Spin-Label-Induced Nuclear Relaxation. Distances between Bound Saccharides, Histidine-15, and Tryptophan-123 on Lysozyme in Solution†

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ABSTRACT: Site-directed paramagnetic nitroxide radicals (spin labels) have been used to perturb the nuclear magnetic resonance spectra of specific protons in lysozyme and in bound inhibitors. The broadening of nuclear resonance lines has been used to calculate distances of up to 20 Å between the nitroxide radical electron and protons of interest. Lysozyme covalently spin labeled at histidine-15 broadens the nuclear resonance spectra of *N*-acetyl-α-D-glucosamine and di-*N*-acetyl-D-glucosamine bound at the active site, and these broadenings are used to estimate the distances from histidine-15 to the acetamido methyl groups of these saccharides. In other experiments, spin-labeled saccharides bound to the active site of lysozyme were found to broaden the resonance of the C-2 proton on histidine-15, providing a measure of the distance from subsite D to histidine-15. In further experiments, *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)acetamide, which binds near tryptophan-123, was used to estimate the distance from that residue to the acetamido methyl groups of bound saccharides, and to the C-2 proton of histidine-15. The distances estimated from the resonance data are in excellent agreement with distances estimated from the molecular model of lysozyme built according to the X-ray crystallographic coordinates of Phillips (*Proc. Nat. Acad. Sci. U. S. 57*, 484 (1967)).

Nuclear magnetic resonance shares with X-ray diffraction the potential for detecting single atoms in a biological macromolecule. For example, every proton in a macromolecule gives a nuclear resonance signal. Although nuclear resonance spectra can detect the interaction of diamagnetic enzymes and their substrates, or inhibitors, and can provide information about the kinetics of these interactions, such resonance spectra rarely give quantitative information concerning the distances between the nuclei of bound substrates and the nuclei of the enzymes. However, experiments which involve the use of paramagnetic ions or molecules to perturb resonance spectra can yield data which allow the calculation of distances between the paramagnetic center and various nuclei. The use of paramagnetic ions to enhance the nuclear relaxation of coordinated water molecules has been used to study enzyme kinetics and geometry (Mildvan and Cohn, 1970). Recently several papers have appeared which describe the use of nitroxide spin labels to perturb the resonances of specific nuclei in biological systems. Mildvan and Weiner (1969) have used a spin-labeled analog of NAD to perturb the nuclear resonance spectrum of ethanol in the presence of liver alcohol dehydrogenase. Krugh (1971) has studied changes in the relaxation rates of purine protons in AMP bound to DNA polymerase (*E. coli*) in the presence of spin-labeled ATP. Still further experiments using paramagnetic metal ions and nitroxide spin labels to enhance nuclear relaxation rates and to estimate intramolecular distances in enzyme complexes in solution are described by Cohn and Reuben (1971), and by Bennick *et al.* (1971). Kornberg and McConnell (1971) have described experiments in which they measured the broadening of the quaternary methyl and phosphorus resonances of phosphatidylcholine molecules in vesicles doped with spin-labeled phosphatidylcholine. In all of these earlier experiments the important line broadening effects are due to interactions at distances that are ~10 Å or less. However, we show here that nitroxide spin labels can enhance the relaxation of protons at least 20 Å away. We have used this effect to estimate distances between spin labels bound (covalently or noncovalently) to lysozyme, and specific nuclei of the enzyme or of its bound substrate.

Experimental Section

Materials

Hen egg-white lysozyme (six-times crystallized, dialyzed, and lyophilized, lot 902) was obtained from Miles Laboratories and was used as received. *N*-Acetyl-α-D-glucosamine (NAG)[1] was a product of Aldrich Chemical Co. Di-*N*-acetyl-D-glucosamine (di-NAG) was a generous gift of Dr. John Rupley.

Synthesis of (1-Oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)-methyl-β-chitobiose (Tempyro-CH_2-NAG-NAG, I). The procedure for the preparation of this compound and also compound II is a modification of the method described by Kuhn and Kirschenlohr (1953). Acetochlorochitobiose (314 mg), prepared by the method of Dahlquist and Raftery (1969), was intimately mixed with 344 mg of IV and 504 mg of mercuric cyanide using a mortar and pestle. This mixture was transferred to a 25-ml flask and dried *in vacuo* for 5 hr. The reaction was initiated by adding 3 ml of chloroform (freshly distilled

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[1] Abbreviations used are: NAG, *N*-acetyl-α-D-glucosamine; di-NAG, di-*N*-acetyl-D-glucosamine; Tempyro, 1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl; Tempo, 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl; SL, spin labeled; IS complex, the molecular complex formed when the paramagnetic species (*e.g.*, spin-labeled enzyme or spin-labeled inhibitor) combines with the molecular species containing the nucleus of interest (*e.g.*, inhibitor or enzyme).

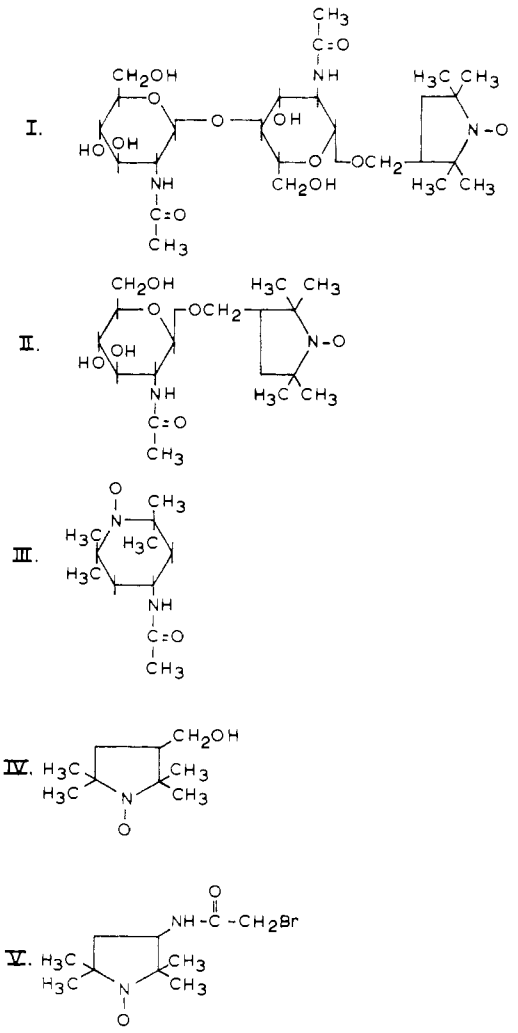

FIGURE 1: Chemical structures of spin labels used in these studies: Tempyro-CH_2-NAG-NAG (I), Tempyro-CH_2-NAG (II), Tempo-acetamide (III), Tempyro-carbinol (IV), and Tempyro-bromoacetamide (V).

from phosphorus pentoxide). The reaction mixture was stirred at room temperature for 72 hr, then evaporated to dryness under reduced pressure at less than 40°. The residue was redissolved in chloroform and excess mercuric cyanide removed by filtration. The yellow filtrate was washed exhaustively with water to remove excess IV, dried over magnesium sulfate, and evaporated to dryness yielding a yellow glass. This was applied to a column (0.6 × 10 cm) of SilicAR, CC-4, 100–200 mesh (Mallinckrodt Chemical Works), equilibrated with methylene chloride. The column was developed by eluting successively with methylene chloride, methylene chloride–chloroform (1:1), chloroform, and chloroform–methanol (10:1). The desired blocked glycoside was eluted last. Evaporation to dryness afforded a yellow glass which could not be crystallized. The infrared spectrum exhibited carbonyl bands at 1745 cm^{-1} (acetate) and 1660 cm^{-1} (acetamide) and no hydroxyl absorption. This material was deacylated by dissolving in 20 ml of methanol saturated with ammonia at 5°. After 24 hr, the reaction mixture was evaporated to dryness at less than 30°. The yellow syrup was completely soluble in water; lyophilization afforded 91 mg of a yellow hygroscopic powder. The infrared spectrum exhibited carbonyl absorption at 1655 cm^{-1} (acetamide) but not at 1730–1750 cm^{-1} (acetate). *Anal.* Calcd for $C_{25}H_{44}N_3O_{12}\cdot NH_3$: C, 50.41; H, 7.95; N, 9.40. Found: C, 50.56; H, 8.07; N, 10.04.

Synthesis of (1-Oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)-methyl β-N-Acetyl-D-glucosaminide (Tempyro-CH_2-NAG, II). To a mixture of 50 ml of dry pyridine and 100 ml of acetic anhydride was added 3.8 g of *N*-acetyl-α-D-glucosamine. The reaction mixture was stirred at 25° for 14 hr during which time it became homogeneous. The mixture was diluted with 100 ml of chloroform and extracted three times with 200 ml of water, three times with 200 ml of 1% sodium bicarbonate, then two times with 100 ml of water, each at 5°. The organic layer was dried over magnesium sulfate, then evaporated to a syrup. Trituration with diethyl ether afforded colorless star-like crystals: yield 3.1 g (46%); mp 133–135°; ir (KBr) bands at 3425, 1735, and 1665 cm^{-1}. *Anal.* Calcd for $C_{16}H_{23}NO_{10}$: C, 49.35; H, 5.95; N, 3.60. Found: C, 49.02; H, 5.88; N, 3.44.

This fully blocked derivative of NAG (480 mg) was added to 5 ml of acetic anhydride saturated with dry HCl (Matheson) at 5°. After sitting at 5° for 3 days, the mixture was diluted with 30 ml of cold chloroform, washed four times with 30 ml of saturated sodium bicarbonate and three times with 30 ml of water (each at 5°), dried over $MgSO_4$, and evaporated to dryness at less than 30° giving a clear syrup, which when triturated with diethyl ether gave crystals of the monosaccharide chloride: yield 300 mg (66%); mp 126–128°; $[\alpha]_D^{20}$ +116.9° (*c* 3.2, chloroform); ir (KBr) bands at 3240, 1740, 1640 cm^{-1}. *Anal.* Calcd for $C_{14}H_{20}ClNO_8$: C, 45.97; H, 5.51; Cl, 9.69; N, 3.83. Found: C, 46.14; H, 5.50; Cl, 9.56; N, 3.55.

The glycosyl halide (544 mg) was intimately mixed with 516 mg of IV and 700 mg of mercuric cyanide by pulverizing with a mortar and pestle. This mixture was then transferred to a 25-ml flask and dried *in vacuo* for 5 hr. To start the reaction 5 ml of chloroform (freshly distilled from phosphorus pentoxide) was added. The mixture was stirred at 25° for 48 hr, then evaporated to dryness. The residue was redissolved in chloroform and filtered. The filtrate was extracted exhaustively with water to remove excess starting alcohol, dried over magnesium sulfate, and evaporated to dryness. Purification by chromatography was carried out similar to that described for I. The purified glycoside was evaporated to dryness, yielding a yellow glass: yield 300 mg, ir (film) bands at 1660 and 1740 cm^{-1}. This material was deblocked by treatment with 10 ml of methanol saturated with ammonia at 5°. After 24 hr, the reaction mixture was evaporated to dryness at less than 30°. The yellow syrup was dissolved in water and lyophilized, yielding 105 mg of a semicrystalline, hygroscopic solid. The infrared spectrum exhibited carbonyl absorption at 1660 cm^{-1} but not in the region 1730–1750 cm^{-1}. *Anal.* Calcd for $C_{17}H_{31}N_2O_7\cdot H_2O$: C, 51.89; H, 8.45; N, 7.12. Found: C, 52.00; H, 8.39; N, 7.44.

N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)acetamide (Tempo-acetamide, III). The label was prepared by acylation of the commercially available amine (Aldrich Chemical Co.) followed by sodium tungstate oxidation after the method of Hamilton and McConnell (1968).

(1-Oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)carbinol (Tempyro-carbinol, IV). The alcohol was prepared by reduction of the precursing carboxylic acid (Rozantzev and Krinitzkaya, 1965) with lithium aluminum hydride, and was a gift from Dr. B. G. McFarland.

N-(1-Oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)bromoacetamide (Tempyro-bromoacetamide, V). The alkylating reagent was prepared by acylation of the precursing amine with bromoacetyl bromide according to the procedure of Morrisett (1969).

Preparation of SL-Ser-195-chymotrypsin. α-Chymotrypsin was labeled at serine-195 with 1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmethyl phosphonofluoridate by the method of Morrisett and Broomfield (1971).

Preparation of 3-SL-His-15-lysozyme. Lysozyme was labeled at N-3 of histidine-15 with the bromoamide spin label IV by a modification of the method described by Piszkiewicz and Bruice (1968). In 1.0 ml of 0.1 M sodium acetate (pH 5.5) was dissolved 70 mg of lysozyme. The solution was adjusted to pH 5.1 with 1 M HCl and then 13.9 mg of IV (Morrisett, 1969) was added. This mixture was stirred at 40° for 36 hr. Initially a fine suspension of the label formed, but within 12 hr the solution became homogeneous. The reaction mixture was then passed through a column (1.2 × 40 cm) of Sephadex G-10 equilibrated with 0.1 M ammonium formate. Protein-containing fractions were pooled, shell frozen, and lyophilized. This material contained 1.25 (average of three preparations) spin labels per lysozyme molecule. The white powder was dissolved in 1 ml of 0.2 M sodium phosphate (pH 7.18) and chromatographed on a column (1.2 × 50 cm) of Bio-Rex 70, −400 mesh (Bio-Rad Laboratories). (Elution profile available on request.) The major (first) peak contained 79% of the total protein eluted from the column. This material exhibited an activity of 66% against *Micrococcus lysodeikticus* cells (Worthington Biochemical Corp.). Amino acid analysis indicated loss of the single histidine residue. No 1-carboxymethylhistidine was detectable, but the integrated value for half-cystine was elevated by about 0.5 residue. Since 3-carboxymethylhistidine elutes at the same position as half-cystine, we conclude that this major peak is lysozyme alkylated on nitrogen-3 of the imidazole ring of histidine-15. The electron paramagnetic resonance spectrum of this material indicated the label was moderately immobilized, the distance between the outermost wings of the spectrum being about 35 G. This material contained 1.02 spin labels/enzyme molecule.

Methods

Protein concentration was determined on a Cary 15 spectrophotometer by measuring the absorption at 280 mμ, using a molar extinction coefficient of 3.85×10^4 (Murachi *et al.*, 1970). This value was not significantly changed by covalently attaching the Tempyro-methyl moiety.

Activity measurements were performed according to a modification of the procedure described in the Worthington technical brochure (Worthington Biochemical Corp.). The enzyme solution (0.1 mg/ml) and substrate solution of *M. leisodeikticus* cells (0.1 mg/ml) were made up in 0.05 M sodium acetate buffer (pH 4.5). Decrease in absorption at 450 mμ was measured as a function of time.

Samples for magnetic resonance measurements were prepared by dissolving the lyophilized protein in D_2O (Stohler Isotope Chemicals, 99.8% pure), adding a known volume of a stock spin label or inhibitor solution (made up in D_2O), adjusting the pH to 4.5 with 1 M DCl or 2 M NaOD, then transferring the solution to a conventional 5-mm nuclear magnetic resonance (nmr) tube.

The pH of solutions was measured at room temperature with an Instrumentation Laboratory Inc. meter equipped with a Beckman 39030 combination electrode standardized against Coleman pH 4.01 standard buffer. All pH values reported have been read directly from the meter (pD = meter reading +0.4, Glasoe and Long, 1960).

Nuclear magnetic resonance measurements were performed using a Varian HA-100 nmr spectrometer. Transverse nuclear relaxation rates T_2^{-1} were estimated from nuclear resonance line widths $\Delta\nu_{1/2}$

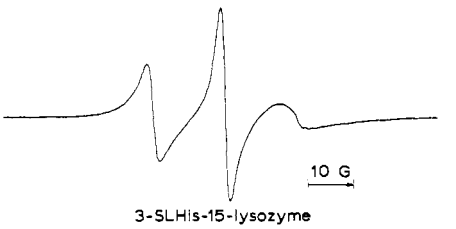

FIGURE 2: Electron paramagnetic resonance spectrum of lysozyme (~1%, w/v) spin labeled at N-3 of histidine-15 with Tempyro-bromoacetamide (3-SL-His-15-lysozyme).

$$T_2^{-1} = \pi\Delta\nu_{1/2} \quad (1)$$

Here $\Delta\nu_{1/2}$ is the nuclear resonance line width (full width at half-height). Equation 1 applies to Lorentzian line shapes, without inhomogeneous line broadening. It was difficult to check the validity of this assumption with the HA-100 instrumentation. For the line-width measurements the spectrometer was operated in the frequency sweep mode while locked on a concentric capillary of tetramethylsilane. This external standard was inadequate for determining *accurate* chemical shifts because of the difficulty in properly correcting each solution for its change in diamagnetic susceptibility caused by addition of the paramagnetic spin labels.

Electron paramagnetic resonance spectra were recorded on samples contained in 100-μl glass capillaries mounted in the variable-temperature accessory of a Varian E-4 spectrometer operated at 9.5 GHz. The concentration of nitroxide was determined by comparing the peak-to-peak height of the low field line of the spectrum of the sample with that of a standard solution of known concentration. In all cases the standard contained the same nitroxyl ring moiety as the sample. Estimation of binding constants for the spin-labeled inhibitors was made using

$$K_a = \frac{[SL_t] - [SL_u]}{[SL_u][E_t] - ([SL_t] - [SL_u])} \quad (2)$$

where SL_t is the total concentration of spin label, SL_u is the unbound spin-label concentration, and E_t is the total enzyme concentration.

A model of lysozyme constructed from Phillips' coordinates (Phillips, 1967) was used to measure the distance between groups on the enzyme. This enzyme model was constructed at Harvard University by Peter Urnes using Kendrew molecular models. Distances on this lysozyme model are compared directly to those obtained from nmr experiments in the present paper.

Theoretical Development

Relaxation times of nuclei in paramagnetic solutions are often affected by electron spin–nuclear spin interactions. Solomon (1955) and Bloembergen (1957) have published treatments of the influence of paramagnetic ions on nuclear relaxation resulting from (1) the nuclear spin–electron spin magnetic dipole–dipole interaction, which depends on nuclear spin–electron spin distance and from (2) isotropic

TABLE I: Experimental Parameters Used in Distance Calculations.

Mixture Components[a]	Resonance Line	Obsd Line Width[b]	Broadening Due to Uncomplexed Nitroxide[b]	Initial Line Width[b]	Net Broadening	K_a	f_M	Calcd r (Å)[e]	Model r (Å)[f]
3-SL-His-15-lysozyme (2 mM) + α-NAG (12 mM)	Acetamido methyl	2.4	0.4[g]	1.2[d]	0.8	50[i]	0.05	15	(18)[j]
β-NAG (8 mM)		3.6	0.4[g]	1.2[d]	2.0	50[i]	0.05	13	(18)[j]
3-SL-His-15-lysozyme (2 mM) + di-NAG (2 mM)	Nonreducing acetamido methyl	8.4	0	5.3[d]	3.1	5000[i]	0.725	18	(28)[k]
Lysozyme (7 mM) + Tempo-acetamide (14 mM)	C-2 proton of His-15	8.0	2.0[h]	4.0	2.0	33[c]	0.286	17	18
Lysozyme (2 mM) + Tempo-acetamide (8 mM) + di-NAG (25 mM)	Nonreducing acetamido methyl	2.6	$\leq$0.2	2.4[d]	$\leq$0.2	5000[i]	0.016	$\geq$15	28
Lysozyme (7 mM) + NAG-CH_2-Tempyro (6 mM)	C-2 proton of His-15	5.0	0	4.0	1.0	185[c]	0.393	20	21
Lysozyme (7 mM) + NAG-NAG-CH_2-Tempyro (3 mM)	C-2 proton of His-15	4.8	0	4.0	0.8	150[c]	0.20	19	21

[a] Components were dissolved in D_2O; the solution was adjusted to pH 4.5. [b] Given in hertz. [c] Given as M^{-1} and determined by paramagnetic resonance. [d] This is the line width of the saccharide in the absence of the nitroxide. For example, in experiments employing 3-SL-His-15-lysozyme, the native enzyme was substituted. [e] These values were calculated using eq 15 and 11 and the assumptions discussed in the Results section. [f] These values were determined by measurements on a Kendrew model of lysozyme built according to the coordinates of Phillips (1967). [g] These values were estimated from line-width measurements of NAG in the presence of SL-Ser-195-chymotrypsin to which the saccharide does not bind. [h] This value was estimated by substituting Tempyro-carbinol (IV) for Tempo-acetamide. The alcohol does not bind to lysozyme. [i] Values reported by Chipman and Sharon (1969). [j] This distance (18.1 Å) is the model distance between C-2 proton of His-15 and the acetamido methyl of α-NAG in subsite C, and cannot be exactly equal to the calculated distance (13.8 or 11.4 Å). See text. [k] This distance (28.4 Å) is the model distance between the C-2 proton of His-15 and the nonreducing acetamido methyl of di-NAG in subsite B, and cannot be exactly equal to the calculated distance of 18 Å. See text.

nuclear spin–electron spin coupling. The Solomon equation for the dipolar-induced transverse relaxation is

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)\gamma_I^2 g^2\beta^2}{r^6}\left[4\tau_c + \frac{\tau_c}{1+(\omega_I-\omega_S)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{6\tau_c}{1+\omega_S^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_I+\omega_S)^2\tau_c^2}\right] \quad (3)$$

Here τ_c is the correlation time for the dipolar interaction, S is the electronic spin quantum number, γ_I is the nuclear magnetogyric ratio, g is the electronic g factor, β is the Bohr magneton, r is the distance between the electron and the nucleus, ω_S is the electronic Larmor precession frequency, and ω_I is the nuclear Larmor precession frequency (radians per second). This equation was derived under the assumptions that the unpaired electron spin moment is a fixed distance from the nucleus and that the vector connecting those spins undergoes isotropic rotational diffusion. We shall refer to $1/T_{2M}$ as the nuclear relaxation rate in the "IS complex." By "IS complex" is meant the molecular complex that is formed when the paramagnetic species (*e.g.*, a spin-labeled enzyme or spin-labeled inhibitor) combines with the molecular species containing the nucleus of interest (*e.g.*, inhibitor or enzyme).

In our experiments, the unpaired electron of the nitroxides, and the nucleus of interest, are widely separated when they form the IS complex. Thus, we omit terms corresponding to relaxation brought about by the isotropic spin-exchange interaction. Further, in our experiments $\omega_S \gg \omega_I$, and τ_c is sufficiently large that $\omega_S^2\tau_c^2 \gg 1$. Under these conditions the nuclear relaxation rate in the IS complex is simply

$$\frac{1}{T_{2M}} = \frac{S(S+1)\gamma_I^2 g^2\beta^2}{15r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right] \quad (4)$$

Clearly, if T_{2M} and τ_c are known, the distance r can be determined. As will be discussed later, τ_c in this equation is equal to the rotational correlation time of lysozyme, which is 10^{-8} sec (Dubin *et al.*, 1971). A convenient expression for r (expressed in Å) in terms of T_{2M} (expressed in seconds) is

$$r = 28.11(T_{2M})^{1/6} \quad (5)$$

when $\tau_c = 10^{-8}$ sec, and the nuclear resonance frequency is 100 MHz. In the remainder of this section we consider the problem of determining T_{2M}. This problem arises from the fact that in general the IS complexes form and dissociate, and the observed nuclear relaxation rate depends on the nuclear relaxation rate in the IS complex, on the nuclear relaxation rate when the nucleus is not in the IS complex, and on certain dynamic effects that the exchange process has on relaxation rates.

It is possible to modify the Bloch equations for nuclear magnetic resonance to include this effect of chemical exchange of nuclei between paramagnetic and diamagnetic environ-

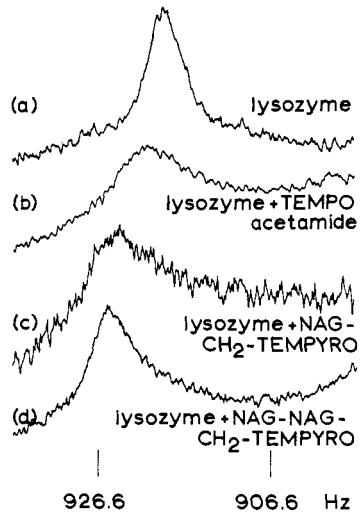

FIGURE 3: Proton magnetic resonance spectrum of the C-2 proton on histidine-15 of 7 mM lysozyme at pH 4.5 (a) alone (653 CAT scans), (b) plus 14 mM Tempo-acetamide (1500 CAT scans), (c) plus 6 mM NAG-CH_2-Tempyro (500 CAT scans), (d) plus 3 mM NAG-NAG-CH_2-Tempyro (850 CAT scans). Vertical lines indicate chemical shift (hertz) from external Me_4Si.

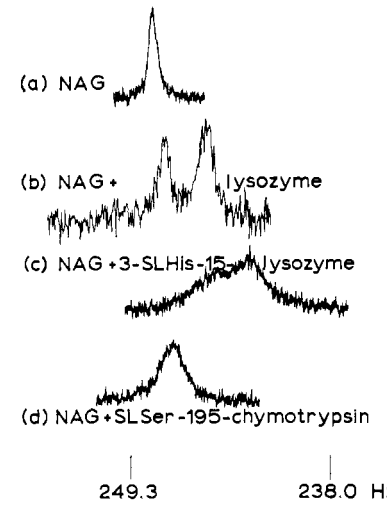

FIGURE 4: Proton magnetic resonance spectrum (methyl resonance) of 20 mM NAG at pH 4.5 (a) alone (single scan), (b) plus 2 mM lysozyme (3 CAT scans; upfield (downfield) line is due to the $\alpha(\beta)$ anomer, (c) plus 2 mM 3-SL-His-15-lysozyme (single scan), (d) plus 2 mM SL-Ser-195-chymotrypsin (single scan). Vertical lines indicate chemical shift (hertz) from external Me_4Si.

ments (McConnell, 1958). If the nucleus of interest is transferred by chemical exchange back and forth between two distinct environments, A and M, then the nuclear resonance spectrum can be calculated from the following expression for the complex magnetization $\hat{M}$

$$\hat{M} = -\gamma H_1 M_0 \frac{f_A f_M \left\{\left[(\omega - \bar{\omega}) - i\left(\frac{f_A}{T_{2A}} + \frac{f_M}{T_{2M}}\right)\right] - \left[(\omega_M - \bar{\omega}) - \frac{i}{T_{2M}}\right] - \left[(\omega_A - \bar{\omega}) - \frac{i}{T_{2A}}\right]\right\} + \frac{i}{\tau}}{\left[f_A(\omega - \omega_A) + i\left\{\frac{f_A}{T_{2A}} + \frac{1}{\tau}\right\}\right]\left[f_M(\omega - \omega_M) + i\left\{\frac{f_M}{T_{2M}} + \frac{1}{\tau}\right\}\right] + \frac{1}{\tau^2}} \quad (6)$$

In this equation, $\hat{M}$ = the complex nuclear magnetization; this is equal to $u + iv$, where u is the in-phase nuclear magnetization, and v is the out-of-phase magnetization; γ = the magnetogyric ratio of the proton; M_0 = the equilibrium nuclear magnetization; H_1 = amplitude of the in-phase rotating component of the radiofrequency field; f_M = the fraction of the time the proton in question is in the IS complex, and $f_A = 1 - f_M$; ω_M = angular resonance frequency of the proton in the IS complex; ω_A = angular resonance frequency of the proton when not in the IS complex; $\tau = \tau_A + \tau_M$, where τ_A^{-1} is the probability per unit time that the proton in question forms IS complex, and τ_M^{-1} is the probability per unit time that this complex dissociates; $\bar{\omega} = f_A\omega_A + f_M \cdot \omega_M$. T_{2M} and T_{2A} are the transverse nuclear relaxation times when the proton is in the IS complex, and when the proton is not in the IS complex, respectively. Equation 6 can be derived from the Bloch equations for nuclear magnetic resonance, modified to include the effects of chemical exchange (McConnell, 1958; Carrington and McLachlan, 1967). Equation 6 is applicable to the case of slow passage and no radiofrequency saturation. In the present work, the term "fast exchange" refers to conditions for which

$$\frac{1}{\tau} \gg \frac{f_A}{T_{2A}}, \frac{f_M}{T_{2M}} \quad (7)$$

$$\frac{f_A}{T_{2A}} + \frac{f_M}{T_{2M}} \gg \tau f_A^2 f_M^2(\omega_A - \omega_M)^2 \quad (8)$$

Under these conditions of fast exchange the observed transverse relaxation time is

$$\frac{1}{T_2} = \frac{f_A}{T_{2A}} + \frac{f_M}{T_{2M}} \quad (9)$$

The fast exchange limit considered here is *not* the same as that used in earlier estimates of intramolecular distances using enhanced nuclear relaxation due to spin labels and paramagnetic metal ions (Mildvan and Cohn, 1970; Mildvan and Weiner, 1969. These earlier studies make use of an approximation introduced by Swift and Connick (1962) and Luz and Meiboom (1964), $\tau_M^{-1} \gg \tau_A^{-1}$. This approximation is not appropriate in the present work, where in some cases $\tau_M^{-1} < \tau_A^{-1}$. Inequalities (eq 7) can be written in an equivalent form, $\tau_M \ll T_{2M}$, and $\tau_A \ll T_{2A}$, the first of which is the definition of fast exchange in the work of Swift and Connick (1962), Luz and Meiboom (1964), and later applications to biophysical problems.

Analysis of Data

Determination of T_{2M}. Table I gives the results of experiments utilizing the enhanced nuclear resonance line widths to calculate distances between sites on the lysozyme molecule. Examples of the observed broadenings are shown in Figures 3, 4, and 5. The captions to these figures give the experimental conditions for each measurement.

The resonance line widths in Table I are analyzed as follows. First, the resonance line shapes are taken to be Lorentzian, with the relation between line width (at half-height) and re-

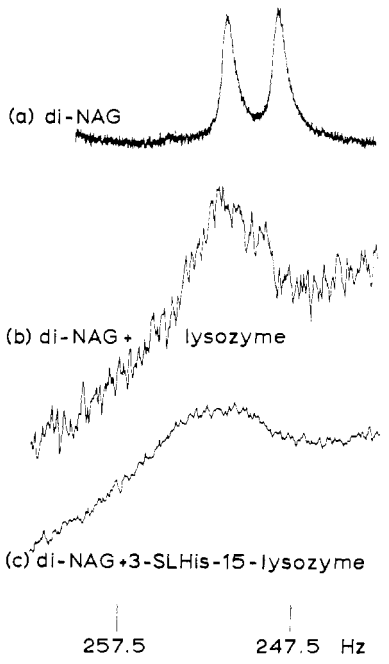

FIGURE 5: Proton magnetic resonance spectrum (methyl resonances) of di-NAG at pH 4.5: (a) 0.1 M di-NAG (single scan); reducing methyl resonance is to the right hand side of the nonreducing methyl resonance; (b) 2 mM di-NAG plus 2 mM lysozyme (400 CAT scans); reducing methyl resonance is broadened beyond detection; (c) 2 mM di-NAG plus 2 mM 3-SL-His-15-lysozyme (1400 CAT scans); only the nonreducing acetamido methyl resonance is observable. Vertical lines indicate chemical shift (hertz) from external Me_4Si.

laxation rate that is given in eq 1. Second, each of the relaxation rates, T_{2A} and T_{2M} (as well as the corresponding Lorentzian line widths), can be thought of as having both paramagnetic and diamagnetic contributions.[2] That is, eq 9 can be written as[3]

$$\frac{1}{T_2} = \left(\frac{f_M}{T_{2M}}\right)_p + \left(\frac{f_A}{T_{2A}}\right)_p + \left(\frac{f_M}{T_{2M}}\right)_d + \left(\frac{f_A}{T_{2A}}\right)_d \quad (10)$$

The first term on the right-hand side of eq 10 is the contribution of the electron spin magnetic moment to the nuclear relaxation when the proton of interest is in the IS complex. The second term is the contribution of the electron spin to the nuclear relaxation when the proton of interest is not in the IS complex. The sum of the last two terms on the right hand side of eq 10 then represents all other contributions to the nuclear relaxation. These other ("diamagnetic") contributions, expressed as Lorentzian line widths, are given in Table I under the column entitled initial line width. The second term in eq 10, $(f_A/T_{2A})_p$, is given in Table I under the heading of broadening due to uncomplexed nitroxide. Since the observed line width corresponds to $1/T_2$, the net broadening in Table I determines T_{2M}

$$\text{net broadening} = \frac{f_M}{\pi T_{2M}} \quad (11)$$

The footnotes to Table I give the methods used to determine, or estimate, the broadening due to uncomplexed nitroxide and the initial line width. Equations 5 and 11, together with the values of f_M and the net broadening given in Table I, yield the calculated distances given in this table.

Fast Exchange Is a Valid Assumption for Spin-Labeled Inhibitors of Lysozyme. For all distance calculations, we have assumed there is fast exchange between the small molecules bound to the enzyme and those free in solution. The values of τ_M for NAG and di-NAG have been determined by nuclear resonance methods to be of the order of 10^{-4} and 10^{-3} sec, respectively (Sykes and Parravano, 1969). Since the "on" rate constants (k_1) for NAG and di-NAG are nearly the same (Sykes and Parravano, 1969), the large difference in their association constants (50 M^{-1} for NAG and 5000 M^{-1} for di-NAG) is due to different "off" rate constants (k_{-1}). If the "on" rate constants for NAG-CH_2-Tempyro, NAG-NAG-CH_2-Tempyro, and Tempo-acetamide are the same as the "on" rate constants for NAG, the "off" rates for these labels are 3×10^{-4} and 8×10^{-5} sec, respectively. For all the experimental conditions given in Table I, the conditions for "fast exchange" in eq 7 and 8 are satisfied. The only exception to this statement occurs for the nuclear magnetic resonance of the nonreducing acetamide. In that case f_A/T_{2A} is only a factor of two less than τ^{-1}. This is of no significance to our conclusions since only a crude lower limit to the corresponding distance was obtained from this particular experiment. The above order-of-magnitude estimates of the rate constants can also be used to show that the frequency shift correction terms to eq 9 are negligible. This can be seen by noting first of all that the "pseudocontact" shift (Robertson and McConnell, 1958) is only of the order of 1 or 2 Hz when the electron and proton are separated by 10 Å. Shifts of this magnitude make a totally negligible contribution to the frequency correction term. Other paramagnetic contributions to resonance line positions act equally, or nearly equally, both in the IS complex and when the proton of interest is not in the IS complex; thus these paramagnetic contributions to the resonance positions tend to cancel in the frequency shift correction. Finally, ordinary chemical shift terms make a negligible contribution to the frequency shift correction terms.

τ_c Is Dominated by τ_2. The correlation time, τ_c, which characterizes the process that modulates the dipolar interaction, is given by the equation

$$\frac{1}{\tau_c} = \frac{1}{\tau_2} + \frac{1}{\tau_S} + \frac{1}{\tau_M} \quad (12)$$

where τ_2 is the correlation time characteristic of the rotational motion of the vector between the spin label and the nucleus, τ_S is the electronic spin relaxation time, and τ_M is the residence time of the nuclear species in the IS complex. Hence τ_c will be determined by whichever time is shortest: τ_2, τ_S, τ_M. The evidence presented below leads to the conclusion that τ_2 determines τ_c.

The rotational correlation time of lysozyme (τ_2) is 1×10^{-8}

[2] The separation of the relaxation rate T_{2M}^{-1} into paramagnetic and diamagnetic contributions is valid as long as the paramagnetic relaxation rate is fast compared to τ_M^{-1} and T_{2M}^{-1} itself, which is the case considered here. Otherwise there can be nonvanishing cross terms ("interference terms") between electron-induced and nuclear-induced proton relaxation of a particular proton, since both processes are governed by the same correlation time, determined by rotational diffusion of the enzyme.

[3] Alkylation of histidine-15 with V decreases the net charge of the enzyme by 1. This charge may well affect the binding of *Micrococcus lysodeikticus* cells without affecting the binding of the small saccharides.

sec (Dubin *et al.*, 1971).[4] The exchange time, τ_M, for lysozyme inhibitors is $\geq 10^{-5}$ sec which is much longer than τ_2. The electronic relaxation time, τ_S, for Fremy's salt (an inorganic nitroxide) is 3.4×10^{-7} sec (Kooser *et al.*, 1969). A power saturation experiment on 3-SL-His-15-lysozyme was carried out by Dr. R. C. McCalley.[5] Calculations based on data obtained from this experiment allow us to set a lower bound on the relaxation time of a nitroxide covalently bound to lysozyme: $\tau_S > 1.4 \times 10^{-7}$ sec. The lower limit on the paramagnetic relaxation time is set by the condition that $\tau_S > 1/\gamma_e H_{1/2}$ where γ_e is the magnetogyric ratio for the electron, and $H_{1/2}$ is the value of the in-phase rotating component of the microwave oscillatory field for which the observed paramagnetic resonance signal intensity is reduced to one-half the value which would prevail at that power level without saturation. This inequality takes into account the possibility that the paramagnetic resonance signal is inhomogeneous, and assumes that $T_1 \geq T_2$. The lower bound is 14 times greater than the rotational correlation time (τ_2) of lysozyme.

At concentrations of nitroxide $\gtrsim 10^{-3}$ M, spin exchange causes detectable broadening of the paramagnetic resonance line. Spin exchange produces an effective shortening of τ_S. For example, a solution of 7 mM (14 mM) Tempo-acetamide plus 3.5 mM (7 mM) lysozyme had a low field line width of 2.1 G (3.2 G). In present work two possible pitfalls are encountered in using high concentrations of nitroxides; the first concerns the possibility that τ_S may become shorter than τ_2. If we take 3.2 G as the peak-to-peak derivative curve width of the widest line observed in such experiments, we obtain

$$8.96 \times 10^6 \lesssim \frac{1}{3\pi\tau_S}$$

$$\tau_S \gtrsim 2.2 \times 10^{-8} \text{ sec}$$

This value is a lower limit on τ_S. All other labels employed in these experiments were used in much lower concentrations. For such cases one expects τ_S to be larger. A second problem encountered in using nitroxide concentrations of the order of 10^{-3} M concerns diminution of paramagnetic resonance peak intensity due to exchange broadening. For example, a 1.0 mM solution of Tempyro-carboxylic acid has a peak intensity only eight times that obtained from a 0.1 mM solution. For this reason all peak-height measurements used in calculating association constants were performed at a concentration of 10^{-4} M or lower.

Discussion

Measurements Using a Spin Label Covalently Bound to Lysozyme. When histidine-15 of lysozyme is labeled with Tempyro-bromoacetamide (V), the resonance spectrum is perturbed to such an extent that no fine structure remains, and only broad envelopes are observed. Addition of ascorbic acid, which reduces the paramagnetic nitroxide (Kornberg and McConnell, 1971), causes the reappearance of the fine structure that is seen in the unlabeled enzyme. These experiments afford no quantitative data concerning distances between specific protons and the covalently linked nitroxide. Experiments involving the labeled enzyme and chemically exchanging inhibitors are fruitful, however. Figure 4 shows that when NAG is bound to 3-SL-His-15-lysozyme, the two methyl peaks (corresponding to the α and β anomers) are differentially broadened; the β resonance (left-hand side in Figure 4c) is broadened 2.0 Hz more than that observed with NAG in the presence of native lysozyme, while the α resonance is broadened only 0.8 Hz more than that observed with NAG in the presence of native lysozyme. The calculated distance (using eq 5) between the free electron on the nitroxide attached to histidine-15 and the acetamido methyl group of α- and β-NAG is 15 and 13 Å, respectively. That the methyl group on each of the two anomers is broadened differently is in accord with the X-ray crystallographic results of Phillips (1967) which indicates that the two anomers bind in different configurations. However, measurements on the enzyme model indicate that an acetamido methyl group on a NAG moiety in subsite C[6] is 18 Å from the middle of the imidazole ring of histidine-15. Clearly, when the Tempyro-acetamido moiety is attached at N-3 of this ring, the nitroxide may be oriented so that the free electron and the center of the ring are not equidistant from the methyl groups. Model studies show that the nitroxyl ring can assume two extreme orientations, one with the ring sticking out into the solvent, and the other with the ring pointed inward and occupying a hydrophobic pocket. Intermediate orientations are also possible. The paramagnetic resonance spectrum (Figure 2) of 3-SL-His-15-lysozyme corresponds to an apparent rotational correlation time that is less than 3×10^{-9} sec. Even in a solution of 15% lysozyme the apparent correlation time for the label at His-15 is certainly less than 5×10^{-9} sec. (These estimates of correlation times are based on recently developed methods of line-shape analysis. See McCalley *et al.* (1972). We are indebted to E. Shimshick for obtaining the resonance spectrum of 3-SL-His-15-lysozyme in 15% w/v lysozyme solution.) Since at this lysozyme concentration (15%) the rotational correlation time for the enzyme is 10×10^{-9} sec (Dubin *et al.*, 1971), there must be some motion of the label relative to the protein. This, of course, does not preclude the label being largely localized in the hydrophobic pocket mentioned above. Moreover, it will be seen that if eq 4 and 5 are used to calculate a distance r in a case where r rapidly takes on a distribution of values, the apparent r will be weighted heavily toward the value of r corresponding to the distance of closest approach. The observed results are then consistent with but do not prove the view that the nitroxide is oriented inward and interacts with other moieties in its vicinity. In this case, the free electron-bearing nitrogen is about 7 Å (the length of the Tempyro-acetamido group) closer to the methyl groups than the imidazole ring. Thus the distance (measured on the model) from the free electron to the methyl group of NAG in subsite C would be about 11 Å. This compares favorably to the present estimated values of 15 Å (α anomer) and 13 Å (β anomer).

An X-ray study of the di-NAG–lysozyme complex has shown that the disaccharide binds in subsites B and C. The sugar moiety on the reducing end is bound in subsite C (Johnson and Phillips, 1965a,b) and its acetamido methyl group is located over the plane of the indole ring of tryptophan-108. The nonreducing terminus is located in subsite B and its

[4] This value was determined by the depolarized light-scattering technique using an enzyme concentration of 15% and pH 4.2 (Dubin *et al.*, 1971).

[5] Dr. Roderick C. McCalley has made a more detailed analysis of the saturation behavior of histidine-15-labeled lysozyme and finds that the central line is nearly homogeneous and that τ_S is about 3.5×10^{-7} sec (private communication).

[6] Subsite C of the model is occupied by one NAG unit of a hexasaccharide placed in the active site.

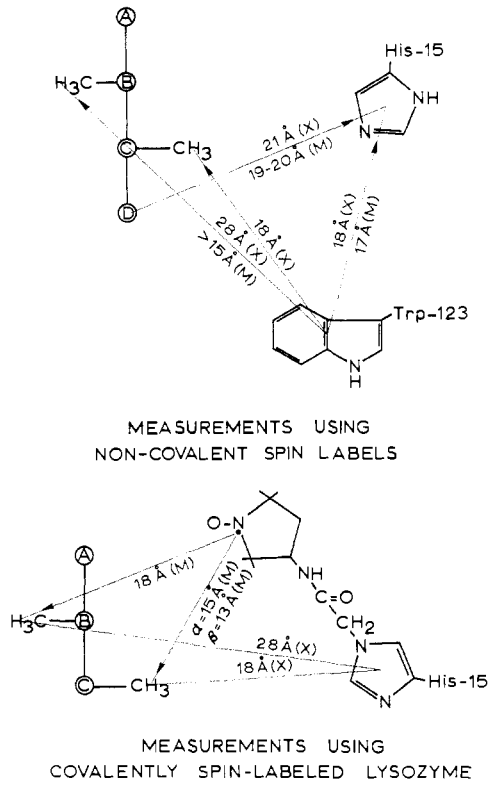

FIGURE 6: Diagram comparing distances between groups on lysozyme as determined by measurements on a model based on X-ray diffraction data (X) and determined from proton magnetic resonance line broadening experiments (M). Lines point *from* the site of the spin label *to* the relaxed proton(s) being observed. In the upper diagram, the paramagnetic effect arises from either Tempo-acetamide bound near tryptophan-123 or the Tempyro moiety held in subsite D. The proton resonance lines arise from the C-2 proton line of His-15 in one case, and from the acetamide methyl groups of di-NAG (or NAG) bound in subsites B and C (or C alone) in the other cases. For the lower diagram, the paramagnetic effect originates at the Tempyro-acetamido moiety attached to histidine-15. The observed resonances are those of the acetamido methyl groups of di-NAG (or NAG) bound in subsites B and C (or C alone). The probable distance between the methylene (attached to N-3 of His-15) and the nitroxyl nitrogen is of the order of 7 Å.

methyl group is oriented away from the enzyme, into solution. Based on these data one might expect the resonance line of the reducing methyl to be broadened more than that of the nonreducing methyl because of greater motional constraints imposed on the former. Thomas has confirmed this expectation by using deuterated analogs of di-NAG (Thomas, 1967). For the spectrum of di-NAG (Figure 5a), the upfield (right-hand) methyl resonance has been assigned to acetamido protons on the reducing end, and the downfield resonance corresponds to the nonreducing acetamido protons. Upon addition of lysozyme, the reducing methyl is shifted upfield and broadened. The nonreducing methyl is not shifted and is broadened less. Both native and spin-labeled lysozyme broadened the methyl resonances to the same extent for di-NAG:enzyme ratios from 50:1 to 10:1. In order to obtain a measurable difference in line broadening, a di-NAG:enzyme ratio of 1:1 was required. Under these conditions, the reducing methyl resonance line was broadened beyond detection. However, the nonreducing resonance was broadened 3.1 Hz more in the sample containing spin-labeled lysozyme than in that containing the native enzyme (compare Figure 5b,c). The calculated distance between the nonreducing methyl group and the free electron is 18 Å. The measured distance (from the model) between the nonreducing methyl group and the imidazole of histidine-15 is 28 Å. If this value is corrected for the length of the label as described above, this distance is reduced to the order of 21 Å.

Measurements Using Spin Labels Noncovalently Bound to Lysozyme. For "distance-measuring" experiments in which the nitroxide is noncovalently bound and the observed resonance line is due to a nucleus on the protein, several properties of that line are required. (1) It must be narrow enough to allow a reasonably accurate width determination. (2) It should be measurably broadened by the noncovalently binding label. (3) Its assignment must be known. The C-2 proton of histidine-15 of lysozyme has a resonance line fulfilling these requirements.

The specificity of lysozyme for the *N*-acetyl side chain (Rupley *et al.*, 1967) suggested to us that a molecule such as Tempo-acetamide (III) might mimic NAG and bind selectively to the C subsite. Berliner (1971) has recently completed a 6 Å crystallographic study of the lysozyme–Tempo-acetamide complex. While the label does appear to be bound to subsite C, the occupancy is low. Furthermore, a similar weak binding site is found at subsite A. More significantly, the pseudosaccharide is more strongly bound at a third site located near tryptophan-123. Since this third site is as close or closer to histidine-15 than the other two sites, and since, on the average it is more highly occupied, the dominant paramagnetic effect originates at this site. A binding constant of 6 M^{-1} for Tempo-acetamide was determined by inhibition experiments using *Micrococcus lysodeikticus* cells. A value of 33 M^{-1} was obtained by paramagnetic resonance. The relative magnitude of these constants is consistent with the mode of binding described above. The resonance line of the C-2 proton of histidine-15 is measurably broader in the presence of Tempo-acetamide than in its absence (Figure 3). Because of the low binding constant for this label, relatively high concentrations ($\geq$7 mM) were required. Under these conditions the concentration of *unbound* label is large enough so that it broadens the histidine line, although not to the extent of bound label. Evaluation of the magnitude of this "bulk broadening effect" was accomplished by measuring the line width of the C-2 histidine resonance of the enzyme in the presence of Tempyro-carbinol (IV). This molecule does not bind to lysozyme. Thus, the net paramagnetic effect of Tempo-acetamide (III) on the line width was taken as the difference between the line width in the presence of Tempo-acetamide and Tempyro-carbinol. Using a net broadening of 2 Hz and the binding constant of 33 M^{-1}, the average distance between the electron bearing nitrogen atom and the C-2 proton was calculated to be 17 Å. Measurements from the model have set the distance from the center of the tryptophan-123 indole ring to the C-2 proton at 18 Å. As expected, the addition of a tenfold excess of NAG (K_a = 50 M^{-1}, Chipman and Schimmel, 1968) as well as a fourfold excess of di-NAG (K_a = 5000 M^{-1}, Chipman and Schimmel, 1968) did not reverse the net broadening effect of Tempo-acetamide. This result is also interesting in that it shows that (on binding NAG or di-NAG) lysozyme undergoes no appreciable conformation change that affects the (His-15)–(Trp-123) distance.

In a separate experiment, the distances between the strong Tempo-acetamide binding site and subsites B and C were estimated by measuring the change in line width of the acetamido methyl signals of di-NAG in the presence of lysozyme and Tempo-acetamide. No observable change in line width was observed, implying that the distance between either

methyl group and the nitroxide moiety of Tempo-acetamide is ≥ 15 Å. Measurements on the model indicate that the distance from the reducing and nonreducing methyl groups to the center of the indole ring of Trp-123 is 18 and 28 Å, respectively. The smaller lower limit of the distance at which the broadening effect on the resonance line may be observed is due to the low concentration of the ternary complex (di-NAG–lysozyme–Tempo-acetamide). Higher concentrations were prohibited by the low K_a for Tempo-acetamide.

Our initial aim in using Tempo-acetamide was to determine the distance from subsite C to histidine-15. Since this label did not strongly bind to subsite C, we were left with the single alternative of synthesizing spin-labeled inhibitors of lysozyme. The molecules of choice were spin-labeled glycosides of NAG and di-NAG with the six-membered *Tempo* moiety as the aglycone. For reasons of instability, these saccharides could not be prepared in satisfactory yield.[7] Hence we were forced to use the less desirable five-membered primary alcohol, Tempyro-carbinol (IV), in place of Tempo alcohol during the coupling reaction.

NAG-NAG-CH_2-Tempyro (I) and NAG-CH_2-Tempyro (II) bind to lysozyme with association constants of 150 and 185 M^{-1} respectively.[8] Based on the known mode of NAG and di-NAG binding (Blake *et al.*, 1967) we expect I to occupy subsites B, C, and D while II should occupy C and D. In both cases we believe the Tempyro ring to be located in the D subsite. The broadening of the C-2 proton of histidine-15 caused by the binding of NAG-CH_2-Tempyro (1.0 Hz) and NAG-NAG-CH_2-Tempyro (0.8 Hz) is illustrated in Figure 3c,d. Calculations based on the results with these labels indicate that the distance from the nitroxyl nitrogen to the C-2 proton is 20 Å for NAG-CH_2-Tempyro and 19 Å for NAG-NAG-CH_2-Tempyro. The measured model distance from the nitrogen of the label located in subsite D to the middle of the imidazole ring of histidine-15 is 21 Å.

In general we believe that there is excellent agreement between distances determined by X-ray crystallography and those obtained here by the enhancement of proton relaxation rates. In the three instances of proton relaxation of histidine-15, the magnetic resonance distances and X-ray distances are the same to within 10%. In the other three cases in Table I involving the spin label covalently attached to histidine-15, the distances determined by magnetic resonance, when corrected for flexibility of the spin-label group, are close to those estimated from the X-ray crystal structure (see discussion above). It is hoped that it will soon be possible to apply magnetic resonance methods to the determination of intramolecular distances on macromolecules in solution where these distances could not be guessed or estimated from biochemical arguments. This state of confidence in the method should be obtainable through the "overdetermination" of intramolecular distances, for example, by the Fourier transform determination of both relaxation times T_1 and T_2, and by the determination of correlation times by methods independent of the nuclear relaxation being studied (as was done in the present work). The agreement is generally better in those experiments employing spin-labeled inhibitors than in those where covalently labeled lysozyme is used. This is probably due largely to the uncertainty concerning the average position of the chemically attached nitroxyl ring.

In principle, one should be able to use spin-labeled substrates or inhibitors to measure the distance to a number of different nuclei on a protein. In practice, this requires that the nucleus of interest have a detectable resonance line. This is not always the case. For example, the C-2 protons of the two histidine residues of chymotrypsin are not easily detected because of their excessive line widths (R. W. Wien, J. D. Morrisett, and H. M. McConnell, unpublished results). However, several of the C-2 proton resonances of human carbonic anhydrase B, a protein of similar molecular weight, are readily detectable (King and Roberts, 1971).

It is clear that nitroxide spin labels can be used for distance measurements greater than those previously determined using paramagnetic metal ions and other spin labels. In the present work, we have estimated distances up to 20 Å. The limit of the method for perturbation of protons might well exceed this by 50%.

Acknowledgments

We are grateful to Dr. B. G. McFarland for the gift of compound IV, Dr. John Rupley for a gift of di-NAG, and Dr. Brian Sykes for making the distance measurements on the lysozyme model. We are especially indebted to Dr. Larry Berliner for making available to us before publication crystallographic data on the lysozyme–Tempo-acetamide complex. We thank Patria Oboza for performing the amino acid analyses. We are also indebted to Dr. Roderick C. McCalley and Mr. Edward J. Shimshick for their determination of the paramagnetic resonance spectra of histidine-15 spin-labeled lysozyme and for their determination of the electron paramagnetic relaxation rate of this spin label (TBTGA).

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[7] Attempts to prepare mannosyl-Tempo have also been unsucessful, apparently for the same reason (Mr. Terry Frey, private communication).

[8] These values were determined by paramagnetic resonance as described in the Methods section. The method assumes that bound labels will not make a significant contribution to the amplitude of the resonance line. In our case this assumption may not always be true; the Tempyro moiety may possess motional freedom independent of the saccharide group. The paramagnetic resonance spectrum of 3-SL-His-15-lysozyme suggests that even a covalently bound label may make a small contribution to the line amplitude by virtue of the small correlation time of lysozyme. For these reasons we feel the K_a values here represent lowest possible limits.

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A Calorimetric Study of the Interaction of Mn^{2+} with Glutamine Synthetase from *Escherichia coli*†

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ABSTRACT: Mn^{2+} is a specific activator and stabilizer of the dodecameric structure of glutamine synthetase from *Escherichia coli*. The interaction of Mn^{2+} with the unadenylylated form of this enzyme has been investigated calorimetrically in the present study. To measure proton release from the enzyme during metal ion binding at pH 7.17 (37°), Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and Tris-chloride buffers were used since these buffering compounds have quite different heats of protonation ($\Delta H = -4.8$ and -11 kcal per mole of H^+, respectively). Although complex thermokinetic curves were observed, the results are consistent with the following interpretation. During the binding of each of the first 12 equiv of Mn^{2+} ($K_{eq}' = 2 \times 10^6$ M^{-1}), two protons are displaced from the enzyme per mole of subunit of ~50,000 molecular weight (with measured values of ΔH differing by 13 kcal/mole subunit in the two buffers). One proton is released instantaneously and the second proton is released in a slow first-order process that has an apparent half-time at 37° of ~55 sec and at 25° of ~230 sec. The heats observed in the slow reaction are less than that due to protonation of either buffer, suggesting an endothermic process. The slow thermal process may be attributed to a conformational change in the protein, with the endothermic contribution not resolved from the associated slow release of one proton equivalent. Since very little net heat is associated with the interaction of Mn^{2+} (or Mg^{2+}) with glutamine synthetase, the binding of these divalent cations to the enzyme involves a rather large entropy increase. For the binding of each Mn^{2+} to unadenylylated glutamine synthetase at 37° and pH 7.2, the following thermodynamic parameters are indicated: $\Delta G' = -8.9$ kcal mole^{-1} (standard state for hydrogen ions at activity of $10^{-7.2}$ M), $\Delta H \simeq +3$ kcal mole^{-1}, and $\Delta S' \simeq +38$ cal deg^{-1} (mole of subunit-Mn)$^{-1}$.

Glutamine synthetase from *Escherichia coli* is a dodecameric aggregate of 12 apparently identical subunits (Woolfolk *et al.*, 1966) arranged molecularly in two superimposed hexagons (Valentine *et al.*, 1968). The enzyme has a molecular weight of 600,000 (Shapiro and Ginsburg, 1968).

A dominant control of glutamine synthetase activity in *E. coli* occurs through enzyme-catalyzed adenylylation and deadenylylation reactions, which in turn are regulated by intracellular metabolite concentrations (Stadtman *et al.*,

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