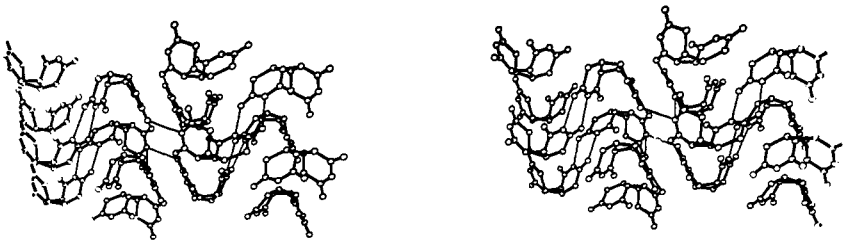

Figure 5. Stereodrawing of the contents of two unit cells. In the a axis, positive direction goes to the right. The c^* direction goes from bottom to top (from 0 to $2c$) and b goes into the page. Hydrogen bonds are represented by narrow lines.

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A Nuclear Magnetic Resonance Study of Lysozyme Inhibition. Effects of Dimerization and pH on Saccharide Binding

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Abstract: Nuclear magnetic resonance methods were used to study the interaction of *N*-acetyl-α- and -β-D-glucosamine with lysozyme. The association constant and bound chemical shift of the acetyl protons of each anomer have been determined as a function of pH. These results are interpreted in terms of the amino acid residues in the active site of lysozyme. In addition, several models for lysozyme dimerization are tested.

Important clues in elucidating the mechanism of action of an enzyme can be gained from a study of the pH dependence of substrate and inhibitor binding to the enzyme. Ribonuclease,[2a] α-chymotrypsin,[2b,c] and lysozyme,[3-6] for example, have received detailed study. In the case of lysozyme, both the rates of cleavage of polysaccharide substrates and the inhibition by saccharides are pH dependent.

Several workers have used ultraviolet spectroscopy methods to study the pH dependence of the inhibition of lysozyme by *N*-acetyl-α-D-glucosamine (α-NAG)[3] and tri-*N*-acetyl-D-glucosamine.[4-6] Their results show that a plot of the association constant *vs.* pH is bell shaped, indicating that at least two ionizable groups are involved in the lysozyme mechanism.

Nuclear magnetic resonance methods have also been used to study the interaction of inhibitors with lysozyme,[7-13] and Dahlquist and Raftery have studied the pH dependence of the β-methyl-*N*-acetyl-D-glucosaminide (β-MNAG) inhibition of lysozyme by these methods.[9] The advantage of nmr methods is that a study of the observed chemical shift or relaxation times of nuclei on the inhibitor molecule as a function of inhibitor and enzyme concentration at a given pH yields not only the association constant at that pH, but also the chemical shift or relaxation times of those nuclei in the enzyme–inhibitor complex. These parameters char-

(1) (a) Harvard University; (b) National Science Foundation Predoctoral Fellow; (c) Stanford University.

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acterize the environment of the bound inhibitor, and their magnitude and pH dependence can often be related to the details of the interactions involved.

The present paper is a nmr study of the pH dependence of the association constant and the bound chemical shift for the interaction of α-NAG with lysozyme and for the interactions of α- and β-NAG with lysozyme in a mutarotated solution of the two anomers. The results for the pH dependence of the α-NAG association constant are compared to those obtained by Kowalski and Schimmel,[8] and the various models proposed by Sophianopoulos[14] for the high pH dimerization of lysozyme are tested. The pH dependence of the association constant and the bound chemical shift for α-NAG and β-NAG are compared with the results for β-MNAG.[9] An interpretation of these results is presented based upon the effects of amino acid residues which have been shown to be in the active site by X-ray crystallography.[15]

Theory

The nuclear magnetic resonance spectrum of nuclei on an inhibitor molecule which rapidly exchanges between solution and the active site of an enzyme is the weighted average of its spectrum in solution and bound to the enzyme. In a solution of two mutarotated sugar anomers and an enzyme to which they bind, the general equation for the observed chemical shift, δ_{oi}, for a nucleus on one of the inhibitor molecules, is

$$\delta_{oi} - \delta_i = \frac{\Delta_{\text{eff},i} K_{\text{eff},i} Z}{1 + K_{\text{eff},i} Z} \tag{1}$$

where

$$Z = \frac{(E_0 - I_0 - (1/\bar{K}_{\text{eff}})) + \sqrt{(E_0 - I_0 - (1/\bar{K}_{\text{eff}}))^2 + 4E_0/\bar{K}_{\text{eff}}}}{2}$$

if only one inhibitor can bind to the enzyme at a time (Appendix I). δ_i is the chemical shift in the absence of enzyme, $i = \alpha, \beta$, and E_0 and I_0 are the initial concentrations of enzyme and inhibitor, respectively. No assumptions are made about the number of ionizable groups or binding sites on the enzyme. $\Delta_{\text{eff},i}$ and $K_{\text{eff},i}$ are appropriate weighted averages of the chemical shifts and association constants for the various sites and ionization states of the enzyme. $\bar{K}_{\text{eff}}$ is an average of the α and β K_{eff}'s, weighted by the fraction of each anomer present at equilibrium

$$\bar{K}_{\text{eff}} = p_\alpha K_{\text{eff},\alpha} + (1 - p_\alpha) K_{\text{eff},\beta}$$

If only one anomer is present, eq 1 reduces to

$$\delta_{oi} - \delta_i = \frac{\Delta_{\text{eff},i} K_{\text{eff},i} Z'}{1 + K_{\text{eff},i} Z'} \tag{2}$$

where Z' is equal to Z with $\bar{K}_{\text{eff}}$ replaced by $K_{\text{eff},i}$. This expression can be shown to be equivalent to the expression derived by Sykes and Parravano for $\delta_{oi} - \delta_i$.[13]

If the enzyme is able to form dimers, the effect of dimerization on δ_{oi} must be taken into account. We have treated three cases under the assumptions that: (1) there is only one ionization in the pH range where dimerization occurs, (2) only the deprotonated form is able to form dimers, and (3) the dimerization process either leaves an active site unaffected or blocks it completely (Appendix II). In the first case, in which both sites are unaffected, eq 1 or 2 applies. In the second case, where one site is unaffected and the other blocked, the appropriate equation for δ_{oi} when only one anomer is present is

$$\delta_{oi} - \delta_i = \frac{\Delta[\text{I}][\text{E}_0]K}{2[\text{I}_0](1 + [\text{I}]K)} + \frac{[\text{I}]}{[\text{I}_0]K_d} \times \left(\left\{1 + \frac{8[\text{E}_0]K_d(1 + [\text{I}]K)}{(1 + [\text{H}]C_{\text{H}})^2(1 + [\text{I}]K_{\text{eff}})^2}\right\}^{1/2} - 1\right) \times \left\{\frac{(1 + [\text{H}]C_{\text{H}})^2(1 + [\text{I}]K_{\text{eff}})^2}{4(1 + [\text{I}]K)}\right\} \times \left\{\frac{K_{\text{eff}}\Delta_{\text{eff}}}{1 + [\text{I}]K_{\text{eff}}} - \frac{\Delta K}{2(1 + [\text{I}]K)}\right\} \tag{3}$$

where K_d is the enzyme dimerization constant, [I] is the concentration of free inhibitor, [H] is hydrogen ion concentration, C_{H} is the ionization constant, and Δ and K are the bound chemical shift and association constant for the dimer site. If both sites are blocked, eq 2 applies with the substitution of

$$E_{0,\text{app}} = \frac{(1 + [\text{H}]C_{\text{H}})^2(1 + [\text{I}]K_{\text{eff}})^2}{4K_d} \times \left(\sqrt{1 + \frac{8[\text{E}_0]K_d}{(1 + [\text{H}]C_{\text{H}})^2(1 + [\text{H}]K_{\text{eff}})^2}} - 1\right) \tag{4}$$

for E_0 in that equation.

Computer programs are used to evaluate all data. In each case, the chemical shifts, $[E_0]$, and $[I_0]$ are read in, and parameters such as K_d are specified. The program calculates a least-squares fit to the observed chemical shifts for each K_{eff} within a specified range of trial values. The slope of the least-squares fit then gives Δ_{eff} for that K_{eff}. The trial K_{eff} which gave the smallest root-mean-square deviation was taken as K_{eff}. There was only one minimum in the computer fit of the data of our experiments.

Experimental Section

Materials and Methods. P-L Biochemicals lysozyme (lot LY-4; crystallized twice, dialyzed, and lyophilized) was used without further purification. α-NAG ($[\alpha]^{25}\text{D}$ +81°, lit.[16] +82°) was obtained from Aldrich Chemical Co. Citrate buffer (0.1 *M*) was used for all experiments, except those above pH 7.5 where 0.1 *M* phosphate buffer was used. Acetone was added as an internal standard (0.6% v/v). All pH's were measured at 32 ± 1° and after the enzyme, typically 25 mg/ml, had been added to the buffer solution.

All spectra were obtained on a Varian HA-100 spectrometer operating in the frequency sweep mode while locked on a concentric capillary of hexamethyldisilazane. The probe temperature of the spectrometer was 32 ± 1°.

The spectra of α-NAG were run quickly in order to minimize mutarotation to the equilibrium mixture of α- and β-NAG. The nmr tubes, enzyme solution, and weighed amounts of solid α-NAG were thermostated at the probe temperature before the run. For each data point, 1.0 ml of enzyme solution was then added to the solid α-NAG, part of the resulting solution put into a nmr tube, and the spectra of the α-NAG acetyl peak were taken at a specific time after the mixing of the enzyme and the α-NAG. This time was

(14) A. J. Sophianopoulos, *J. Biol. Chem.*, 244, 3188 (1969).

(15) (a) D. C. Phillips, *Proc. Nat. Acad. Sci. U. S.*, 57, 484 (1967); (b) D. C. Phillips and V. R. Sarma, *Proc. Roy. Soc., Ser. B*, 167, 378 (1967).

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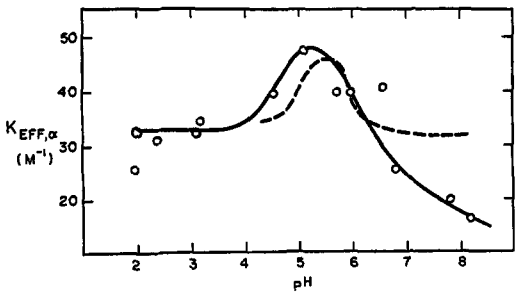

Figure 1. The association constant, $K_{eff,\alpha}$, for α-NAG *vs.* pH: ——, data analyzed using eq 2;– – –, results of Kowalski and Schimmel.[3]

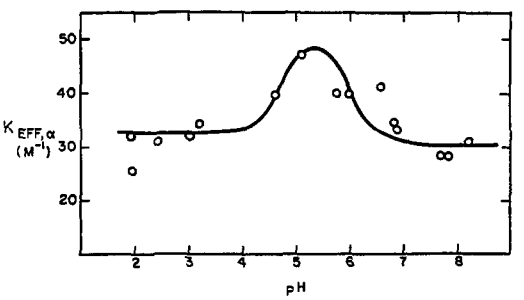

Figure 2. The association constant for α-NAG *vs.* pH, data analyzed using eq 3.

always less than 3 min. During this time no resonance corresponding to β-NAG was observed. The spectrum of acetone, the internal standard, was then taken at a constant time later.

The mutarotated solutions, prepared from α-NAG, were allowed to stand for at least 3 hr before each run. The relative concentrations of α- and β-NAG were determined at each pH from the measured optical rotations.

Lysozyme. Results

I. α-NAG and Lysozyme. The observed chemical shift of the acetyl resonance of α-NAG as a function of α-NAG and lysozyme concentrations was analyzed using eq 2. The results for the association constant of the α-NAG–lysozyme interaction are plotted as a function of pH in Figure 1. For comparision, the association constant curve reported by Kowalski and Schimmel[3] is also shown in Figure 1. Both curves are bell shaped, but the two deviate substantially at high pH. One expects to correct the results of the present work for dimerization at high pH, since the enzyme concentration is higher in the present work than in the ultraviolet spectroscopy work. Three models for dimer binding of α-NAG were tested (Appendix II). The results for the association constant, analyzed using eq 3, are presented in Figure 2. This brought the present results into agreement with those of Kowalski and Schimmel, while agreement was not obtained using eq 4. The value of the dimerization constant was taken from Sophianopoulos.[14] The plot of Δ_{eff} *vs.* pH obtained using eq 3 is shown in Figure 3.

II. Mutarotated Solutions. Figure 4 shows spectra of mutarotated solutions of approximately constant NAG and lysozyme concentrations at pH 2.05, 3.93, 5.51, and 7.69, showing the large differences between α- and β-NAG as a function of pH. Coincidence of the acetyl resonances of α- and β-NAG was observed at all concentrations of NAG and lysozyme at pH 2.05 and

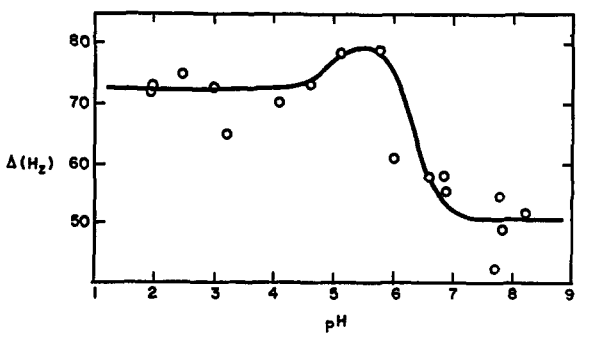

Figure 3. The bound chemical shift of the acetyl protons of α-NAG *vs.* pH.

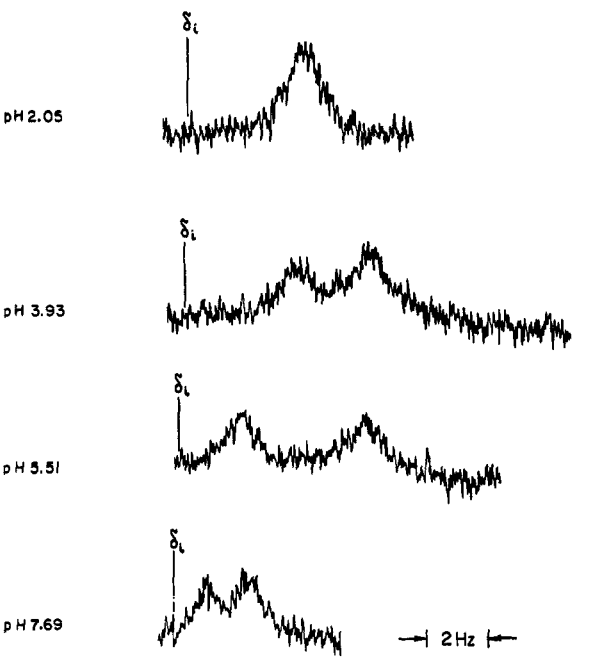

Figure 4. Typical spectra of acetyl resonances of α- and β-NAG in the presence of lysozyme as a function of pH; [NAG] $\simeq$ 0.015 *M*; [lysozyme] $\simeq$ 0.0025 *M*.

3.00. Association constants for β-NAG were calculated using the effective α-NAG association constants from Figure 2, the observed $\bar{K}_{eff}$ from an analysis of the data using eq 1, and the anomeric equilibrium constant measured from optical rotation data at each pH. The equation used is $K_{eff,\beta} = (\bar{K}_{eff} - p_\alpha K_{eff,\alpha})/(1 - p_\alpha)$. Putting $K_{eff,\alpha} = 31\ M^{-1}$ and the β shifts at low pH into eq 1, one obtains $K_{eff,\beta} = 31\ M^{-1}$ and $\Delta_{eff,\beta} = 73$ Hz. Thus, as expected from the peak coincidence at low pH, $K_{eff,\beta} = K_{eff,\alpha}$ and $\Delta_{eff,\beta} = \Delta_{eff,\alpha}$ come out of the mutarotated solution analysis. The values obtained for $K_{eff,\beta}$ and $\Delta_{eff,\beta}$ at pH 5.5 from the β shifts are 46 M^{-1} and 39 Hz, respectively. The same value of $K_{eff,\beta}$ was not obtained from the α shifts, however. The source of this discrepancy is not known, but may indicate some deviation from 1:1 binding of NAG to lysozyme. A detailed analysis of the high-pH binding of β-NAG was not attempted because of the complications arising from dimer formation. It is obvious however from the observed chemical shifts (Figure 4) that, as in the case of α-NAG, the association constant and/or bound chemical shift of β-NAG falls off sharply above pH 6.

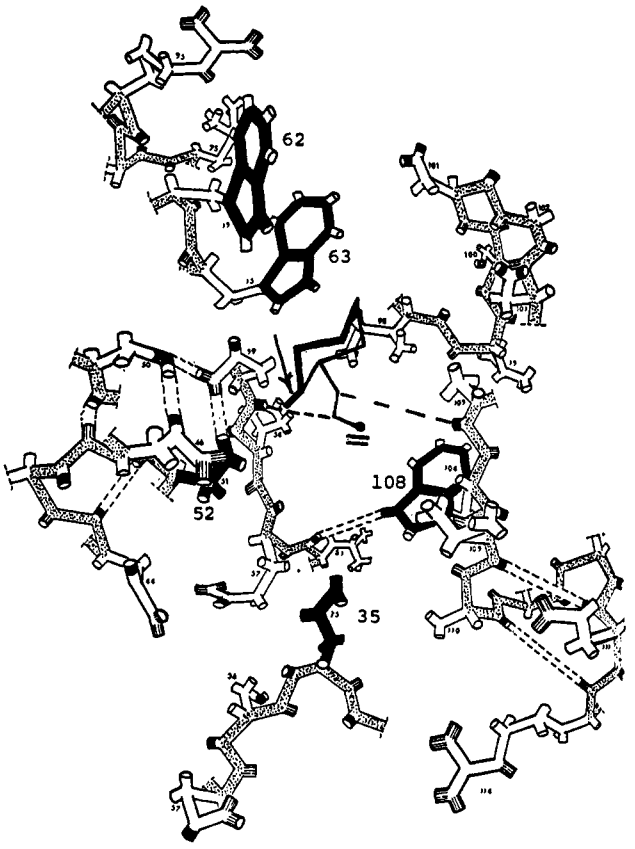

Figure 5. Binding position of NAG at low pH.[15] The direction of the β-NAG anomeric OH is indicated by the arrow and the acetyl protons by the double underline.

Discussion

Interpretation of Nmr Results Based upon the X-Ray Diffraction Model for Lysozyme. The plot of the association constant for α-NAG *vs.* pH is bell shaped, having inflection points at $pK_A = 4.7$ and 6.1, and maximal binding at pH ≅ 5.2. The same plot for β-MNAG is different, having only one break at $pK_A = 6.1$, with better binding at the lower pH.[9] The results for Δ_{eff} *vs.* pH are also different. For α-NAG, Δ_{eff} increases slightly in going from pH ≅ 3 to pH ≅ 5.2, and then decreases sharply on going to pH ≅ 7. For β-MNAG, Δ_{eff} decreases through both pH ranges.[9]

An interpretation of these results can be made, based upon a model for lysozyme constructed from Phillips' coordinates.[15] Three features of the binding of monosaccharides to site "C" in lysozyme are important to this interpretation. One is the relative closeness of Asp-52 and Glu-35 to site "C." The second is that the direction of the anomeric OH group may be very important in determining hydrogen bonds to residues in the cleft of lysozyme. The third is the fact that the changes in hydrogen bonding and the position of the monosaccharide can result in large changes of the position of the acetyl protons with respect to Trp-108. The ring-current effects of Trp-108 are expected to be the cause of the observed bound chemical shifts.[17] Phillips enumerates the following binding contacts for NAG in the site of lysozyme at "pH 4.7."[15] Both α- and β-NAG make specific hydrogen bonds between the acetamido NH and carbonyl oxygen and the main chain CO and NH groups of residues 107 and 59, respectively. β-NAG also makes hydrogen bonds between its O-3 and O-6 atoms and the NH groups on the indoles of Trp-62 and -63, respectively, whereas α-NAG is rotated about the acetamido hydrogen bonds, making a hydrogen bond from O-1 to the backbone NH of residue 109.

The result that at low pH the K_{eff}'s for α-NAG and β-NAG are equal ($K_{eff} = 31\ M^{-1}$) and that the Δ_{eff}'s for α-NAG, β-NAG, and β-MNAG[9] are also equal ($\Delta_{eff} = 73$ Hz) indicates that interactions at the anomeric position are unimportant at low pH. The monosaccharide binding orientation which seems most reasonable for low pH therefore is that suggested for β-NAG by the X-ray diffraction results (Figure 5).

In going to pH ≃ 5.2, the value of Δ_{eff} for α-NAG is only slightly changed while K_{eff} for α-NAG increases, whereas the value of Δ_{eff} for β-MNAG drops with K_{eff} for β-MNAG remaining constant. The value of Δ_{eff} for β-NAG also drops through this pH range. In addition, K_{eff} and Δ_{eff} for α-NAG ($48\ M^{-1}$ and 78 Hz) are equal to K_{eff} and Δ_{eff} for *N*-acetyl-α-D-glucopyranosyl fluoride ($46\ M^{-1}$ and 78 Hz).[18] These results indicate that the interactions made with the α anomer upon the deprotonation of the $pK_A = 4.7$ residue, which has been identified as Asp-52,[19] can be made equally well with O-1 or F-1 and that these interactions do not change the position of the acetyl protons with respect to Trp-108. The creation of a hydrogen bond to O-1 of α-NAG and the subsequent rotation of the α anomer around the acetamido hydrogen bonds[15] leave the acetyl protons near the center of the face of Trp-108, and thus $\Delta_{eff,\alpha}$ would not be substantially affected. Interactions with the β anomers, however, have a substantial influence on the orientation of β saccharides in the active site in the direction of lifting the acetyl protons off the center of the face of Trp-108.

The changes occurring with the $pK_A = 6.1$ ionization, which has been assigned to Glu-35, would appear to be more general. The Δ_{eff}'s of α-NAG, β-NAG, and β-MNAG all decrease as this ionization occurs, and Glu-35 is not close enough to site "C" to form any direct bonds with monosaccharides bound there. Changes in water structure or enzyme conformation probably give rise to these changes, quite possibly through effects upon nearby Trp-108.

Dimerization. Of the three models for the binding of an inhibitor by the lysozyme dimer, only the "one site blocked" model brings the α-NAG association constant curve of the present work into agreement with that of Kowalski and Schimmel.[3,20] This result indicates that the lysozyme dimerization is a "head-to-tail" association, with the active site of one molecule in the dimer binding some complementary part of the other part of the dimer.[21] Sophianopoulos found this

(17) C. E. Johnson, Jr., and F. A. Bovey, *J. Chem. Phys.*, 29, 1012 (1958).

(18) B. D. Sykes, unpublished results.

(19) S. M. Parsons and M. A. Raftery, *Biochem. Biophys. Res. Commun.*, 41, 45 (1970).

(20) While the high pH inflection point agrees closely with the results of Kowalski and Schimmel, there is a shift of the low pH inflection point. This shift results from the difference in temperature between the two studies, the trend being the direction determined in ref 3.

(21) It is not possible to rule out, however, that enzyme self-association may not stop at dimers but rather involves infinite "head-to-tail" oligomers.[22]

(22) R. C. Deonier and D. W. Williams, *Biochemistry*, 9, 4260 (1970).

model to fit his results best,[14] and found the pK of the ionization necessary for dimerization to be 6.1, which corresponds to glutamic acid 35.

Acknowledgments. The authors would like to acknowledge the support of the National Institutes of Health through Grants GM-17190-01 and GM-14752, the National Science Foundation through Grant No. GP-4924, the Research Corporation, the Milton Fund (Harvard University), and the Center for Materials Research (Stanford University). The authors would also like to acknowledge the technical assistance of Alice Studebaker, and Peter Urnes for constructing the lysozyme model.

Appendix I

Consider a solution of α-NAG, β-NAG, and lysozyme in equilibrium with each other. The lysozyme may exist in several states of protonation, E, EH, EH_n, which are related by the equilibrium expressions

$$\frac{[EH]}{[E][H]} = C_1$$

$$\frac{[EH_2]}{[E][H]^2} = C_2$$

$$\vdots$$

$$\frac{[EH_n]}{[E][H]} = C_n$$

There may also be more than one site to which α- and β-NAG can bind, and let us consider the special case in which only one NAG molecule can bind to a given lysozyme molecule at a time. A set of equilibrium equations may be written to express the affinity of α-NAG or β-NAG for a particular site when the enzyme molecule is in a particular state of protonation. In the case of binding sites $j = 1,2,3 \ldots m$

$$\frac{[E_jI_\alpha]}{[E][I_\alpha]} = K_{\alpha,j} \qquad \frac{[E_jI_\beta]}{[E][I_\beta]} = K_{\beta,j}$$

$$\frac{[E_jHI_\alpha]}{[EH][I_\alpha]} = K_{\alpha,j,H} \qquad \frac{[E_jHI_\beta]}{[EH][I_\beta]} = K_{\beta,j,H}$$

$$\vdots \qquad\qquad \vdots$$

$$\frac{[E_jH_nI_\alpha]}{[EH_n][I_\alpha]} = K_{\alpha,j,H_n} \qquad \frac{[E_jH_nI_\beta]}{[EH_n][I_\beta]} = K_{\beta,j,H_n}$$

The concentrations of free α- and β-NAG are related to the total free NAG by the anomeric equilibrium

$$[I_\alpha] = p_\alpha([I_\alpha] + [I]_\beta)$$

$$[I_\beta] = (1 - p_\alpha)([I_\alpha] + [I_\beta])$$

In the limit of fast chemical exchange, the observed shift is

$$\delta_{oi} = \frac{[I_i]}{[I_{Ti}]}\delta_i + \sum_{j=1}^{m}\left(\frac{[E_jI_i]}{[I_{Ti}]}\delta_{ij_0} + \cdots \frac{[E_jH_nI_i]}{[I_{Ti}]}\delta_{ij_n}\right) = \frac{[I_i]}{[I_{Ti}]}\delta_i + \sum_{j=1}^{m}\sum_{k=0}^{n}\frac{[E_jI_iH_k]}{[I_{Ti}]}\delta_{ijk}$$

where $[I_{Ti}]$ is the sum of all forms of the anomer i and $i = \alpha,\beta$. Writing each bound chemical shift as $\delta_{ijk} = \delta_i + \Delta_{ijk}$ allows one to write

$$\delta_{oi} = \delta_i + \sum_{j=1}^{m}\sum_{k=0}^{n}\frac{[E_jI_iH_k]}{[I_{Ti}]}\Delta_{ijk}$$

since

$$[I_{Ti}] = [I_i] + \sum_{j=1}^{m}\sum_{k=0}^{n}[E_jI_iH_k]$$

Therefore

$$\delta_{oi} - \delta_i = \frac{[I_i]}{[I_{Ti}]}\sum_j\sum_k[EH_k]K_{ijk}\Delta_{ijk} = \frac{[I_i]}{[I_{Ti}]}[E]\sum_j\sum_k[H]^kC_kK_{ijk}\Delta_{ijk}$$

The double sum looks like a weighted average of the chemical shifts, except that it is not divided by the sum of the weighting factors. Defining

$$\Delta_{eff,i} = \frac{\sum_j\sum_k[H]^kC_kK_{ijk}\Delta_{ijk}}{\sum_k\sum_j K_{ijk}[H]^kC_k}$$

then

$$\delta_{oi} - \delta_i = \frac{[I_i]}{[I_{Ti}]}[E]\Delta_{eff,i}\sum_j\sum_k K_{ijk}[H]^kC_k$$

The double sum of this expression may be divided by $\Sigma_k[H]^kC_k$ and defined as the effective association constant

$$K_{eff,i} = \sum_j\sum_k K_{ijk}[H]^kC_k\sum_k[H]^kC_k$$

$$\delta_{oi} - \delta_i = \frac{[I_i]}{[I_{Ti}]}[E]\sum_k[H]^kC_kK_{eff,i}\Delta_{eff,i}$$

But

$$\frac{[I_i]}{[I_{Ti}]} = \frac{[I_i]}{[I_i](1 + \sum_j\sum_k[E][H]^kC_kK_{ijk})} = \frac{1}{1 + K_{eff,i}[E]\sum_kC_k[H]^k}$$

Therefore

$$\delta_{oi} - \delta_i = \frac{\Delta_{eff,i}K_{eff,i}[E]\sum_k[H]^kC_k}{1 + K_{eff,i}[E]\sum_k[H]^kC_k}$$

In order to calculate $\delta_{oi} - \delta_i$ in terms of known quantities, it is necessary to calculate $[E]\Sigma_k[H]^kC_k$

$$[E_0] = \sum_k[EH_k] + \sum_j\sum_k([E_jH_kI_\alpha] + [E_jH_kI_\beta]) = [E]\sum_k[H]^kC_k(1 + [I_\alpha]K_{eff,\alpha} + [I_\beta]K_{eff,\beta})$$

Recalling the anomeric equilibrium, it is possible to write

$$[I_\alpha]K_{eff,\alpha} + [I_\beta]K_{eff,\beta} = [I](p_\alpha K_{eff,\alpha} + (1 - p_\alpha)K_{eff,\beta}) = \bar{K}_{eff}[I]$$

where $[I] = [I_\alpha] + [I_\beta]$ and $\bar{K}_{eff} = p_\alpha K_{eff,\alpha} + (1 - p_\alpha)\cdot K_{eff,\beta}$. Thus, $[E_0] = [E]\Sigma_k[H]^kC_k(1 + [I]\bar{K}_{eff})$. $[I_0] = [I] + \Sigma_j\Sigma_k([E_jH_kI_\alpha] + [E_jH_kI_\beta)] = [I](1 + [E]\Sigma_k[H]^kC_k\bar{K}_{eff})$. Let

$$[E]\sum_k[H]^kC_k = x$$

$$[E_0] = x\frac{(1 + \bar{K}_{eff}[I_0])}{1 + x\bar{K}_{eff}}$$

$$x = \frac{(E_0 - I_0 - 1/\bar{K}_{eff}) \pm ((E_0 - I_0 - 1/\bar{K}_{eff})^2 + 4E_0/\bar{K}_{eff})^{1/2}}{2}$$

The positive root is always the physically meaningful one. Thus

$$\delta_{oi} - \delta_i = \frac{\Delta_{eff,i}K_{eff,i}x}{1 + K_{eff,i}x}$$

where

$$x = \frac{(E_0 - I_0 - 1/\bar{K}_{eff}) + ((E_0 - I_0 - 1/\bar{K}_{eff})^2 + 4E_0/\bar{K}_{eff})^{1/2}}{2}$$

Appendix II

Many models are possible for the interaction of an enzyme dimer with an inhibitor of the monomer. We have chosen, however, to limit our analysis to three models which have the common feature that an active site on the dimer either binds the inhibitor molecule in the same manner as a site on the monomer or is completely unable to bind it. The three models are thus one in which both sites on the dimer are unaffected by dimerization, one in which one site is unaffected and the other completely blocked, and one in which both are completely blocked.

1. In the first model, dimerization and inhibitor binding are independent processes. Consequently, "a monomer or dimer active site" is equivalent to "enzyme" in the equation for the monomeric enzyme alone. Thus, the values of Δ_{eff} and K_{eff} obtained in this model are the same as those obtained in the one in which the enzyme is entirely in the monomeric form.

2. In order to derive the expression for the model in which one dimer site is blocked, we define $[E_2]/[E][E] = K_d$, $[EI]/[E][I] = [E_2I]/[E_2][I] = K$, $[EHI]/[E][H] = C_H$, $[EHI]/[EH][I] = K_H$. The bound chemical shift of I in EI and E_2I is Δ; that in EHI is Δ_H.

$$\delta_{oi} - \delta_i = \frac{[EI]}{[I_0]}\Delta + \frac{[EHI]}{[I_0]}\Delta_H + \frac{[E_2I]}{[I_0]}\Delta = \frac{[EI]}{[I_0]}\left\{\Delta_{eff}\left[1 + \frac{[H]C_HK_H}{K}\right]\right\} + \frac{[E_2][I]}{[I_0]}K\Delta$$

where

$$\Delta_{eff} = \frac{K\Delta + [H]C_HK_H\Delta_H}{K + [H]C_HK_H}$$

A. Calculation of $[E_2]$. $[E_0] = [E] + [EI] + [EH] + [EHI] + 2[E_2] + 2[E_2I] = [E]\{1 + K[I] + [H]C_H(1 + [I]K_H)\} + 2[E_2](1 + [I]K)$. If we define

$$K_{eff} \equiv \frac{K + K_H[H]C_H}{1 + [H]C_H}$$

$$\gamma \equiv (1 + [H]C_H)(1 + [I]K_{eff})$$

then

$$E_0 - 2[E_2](1 + [I]K) = \left(\frac{[E_2]}{K_d}\right)^{1/2}\gamma$$

$$[E_2] = \frac{\gamma^2}{K_d} + 4[E_0](1 + [I]K) \pm \frac{\{((\gamma^2/K_d) + 4[E_0](1 + [I]K))^2 - 16[E_0]^2(1 + [I]K)^2\}^{1/2}}{8(1 + [I]K)^2}$$

The negative root is the physically meaningful one, since $[E_2](1 + [I]K) = [E_2] + [E_2] \leq {}^1/_2[E_0]$.

B. Calculation of [EI]

$$[E_0] - 2[E_2](1 + [I]K) = [EI]\left(1 + \frac{1}{[I]K} + \frac{[H]C_H}{[I]K} + \frac{K_H}{K}[H]C_H\right)$$

$$[EI] = \frac{[I]K}{(1 + [H]C_H)(1 + [I]K_{eff})} \times \{[E_0] - 2[E_2](1 + [I]K)\}$$

Combining this equation with the ones for $[E_2]$ and $\delta_{oi} - \delta_i$ yields

$$\delta_{oi} - \delta_i = \frac{\Delta[I][E_0]K}{2[I_0](1 + [I]K)} + \frac{[I]}{[I_0]K_d} \times \left\{1 + \frac{8[E_0]K_d(1 + [I]K)}{(1 + [H]C_H)^2(1 + [I]K_{eff})^2}\right\}^{1/2} - 1\Big) \times \left\{\frac{(1 + [H]C_H)^2(1 + [I]K_{eff})^2}{4(1 + [I]K)}\right\} \times \left\{\frac{K_{eff}\Delta_{eff}}{1 + [I]K_{eff}} - \frac{\Delta K}{2(1 + [I]K)}\right\}$$

Now consider the high pH limits, in which [EH] becomes negligible. Then $\Delta_{eff} \rightarrow \Delta$, $K_{eff} \rightarrow K$, and $[H]C_H \rightarrow 0$

$$\delta_{oi} - \delta_i = \Delta\left\{\frac{[E_0][I]K}{2[I_0](1 + [I]K)} + \frac{[I]K}{8[I_0]K_d}\right\} \times \left(\left\{1 + \frac{8[E_0]K_d}{(1 + [I]K)}\right\}^{1/2} - 1\right)$$

We have used the high pH (8.2) data and Sophianopoulos' dimerization constant[14] in this equation to obtain K and Δ. Our computer program first calculates K and Δ using $[I] = [I_0]$, then calculates them again using a first-order correction to I. The data for pH's at which [EH] may not be negligible can then be analyzed using eq 3 of the theory section.

3. For the model in which the dimer does not bind the inhibitor at all, we define K_d, K, K_H, C_H, Δ, and Δ_H as in section 2 of Appendix II

$$E_0 = 2[E_2] + [E] + [EH] + [EHI] + [EI] = 2[E_2] + \left(\frac{[E_2]}{K_d}\right)^{1/2}(1 + [I]K_{eff})(1 + [H]C_H) = 2[E_2] + \left(\frac{[E_2]}{K_d}\right)^{1/2}\gamma$$

defining γ and K_{eff} as in section 2

$$[E_2] = \frac{[E_0]}{2} + \frac{\gamma^2}{8K_d} - \left(\frac{\gamma^4}{K_d^2} + \frac{8[E_0]\gamma^2}{K_d}\right)^{1/2}$$

$$[E_2] = \frac{[E_0]}{2} + (1 + [H]C_H)^2(1 + [I]K_{eff})^2 \times \frac{\left\{1 - \left(1 + \frac{8[E_0]K_d}{(1 + [H]C_H)^2(1 + [I]K_{eff})^2}\right)^{1/2}\right\}}{8K_d}$$

In the high pH limit

$$[E_0] - 2[E_2] = \frac{(1 + [I]K)}{4K_d}\left(\left\{1 + \frac{8[E_0]K_d}{(1 + [I]K)^2}\right\}^{1/2} - 1\right)$$

Since $E_0 - 2E_2$ equals the sum of monomeric species, one may calculate $E_{0,app} = E_0 - 2E_2$ and proceed as in the case at the simple monomer calculation (eq 2). The program for pH's at which E, EH, and E_2 are significant calculates values of both $[H]C_H$ and K_{eff}.

Deoxyribonucleic Acid Replication. A Theoretical Study of Löwdin's Mechanism

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Abstract: Löwdin has proposed a mechanism, based on the stability of the DNA "replication plane," to explain the exclusion of rare tautomeric forms of nucleotide bases from double-stranded DNA. A quantitative theoretical test of this mechanism was carried out using a simple electrostatic model to compute the stability of replication planes. Atomic charges used in the model were obtained by adding π charges calculated by the Pariser–Parr–Pople method to σ charges from Del Re calculations. The model does predict the proper degree of incorporation of adenine and thymine rare forms, but fails for the rare forms of guanine and cytosine. The model further predicts the ratio of bromouracil to thymine incorporation to be 0.2 in agreement with the experimental value of 0.23.

Very early Watson and Crick[1] suggested that DNA replication might take place as a result of the unwinding of the two strands of an original DNA molecule and the synthesis of new strands using the old as templates. In particular, they suggested that the separated original strands would be freely exposed to the environment, and that each of the new strands would grow independently of the other, extending itself by another nucleotide unit only after the correct complementary nucleoside triphosphate happened to drift into place.

However, there is a difficulty with this simple mechanism. In the normal DNA double strand, the nucleotide base guanine (G) is paired with cytosine (C) and thymine (T) with adenine (A). Each of these four is in tautomeric equilibrium with a rare form as shown in Figure 1, and each rare form has the necessary hydrogen bonding and steric requirements to fit into the DNA double helix in the pairs: T-G*, A-C*, G-T*, and C-A* (where the asterisk denotes the rare tautomeric form). Incorporation of rare forms leads to mutations, and if the relative rates of incorporation were simply in proportion to their concentrations in the environment of the separated DNA strand, then the rate of mutation predicted from the tautomerization equilibrium constants[2,3] would be at least 10^3 times as great as observed.[4]

To account for the extra exclusion of the rare forms, Löwdin[5-8] proposed a mechanism in which new strands are built onto the old as they separate (Figure 2). The "replication plane," a cross section through the DNA helix at the point of separation and new growth, is shown schematically in Figure 3. Replication takes place by rotation of the two original strands to expose the hydrogen-bonding areas of the bases (I to II in Figure 3), followed by addition of new bases from the environment (III in Figure 3), and completed by separation of new pairs of strands. Löwdin suggests that rate of incorporation of rare tautomers is governed by the relative stability of the various sets of four bases in the replication plane (III in Figure 3). A drawing (see figures on p 183 of ref 7, but note that the labels of the first and third are interchanged) shows that one hydrogen bond in the set of four normal bases is replaced by a repulsive interaction, either between two protons or between two lone pairs, if a normal base is replaced by a correct rare form. This interaction is between daughter helices, not between base pairs within a daughter helix.

The present paper provides a quantitative test of Löwdin's proposal. A simple quantum mechanical model is set up, and the relative stability of the various four-base replication planes is calculated. As a further check, the rate of incorporation is computed for bromouracil, a molecule which has been found experimentally to take the place of thymine.

Computational Methods

The model adopted assumes that each molecule can be represented as a system of point charges located at the nuclear positions. The interaction energy for a four-base system in a particular configuration is obtained by summing the Coulomb interactions between the sets of point charges, having taken care that no van der Waals contact distances between point charges on different molecules are violated. Hydrogen bonds are allowed to reach a minimum length of 2.90 Å.

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