A. Raftery, *Biochemistry*, **8**, 713 (1969).

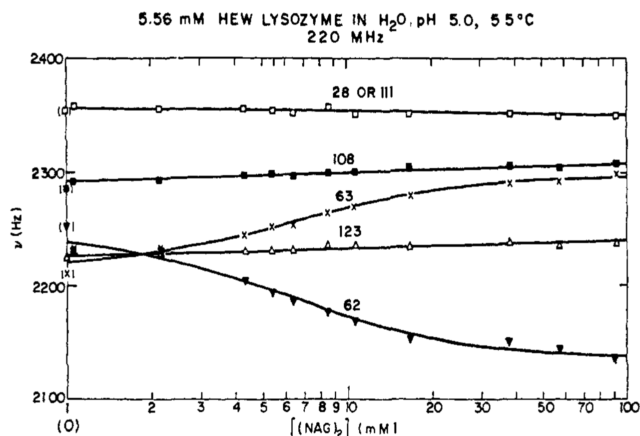


Figure 3. Tryptophan indole NH chemical shifts (internally referenced to DSS) of 5.56 mM HEW lysozyme at various total concentrations of $(NAG)_2$ (solvent H_2O, pH 5.0, 0.1 M citrate buffer), and 55°. The curves associated with tryptophan residues 62 and 63 were computed using least-squares parameters quoted in the text.

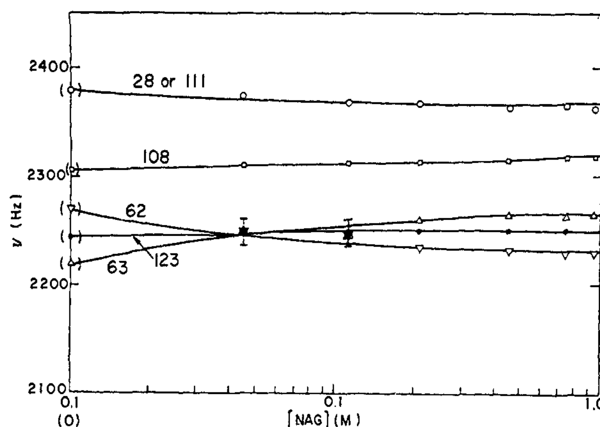


Figure 4. Tryptophan indole NH chemical shifts of 5 mM HEW lysozyme at various total concentrations of NAG (solvent H_2O at pH 4.0 and 45°).

$[S]_0$ and $[E]_0$ are the total concentrations of E and S, respectively, and α is the degree of binding. Let the

$$K = \frac{1}{[S]_0 - \alpha[E]_0} \frac{\alpha}{1 - \alpha} \tag{2}$$

resonance of a single proton of E have chemical shifts ν_0 and ν_S in the free and bound states. If the first-order rate constants for formation and dissociation of the 1:1 complex are much greater than $\nu_S - \nu_0$ (*i.e.*, rapid exchange on the nmr time scale), then the chemical shift ν averaged over the free and bound states is given by

$$\nu = \nu + \alpha(\nu_S - \nu_0) \tag{3}$$

Where there are n different binding sites on E, but only one of these sites can be occupied at one time, eq 2 and 3 are still valid, but K and ν_S are functions of K_i and $\nu_S{}^i$ ($i = 1, 2 \ldots n$), the binding constant, and ν_S for the ith binding site, respectively (eq 4). Hence

$$K = \sum_{i=1}^{n} K_i$$
$$\nu_S = \left(\sum \nu_S{}^i K_i\right) \Big/ \sum_{i=1}^{n} K_i \tag{4}$$

chemical-shift changes alone do not distinguish between single and multiple modes of inhibitor binding so long as only one inhibitor molecule can be bound to the protein at one time.

The α and β anomers of NAG and $(NAG)_2$ are believed to form 1:1 complexes with lysozyme.[14,15] Equations 2 and 3 still describe the binding equilibrium with

$$K = (K_\alpha + K_\beta K_{\alpha\beta})/(1 + K_{\alpha\beta})$$
$$\nu_S = (\nu_S{}^\alpha K_\alpha + \nu_S{}^\beta K_\beta K_{\alpha\beta})/(K_\alpha + K_\beta K_{\alpha\beta}) \tag{5}$$

where K_α and K_β are binding constants for the α and β anomer (denoted as S_α and S_β), respectively, $K_{\alpha\beta} = [S_\beta]/[S_\alpha]$ is the anomeric equilibrium constant, and $\nu_S{}^\alpha$ and $\nu_S{}^\beta$ are the saturation chemical shifts of the α and β anomer, respectively. The method used for calculating K, ν_0, and ν_S from experimental data is described in Appendix II.

The large chemical-shift changes of the H_{62} and H_{63} resonances resulting from $(NAG)_2$ binding (Figure 3) are consistent with the formation of hydrogen bonds between these protons and oxygen atoms on the reducing residue of the β anomer of this inhibitor.[14-16] The titration curves associated with H_{62} and H_{63} in Figure 3 represent a least-squares fit of eq 2 and 3 to the experimental data (using the procedure of Appendix II). The least-squares values of K, ν_0, and ν_s for H_{62} were 372 ± 54 M^{-1}, 2252 Hz, and 2136 Hz, respectively. The corresponding values for H_{63} were 266 ± 40 M^{-1}, 2212 Hz, and 2300 Hz. Optically determined values of K are larger, *ca.* 700 M^{-1}.[32] Binding of NAG to lysozyme causes similar but less pronounced spectral changes (Figure 4). Overlap between the H_{62}, H_{63}, and H_{123} resonances prevented determination of K, ν_0, and ν_S from the H_{62} and H_{63} resonances of lysozyme titrated with NAG. The negligible perturbations of $H_{28(111)}$, H_{108}, and H_{123} suggest that binding of NAG and $(NAG)_2$ at the nonproductive binding site produces conformation changes limited to the immediate vicinity of this binding site. That the productive binding site is at most infrequently populated is indicated by the absence of significant hydrolysis or transfer reactions of $(NAG)_2$,[33] and by the absence of any marked perturbation of the H_{108} resonance (Trp-108 is very close to the active site). Because Trp-108 lies just below the nonproductive binding site,[14-16] the insensitivity of the H_{108} chemical shift to inhibitor binding is somewhat surprising, but may be explained by the dominant influence of a hydrogen bond between H_{108} and the amide carbonyl of Leu-56 that remains intact in both the free and inhibitor-bound enzyme.[14-16] Also, because the ring and ring nitrogen of Trp-108 are considerably less exposed in the free protein than those of residues 62 and 63, saccharide binding alters the environment of 108 to a correspondingly smaller extent.

Deuterium Exchange Kinetics. Since the pioneering studies of Linderstrøm-Lang,[34] isotope exchange of labile hydrogen atoms has been employed to elucidate

(32) J. A. Rupley, unpublished data.

(33) Only the $(NAG)_2$ peak was observed upon chromatography on a charcoal–Celite column of the solution of $(NAG)_2$ and lysozyme employed in the pmr experiments.

(34) K. Linderstrøm-Lang in "Symposium on Protein Structure," A. Neuberger, Ed., Methuen, Paris, 1958.

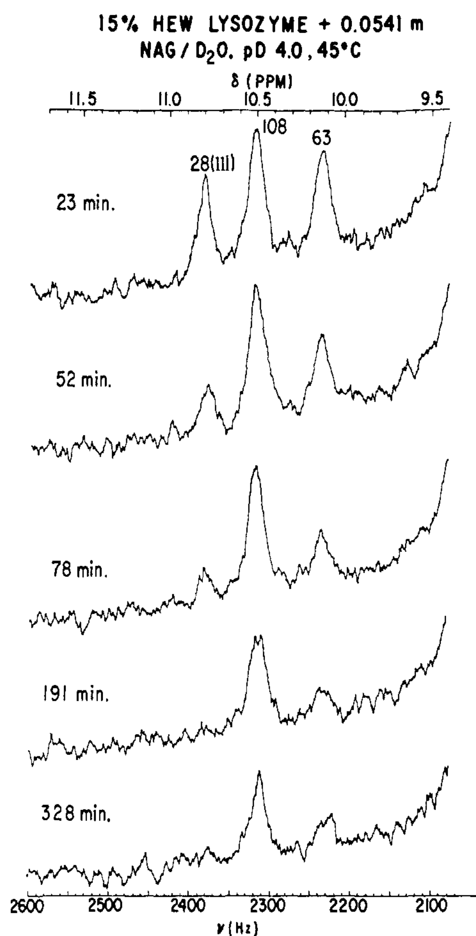


Figure 5. 220-MHz pmr spectra of the tryptophan indole N*H* resonances of 10.5 m*M* HEW lysozyme plus 0.0541 *M* NAG in D_2O, at pD 4.0 and 45° at various times after solution was initiated. Each spectrum (60 passes on the CAT) was accumulated over 25 min, and the time refers to the mean of the time at which spectral accumulation was initiated and completed.

the structure of proteins. The freeze-drying, infrared, and Sephadex techniques applied heretofore in studying isotope exchange rates in proteins have been limited to observation of the exchange kinetics of large subgroups of protons.[35,36] Earlier pmr studies of protein deuterium exchange,[37] while demonstrating the potential of the approach, were limited by the resolution of the available spectrometers. Partial resolution of the N*H* region of the pmr spectra of proteins is achieved with currently available spectrometers, thereby making possible study of exchange kinetics of individual protons. We concentrate here on relating the exchange characteristics of the resolved indole N*H* protons of lysozyme with such features as intramolecular hydrogen bonding, solvent access, and inhibitor binding.

Model compound studies have greatly facilitated interpretation of amide hydrogen exchange kinetics. Interpretation of the exchange of indole N*H* protons is hampered by the absence of such results on model indoles. Klotz and Frank[38] have demonstrated that exchange of protein amide N*H*'s may be strongly catalyzed by acidic and basic side chains of local amino acid residues. Similar general acid–base catalysis may complicate the interpretation of indole N*H* exchange kinetics. We assume, however, that exchangeability reflects primarily the rate and extent of exposure of tryptophan indole N*H*'s to the solvent. This assumption is widely accepted in interpretation of amide hydrogen-exchange kinetics.[35] Future studies may indicate the relative importance of side-chain catalysis on protein hydrogen-exchange kinetics.

With existing instrumentation, we are able to measure rate constants between 10^{-3} and 2×10^{-2} min^{-1} (for first-order or pseudo-first-order reactions). The Fourier transform method promises to extend this range to more rapid rates.[39] Temperature and pD had to be adjusted to obtain exchange rates in the measurable range. Minimum exchange rates of $H_{28(111)}$, H_{108}, and H_{63} (the only indole N*H*'s exchange rates slow enough to measure) occurred at about pD 3.8, 4.0, and 4.7, respectively. Most measurements were made at pD 4.0. At this pD the reaction was studied at 45° to permit simultaneous determination of the maximum number of exchange rates—at higher temperatures $H_{28(111)}$ and H_{63} exchanged too rapidly, whereas at lower temperatures, H_{108} exchanged too slowly.

Anticipating that binding of NAG should preferentially slow the exchange of H_{62}, H_{63}, and H_{108} (Table I), exchange rates of the indole N*H* hydrogens were measured at various NAG concentrations (the inhibitor binds by hydrogen bonds to H_{62} and H_{63}, and blocks access of solvent to H_{108}).[14–16] In the absence of inhibitor under these reaction conditions only $H_{28(111)}$ and H_{108} exchanged slowly enough to observe the time dependence of their resonance intensities. (At pD 4–5 and 23° the exchange of H_{63} could also be followed.) Addition of NAG slowed the exchange of H_{63} sufficiently to permit measurement of the kinetics of its replacement by deuterium. While the deuterium exchange rate of H_{62} was slowed by NAG, the reduction was not sufficient to permit accurate kinetic determinations. In any event, the effects of NAG on the exchange kinetics of H_{62} and H_{63} were qualitatively similar. Even at saturating inhibitor concentrations, H_{123} exchanged too rapidly for its resonance to be observed under the conditions employed (even at 23° the resonance of this proton could not be observed in D_2O).

The time course for deuterium exchange of $H_{28(111)}$, H_{108}, and H_{63} of HEW lysozyme in a typical experiment is shown in Figure 5. These spectra illustrate the resolution and signal-to-noise ratios encountered in these studies. At higher concentrations of NAG a weak H_{62} peak was observed on the high-field side of the H_{63} peak during the initial stages of the reaction. Taking advantage of the symmetry and greater intensity under exchange conditions of the H_{63} peak, accurate estimates of its area were obtained even when partially overlapped with the H_{62} resonance.

Qualitatively, the two most rapidly exchanging of the five resolved indole N*H* protons of lysozyme are associated with the two exposed tryptophan residues at sequence positions 62 and 123 (Table I). Resonances of the indole N*H* protons of these residues are readily distinguished by perturbations of the H_{62} chemical

(35) A. Hvidt and S. O. Nielsen, *Advan. Protein Chem.*, **21**, 287 (1966).
(36) S. W. Englander in "Biological Macromolecules," Vol. 1, G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1967.
(37) A. Wishnia and M. Saunders, *J. Amer. Chem. Soc.*, **84**, 4235 (1962).
(38) I. M. Klotz and B. H. Frank, *Science*, **138**, 830 (1962).
(39) R. R. Ernst and W. A. Anderson, *Rev. Sci. Instrum.*, 37, 93 (1966).

shift, but not the H_{123} chemical shift upon specific oxidation of Trp-62 and the binding of NAG and $(NAG)_2$. The previously discussed diminution of the rate of deuterium replacement of H_{62} by inhibitor binding, and the absence of such effects on H_{123}, are compatible with this assignment.

The foregoing observations accord fully with crystallographic data (Table I). Tryptophan 62 is more exposed than 63 in both free protein and the saccharide complexes. Trp-123 has exposure intermediate between 63 and 62 and is not affected by saccharide binding. The structural basis for slow exchange of Trp-108 and the relatively small effect of saccharide binding on it were discussed above. It is of interest that the proton $H_{28(111)}$ exchanges more rapidly than H_{108}. Disruption of the structure about Trp-28 should be more difficult than for that about 108. Residues 108 and 111 have about equal ring exposure, and it is not difficult to imagine how the latter ring nitrogen might be brought into contact with solvent. Thus one can tentatively assign the $H_{28(111)}$ resonance to Trp-111.

Time courses of deuterium replacement of H_{108}, H_{63}, and $H_{28(111)}$ are given in Figure 6. The exchange of $H_{28(111)}$ and H_{108} followed first-order kinetics over the entire observed reaction range. Even at the low NAG concentration employed, the exchange of H_{108} was too slow to be conveniently followed to completion. In the absence of inhibitor, the exchange of H_{108} progressed by first-order kinetics for at least 90% of the reaction.

There appear to be definite deviations from first-order kinetics in the exchange of H_{63} (Figure 6). The first four points fall on a straight line for which the apparent rate constant is $(16.6 \pm 1.5) \times 10^{-3}$ min^{-1}, and the extent of reaction is 82%. If we include the next two points, we still have a reasonable fit to first-order kinetics with a rate constant of 11.8×10^{-3} and an extent of first-order kinetics of 85%. It is tempting to ascribe the deviations of the remaining points to the low signal-to-noise expected for weak peaks, but although the area estimates of these peaks may indeed by inaccurate, the persistence of these peaks is unmistakable (see the 191- and 328-min spectra in Figure 5). For neither of the first-order rate constants quoted above should the H_{63} peak be observed at 328 min. Hence, there appear to be real deviations from first-order kinetics in the terminal stages of the replacement of H_{63} by deuterium.

The effects of NAG concentration on the exchange rates of $H_{28(111)}$, H_{63}, and H_{108} are presented in Table III. The rates quoted for H_{63} were obtained from

Table III. Deuterium Exchange Rates of Tryptophan Indole N*H*'s (40°, pD 4.0)

[NaG], *M*	$k_{obsd} \times 10^3$ min^{-1} Trp-28 (111)	Trp-108	Trp-63
0.0000	19.9 ± 1.6	4.08 ± 0.25	
0.0292	18.2[a]	2.89 ± 0.14	21.8 ± 1.1
0.0541	24.9 ± 1.3	2.54 ± 0.16	16.6 ± 1.5
0.107	25.4[a]	3.26 ± 0.13	16.6 ± 1.5
0.204		1.83 ± 0.19	9.96 ± 1.76
0.260	19.3 ± 2.2	1.86 ± 0.20	6.10 ± 0.55
0.307	18.0 ± 2.3	1.19 ± 0.34	5.52 ± 1.02
0.314	17.8 ± 2.4	1.34 ± 0.15	6.30 ± 0.15

[a] No estimate of the error was made, since the rate was determined from two points.

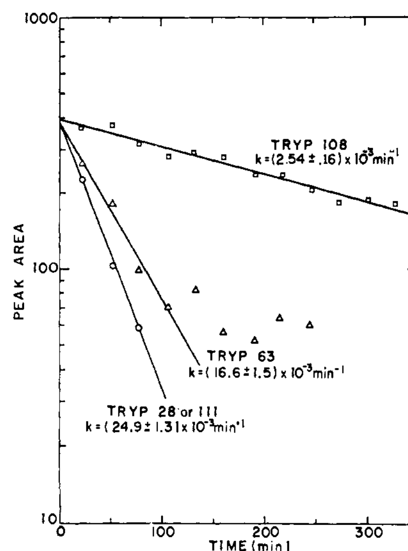


Figure 6. First-order kinetics plots for the deuterium exchange of the slowly exchanging tryptophan indole N*H*'s of 10.5 m*M* HEW lysozyme plus 0.0541 *M* NAG in D_2O at pD 4.0 and 45°. Least-squares rate constants with their standard deviations are indicated. For Trp-63 only the initial linear data was used in computation of rate constants. Peak areas are expressed in arbitrary units.

the initial linear portion of the exchange curve. While they may not be true first-order rate constants, they at least qualitatively reflect the effect of inhibitor binding on the exchange of this proton. The absence of any effect of inhibitor binding on the exchange rate of $H_{28(111)}$ is reasonable since neither Trp-28 nor Trp-111 is near the inhibitor binding site. Diminution of exchange rates of H_{108} and H_{63} by inhibitor binding demonstrates the proximity of both of these protons to the binding site. Since H_{62} has already been assigned to Trp-62, H_{63} and H_{108} must be associated with the remaining active-site cleft tryptophan residues 63 and 108. The more intimate involvement of H_{63} with NAG and $(NAG)_2$ binding, which was demonstrated by the much greater perturbation of its chemical shifts (Figures 3 and 4), distinguishes this proton's resonance from that of H_{108} (H_{63} is hydrogen bonded to these inhibitors, but H_{108} is not).[14-16]

The failure of saccharide to reduce the rate of exchange of those indole protons that are not in the cleft suggests that various parts of the lysozyme molecule can unfold independently of each other to give the fluctuant conformations presumably important for exchange of buried residues. Earlier results—the failure of bound saccharide and of crystallization to significantly reduce the rate of exchange of amide protons—have led to the same conclusion.[40]

Summary of Assignment. The assignment of the five low-field resonances of HEW lysozyme to tryptophan indole N*H*'s is supported by the following evidence. (a) That these resonances are probably N*H* resonances was indicated by the ease of their replacement with D_2O. Labile C*H* protons, such as the C_2 proton of histidine residues, exchange measurably only at temperatures in excess of 40°.[1] O*H* and S*H* protons generally exchange too rapidly with water to yield

(40) M. Praissman and J. A. Rupley, *Biochemistry*, 7, 2446 (1968).

resolvable resonances. (b) The chemical shifts of these NH protons were in the range observed for the NH resonances of model indoles, but were outside the range of the chemical shifts of other NH's.[24] (c) The presence of similar residues in pmr spectra of other proteins which contain tryptophans, and their absence in pmr spectra of proteins that lack tryptophan, are consistent with this assignment.

Specific perturbation of the chemical shifts and deuterium exchange rates of the H_{62} and H_{63} peaks by binding of NAG and $(NAG)_2$ associated these resonances with tryptophan residues at the active site. The remaining active-site tryptophan indole NH (H_{108}) was identified by diminution of its deuterium exchange rate as a result of inhibitor binding, and by perturbation of its chemical shift when Trp-108 was specifically oxidized. The H_{62} resonance was unambiguously assigned by its perturbation as a result of specific oxidation of Trp-62, and by its rapid exchange rate in the absence of inhibitor (it is the most exposed active-site indole NH). Since H_{63} is hydrogen bonded to the inhibitor, but H_{108} is not, the former proton must be associated with the other resonance (besides that of H_{62}) whose chemical shift is perturbed by inhibitor binding. The remaining active-site indole NH must be associated with H_{108}. The rapidly exchanging indole NH resonance, other than H_{62}, is associated with H_{123}, the remaining exposed indole NH. The remaining resolved resonance is then assigned to either tryptophan 28 or 111, with Trp-111 being preferred.

Acknowledgment. We thank Drs. C. C. McDonald and T. Imoto for their aid in this investigation. We are indebted to Mr. Francis Ferrari for assistance in obtaining spectra.

Appendix I

Determination of First-Order Kinetics Rates from Computer-Averaged Data. If a large number of spectral traces are accumulated on the CAT, each pass of short duration compared to the total accumulation time Δt, then the area A under a computer-averaged peak of a reactant consumed by a process obeying first-order kinetics is, to a good approximation

$$A = \frac{A_0}{\Delta t}\int_{t_0}^{t_f} e^{-kt}\mathrm{d}t \qquad (6)$$

where A_0 is the area at t_0, the time at which accumulation of the spectrum was initiated, t_f is the time at which the accumulation was completed (*i.e.*, $\Delta t = t_f - t_0$), and k is the first-order rate constant associated with the process. Carrying out the integration in eq 6 yields

$$A = \frac{A_0}{k\Delta t}(1 - e^{-k\Delta t})e^{-kt_0} = \frac{2A_0}{k\Delta t}\sin h\left(\frac{k\Delta t}{2}\right)e^{-k\bar{t}} \qquad (7)$$

where $\bar{t} = (t_0 + t_f)/2$. If Δt is kept constant for each spectrum, then A is proportional to e^{-kt_0} or $e^{-k\bar{t}}$. Hence, a plot of log A against t_0 or $\bar{t}$ will be linear with slope $-k/2.303$. Therefore, if a large number of scans are taken, and if the scanning time per spectrum is kept constant, time-averaged data can be treated by the usual method for obtaining first-order rate constants.

Appendix II

Calculation of Binding Constants. The error function Q is

$$Q = \sum_{i=1}^{n}(\nu_i - \bar{\nu}_i)^2 W_i \qquad (8)$$

where ν_i is the ith experimental value of ν, $\hat{\nu}_i$ is the corresponding value calculated from eq 2 and 3, and the weighting factor $W_i = 1/N$. When the standard deviation σ_i of ν_i is known, $W_i = 1/\sigma_i^2$. Marquardt's[41] algorithm for minimizing Q by a combined Taylor series–steepest descent iterative procedure was used to find the least-squares values of K, ν_0, and ν_S. This algorithm is embodied in the Fortran IV computer program NLIN2 (IBM Share Library, Distribution No. 309401). Initial estimates of ν_0 and ν_S were obtained from inspection of the titration curve, and the initial estimate of K was obtained from a Scatchard plot.[42,43] K could also be estimated from the midpoint of the titration curve.

Reasons for employing the above procedure for evaluating binding constants rather than more conventional methods were (a) it weights all experimental points equally, or by the reciprocal of their variances, whereas reciprocal plots, such as the Scatchard plot, weight points at low α more heavily than points at high α; (b) this procedure avoids expenditure of large quantities of inhibitors by not requiring accurate estimates of ν_S; and (c) where overlap makes direct determination of ν_0 and/or ν_S difficult, this method permits evaluation of these parameters.

(41) D. W. Marquardt, *J. Soc. Ind. Appl. Math.*, **11** (2), 431 (1963).
(42) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
(43) I. M. Klotz in "The Proteins," H. Neurath and K. Bailey, Ed., Academic Press, New York, N. Y., 1953.