Temperature and pH Dependence of the Binding of Oligosaccharides to Lysozyme*

SURLATH K. BANERJEE AND J. A. RUPLEY†
From the Department of Chemistry, The University of Arizona, Tucson, Arizona 85721

SUMMARY

Association of lysozyme with the following ligands was measured at 0.1 ionic strength, using absorbance and fluorescence procedures: (a) the β(1 → 4)-linked trimer of N-acetylglucosamine, at pH 0 to 9 and 8–60°; (b) the dimer, at pH 0 to 9 and 30° and at 9–60° and pH 5.3; (c) N-acetylglucosamine, at 9–60° and pH 5.3. The complex-free protein difference spectrum varied with temperature and pH. Analysis of the association data indicated that trimer binding perturbs the pK of three ionizations, glutamic 35, 6.1 to 6.4; aspartic 101, 4.3 to 3.4; aspartic 66, 1.9 to 1.5. Since aspartic 66 does not interact directly with the saccharide, its pK shift presumably reflects the change in conformation that has been seen in crystallographic analysis (Blake, C. C. F., MAIR, G. A., NORTH, A. C. T., PHILLIPS, D. C., AND SHARMA, V. R. (1967) Proc. Roy. Soc. London B Biol. Sci. 167, 365; Blake, C. C. F., JOHNSON, L. N., MAIR, G. A., NORTH, A. C. T., PHILLIPS, D. C., AND SHARMA, V. R. (1967) Proc. Roy. Soc. London B Biol. Sci. 167, 378) and involves this carboxyl. Aspartic 66 is buried as a carboxylate that is not part of an ion pair. The enthalpy of association increases by 5.9 kcal between pH 5 and 2 and is compensated by a nearly equal increase in entropy. This behavior may be related to de-solvation of aspartic 101 in complex formation. The apparent average enthalpy of hydrogen-bond formation in the trimer complex is −1.5 kcal.

A considerable number of publications have reported data on the free energies of binding of oligosaccharides to lysozyme (References 1–5; other data are reviewed in Imoto et al. (6)). Several reports have given enthalpy values (1, 7–11) at pH near 5. This paper describes the dependence on both pH and temperature of the association of lysozyme with the β(1 + 4)linked dimer and trimer of N-acetylglucosamine. Particular attention is paid to the pH-dependence of the enthalpy of binding of trisaccharide, and to changes in free energy of binding at pH below 2. The enthalpy of association changes much more strongly between pH 2 and 5 than does the free energy. This behavior appears to be associated with the withdrawal of aspartic 101 from solvent when saccharide binds.

EXPERIMENTAL PROCEDURES

Materials

Twice crystallized lysozyme was obtained from Worthington. The di- and trisaccharides of N-acetylglucosamine ((GlcNAc)_2 and (GlcNAc)_3) were prepared as described previously (12), vacuum dried, and kept over CaCl₂; charcoal-celite columns detected no traces of other components. Stock solutions of protein and saccharides were prepared fresh for each experiment. Chemicals were reagent grade. Water was deionized and freed of organics using a system supplied and maintained by Continental Service and consisting of two deionizer and one charcoal cartridge in series.

Methods

pH was measured at room temperature (24 ± 2°) with a Leeds and Northrup or Radiometer TTT1C instrument. Standard buffers were of pH 4.01 (0.05 m potassium acid phthalate) and 6.50 (Radiometer type S1001 concentrated buffer). A universal buffer of 0.1 ionic strength (Reference 13; phosphate, borate, and acetate at 0.02 to 0.04 m with KCl added to 0.1 ionic strength) was used to control pH of reaction mixtures over the range 2 to 10. For pH 1 to 2, solutions of ionic strength 0.1 were prepared using appropriate amounts of HCl and KCl; at pH below 1, ionic strength was that of the HCl solution. pH was determined before and after each experiment. Measurements of thermostatted solutions of pH 2, 5, and 7.5 showed that there was less than 0.05 pH difference between 15° and 45°. This accords with the reported temperature variation of the pK values of the buffer components (14).

Protein concentration was determined by ultraviolet absorbance at 280 nm (molar extinction coefficient = 36,500; (15)), using a Zeiss PMQII spectrophotometer. Routinely, two determinations were averaged.

Fluorescence—Fluorescence was determined using an Aminco-Bowman Spectrofluorimeter equipped with thermostatted 1-cm cell holder and Sargent model SR recorder. The excitation wave length was 285 nm (2, 4). The difference in fluorescence intensity between the complexed and free protein varies with pH (16); formation of the trimer complex was followed at 340 nm for pH greater than 7 or 370 nm for pH below 7, and formation of the dimer complex at pH below 5 was measured at 360 nm. For determination of equilibrium binding parameters the

* This work was supported by research grants from the American Cancer Society and the National Institutes of Health.

† To whom inquiries concerning this paper should be addressed.
fluorescence intensities of lysozyme solutions (approximately 0.05 mg per ml) were determined as a function of increasing saccharide concentration. The highest concentration of ligand always gave at least 80% saturation. The change in fluorescence was assumed to depend linearly on the amount of complex formed. Association constants were calculated for simple bimolecular association between lysozyme and saccharide:

\[
K_{assoc} = \frac{ES}{(E_0 - ES)(S_0 - ES)}
\]

\(ES\) is the molar concentration of complex, and \(E_0\) and \(S_0\) are the total molar concentrations of lysozyme and saccharide. The association constant and change in fluorescence at saturation (\(\Delta F_{max}\)) were obtained from a Scatchard plot, of \(\Delta F\) versus \(\Delta F/(S_0 - ES)\).

Absorbance—Difference spectra (lysozyme-saccharide versus lysozyme) were measured with a Zeiss PMQII or Cary model 14 or 15 spectrophotometer equipped with thermostatted cell holder. Protein concentration was 1 mg per ml in a 1-cm cell or, for measurement with \((\text{GlcNAc})_3\), 0.1 mg per ml in a 10-cm cell. Fig. 1 gives typical spectra for trisaccharide complexes. The 293 to 289 nm peak-trough difference is more precisely determined than the height of the 293 nm peak above a long wavelength base, and the former value routinely was the measure of extent of association. Values of \(K_{assoc}\) and \(\Delta F_{max}\) were calculated as for fluorescence measurements.

Proton Binding—The pH dependence of an association process can be determined by measurements of the effect of complex formation upon proton binding (17). A Radiometer Titrator (model TTTIC) was equipped with a thermostatted and magnetically stirred cell of 4- to 5-ml volume. Saccharide (routinely 0.012 m final concentration) was added to protein (0.001 M) in 0.1 M KCl, and the change in protons bound determined by back titration with 0.02 M HCl-NaOH, with appropriate correction for blanks. Measurements at pH above 6.0 were made under nitrogen freed of CO\(_2\) and equilibrated with buffer.

RESULTS

Spectra—Fig. 1 gives lysozyme-saccharide difference spectra over the pH range 0 to 11. The spectra resemble that described for pH near 5 by Hayashi et al. (18). Considering the three tryptophans in the lysozyme active site (19), it is not surprising that the data typify tryptophan perturbation (20), with peaks at 293, 285, and 276 nm. The difference spectra are more sharply structured than those obtained through solvent perturbation of lysozyme (e.g. using glucose or ethylene glycol), in particular with regard to the depth of the trough at 289 nm. The shallow trough at 304 nm found at low pH disappears as the pH goes to 5. At high pH a prominent peak develops at 302 nm. These features correlate with carbonyl ionizations of pK approximately 3.5 and 6.5 (21).

Fig. 2 shows the temperature and pH dependence of \(\Delta F_{max}\). The effect of increased temperature accords with the expected broadening of a spectrum line. An ionization of pK 3 to 4 like that seen in the 304 nm trough also affects \(\Delta F_{max}\). The slight pH dependence of \(\Delta F_{max}\) in very acid solution may be associated with a group of lower pK. In this regard, the pH dependence of a saccharide binding (see below) and other experimental approaches determine an ionization of pK 1.5 to 2.

Treatment of Association Data—The pH dependence of an association constant can be described (17):

\[
\frac{\partial \log K_{assoc}}{\partial \text{pH}} = - \left( \frac{\rho^0_{-H^+} - \rho^0_{+H^+}}{T} \right)
\]

\(K_{assoc}\) is the association constant for the experimental pH and \(T\); \(\rho^0_{-H^+}\) 8 moles of protons bound per mole of enzyme (E) or...
enzyme-saccharide complex (ES). $\Delta \delta_{\text{H}^+}$ contains contributions from each of the groups perturbed by saccharide binding. By separating $\Delta \delta_{\text{H}^+}$ into the individual contributions and writing these in terms of group ionization constants for enzyme and enzyme-saccharide complex at the temperature of the experiment ($K_{i, T}$, $K_{\text{ac}, T}$), we obtain:

$$\Delta \delta_{\text{H}^+} = \sum_{i=1}^{n} \left( \frac{(H^+)}{(H^+) + K_{\text{ac}, T}} - \frac{(H^+)}{(H^+) + K_{i, T}} \right) \tag{2b}$$

Integrating Equation 2a with respect to pH:

$$\log K_{\text{assoc}} = \frac{H^+}{H^+} \log K_{\text{ac}, T} - \frac{H^+}{H^+} \log K_{i, T} + \sum_{i=1}^{n} \log \left( \frac{(H^+) + K_{\text{ac}, T}}{(H^+) + K_{i, T}} \right) + \sum_{i=1}^{n} \frac{\Delta H^\theta_{i}}{1 + (H^+/K_{\text{ac}, T})} - \frac{\Delta H^\theta_{i}}{1 + (H^+/K_{i, T})}$$

$$f(T) = \frac{(298.2 - T)}{(298.2 \times 2.3122)^2}$$

Equation 2c with respect to $1/T$:

$$\Delta H_{\text{assoc}} = \Delta H_{\text{ac,assoc}} + \sum_{i=1}^{n} \frac{\Delta H^\theta_{i}}{1 + (H^+/K_{\text{ac}, T})} - \frac{\Delta H^\theta_{i}}{1 + (H^+/K_{i, T})}$$

The derivation of Equations 2e and 3 assumes that the mode of ligand binding is pH independent. Dalhquist and Raftery (22) interpret NMR data for (GlcNAc)$_3$ at pH 2 to 10 and 31-45° in terms of a single complex. There is no evidence suggesting more than one mode of (GlcNAc) binding (6). Equations 2e and 3 also require that each ionizing group affected by saccharide binding have constant pH and enthalpy of ionization, at least over the range in which it contributes significantly to the pH dependence of $\log K_{\text{assoc}}$. These parameters might be altered owing to (a) a change in protein conformation; (b) a change in the state of ionization of a neighboring group; or (c) a non-zero value of $\Delta C_p$ (in the case of enthalpy parameters). A variety of physical measurements have shown the lysozyme conformation to be invariant over the pH and temperature range of this study (6). The close charge pairs in the active site (aspartic 52-glutamic 33 and aspartic 60-arginine 68, each at about 7 Å separation) have pH well separated and any interaction should be constant throughout the titration range of each. Calorimetric data of Bjurulf and Wadsö showed a near zero value of $\Delta C_p$ at pH 5.

Equation 2c, with $4n + 2$ variable parameters (enthalpies and equilibrium constants), was fit to association data using a least squares program for nonlinear functions that is a modification of one developed at Los Alamos Laboratory (23) and the Courant Institute, New York University. Different sets of initial parameter estimates, used to test uniqueness of the fit, gave the same final values within the limits of the standard deviation. Because $\Delta H^\theta$ for ionization of carboxylic acids is near zero, for convenience we routinely fixed $\Delta H^\theta_{i}$ at zero in computations using Equation 2c.

**Association Data and Fitting of Constants—** Table I gives $\log K_{\text{assoc}}$ as a function of pH and temperature for the (GlcNAc)$_5$-lysozyme system, and similar results for (GlcNAc)$_3$ as a function of pH, at 30°. The $\log K_{\text{assoc}}$-pH profile has three distinguishable regions (Fig. 3, pH 0 to 2.5, 2.5 to 5, and 5 to 7). At least three protein groups are perturbed by saccharide binding.

Table II gives values for the parameters of Equation 2c that fit the data of Table I. The deviations of calculated values of $\log K_{\text{assoc}}$ from experimental data are listed in Table I. The standard deviation of the fit is 0.05 units in $\log K_{\text{assoc}}$ for the trisaccharide data and 0.02 for the disaccharide data. The estimated experimental error is 0.05 units. Reproducibility was 0.02 units. No group of data points deviates systematically and substantially from the mass and so biases the fit; this was demonstrated through several tests. There was no significant change in the parameters calculated if (a) a subset of the data (15 and 30° values) was used; (b) reduced weight was given to data points that deviated more than the average; or (c) random error of range 0.1 in $\log K_{\text{assoc}}$, 0.2 in temperature, and 0.1 in pH was introduced into each data point.

It is important to note that data that are as accurate as can be obtained in measurements on protein systems (2 to 5% error in $K_{\text{assoc}}$) cannot define pK values better than ±0.2 to 0.5 unit (or $\Delta H$ values to ±2 to 5 kcal), if the titration ranges of the groups overlap and if three or more groups are involved. However, although complex pH profiles cannot accurately define values of pK and $\Delta H$, the changes in pK ($\Delta \text{pK}$) and $\Delta H$ ($\Delta \text{AH}$) brought about by saccharide binding depend little on the particular pK estimates.

Literature data have suggested that ionizations with pK different than given above also may be in the active site. NMR measurements have indicated a group of pK 4.7 that is in or near the active site but is not perturbed by binding of β-methyl-GlcNAc (8). A group of pK about 3.2 affects the lysozyme absorbance spectrum (21) and this effect is enhanced by saccharide binding without change in the observed pK (Fig. 2). Fluorescence measurements (16) show pK 3 to 3.5. Assuming three ionizing groups, satisfactory fits could not be obtained with pK 3.2, nor with pK 4.7 if pK was less than 2.5. The dependence of $\log K_{\text{assoc}}$ on pH does not require that saccharide binding perturb more than three ionizable groups, but the crystallographic information indicates that four (glutamine 35 and aspartics 52, 66, and 101) might in fact be affected. No nontrivial four group trial gave as good fit as the three groups.

**Enthalpies of Association and Ionization—** Fig. 4 shows $\Delta H^\theta_{i}$ as a function of pH. The curve was computed using Equation 3 and the parameter estimates of Table II. Values of $\Delta H^\theta_{i}$ calculated from van ‘t Hoff plots of data for pH 2, 5, and 7 agreed within 0.5 kcal with the results of Fig. 4. The compensation between $\Delta H^\theta$ and $T\Delta S^\theta$ and the much greater changes in these parameters than in $\Delta F^\theta$ are striking.

---

1. Equation 2a is rigorous and independent of models used for interpretation.

2. Buffer effects can be neglected. Buffer pH is essentially independent of temperature (see “Experimental Procedure”); the nominal value (measured at room temperature) is within 0.05 pH of the true over the temperature range of this work. Specific ion effects were not observed; association was the same in KCl, NaCl, sodium acetate-acetic, and the buffer system used in this work, at several pH, 30°, and 0.1 ionic strength.

3. C. Bjurulf and I. Wadsö, personal communication.

4. T. Imoto and J. A. Rupley, unpublished data.
Table I. Association of (GlcNAc), and (GlcNAc), with lysozyme. Data for the temperature and pH indicated, at ionic strength 0.1 (except for pH below 1). The deviations ΔY (calculated value - data value) are given for the parameter set of

Table II. Method of measurement: A, fluorescence; B, absorbance using 10 cm cells; C, absorbance using 1 cm cells;

D, data given in Kupfer et al (3).

| PH  | T   | LOGK | ΔY  | PH  | T   | LOGK | ΔY  | PH  | T   | LOGK | ΔY  | PH  | T   | LOGK | ΔY  |
|-----|-----|------|-----|-----|-----|------|-----|-----|-----|------|-----|-----|-----|------|-----|
| 5.00 | 8.0 | 8.0% | 0.13 A | 4.20 | 20.0 | 5.14 | -0.08 A | 6.00 | 30.0 | 5.04 | -0.01 B | 6.00 | 30.0 | 5.04 | -0.01 B |
| 4.90 | 8.0 | 8.8% | 0.07 A | 4.50 | 20.0 | 4.16 | -0.06 A | 6.50 | 30.0 | 4.95 | -0.03 A | 7.00 | 30.0 | 4.95 | -0.03 A |
| 5.30 | 9.0 | 8.79 | -0.09 B | 0.00 | 20.0 | 4.06 | -0.01 A | 5.30 | 20.0 | 4.07 | -0.02 D | 7.90 | 30.0 | 4.87 | -0.08 B |
| 4.00 | 15.0 | 4.82 | -0.00 A | 0.00 | 25.0 | 4.10 | -0.03 A | 1.50 | 25.0 | 4.12 | -0.02 C | 1.00 | 30.0 | 3.16 | 0.00 A |
| 1.50 | 15.0 | 4.50 | -0.01 A | 2.00 | 25.0 | 4.00 | -0.01 A | 7.00 | 30.0 | 4.80 | -0.01 B | 1.00 | 30.0 | 3.10 | -0.03 C |
| 1.00 | 15.0 | 4.35 | -0.01 A | 2.00 | 25.0 | 4.10 | -0.03 A | 7.60 | 30.0 | 4.92 | -0.01 B | 2.00 | 30.0 | 3.21 | -0.02 C |
| 1.00 | 15.0 | 4.50 | -0.01 A | 2.00 | 25.0 | 4.00 | -0.01 A | 7.60 | 30.0 | 4.92 | -0.01 B | 2.00 | 30.0 | 3.21 | -0.02 C |

(GlcNAc)3

(GlcNAc)2

A, fluorescence; B, absorbance using 10 cm cells; C, absorbance using 1 cm cells; D, data given in Kupfer et al (3).
TABLE II

Fit of Equation Bc to association data

$pK_i$ values are for 25° for (GlcNAc)$_2$ (III) and 30° for (GlcNAc)$_3$ (II). Parameters of Equation 2c for the free enzyme (E) were held fixed in the least squares fitting. Standard deviations are given in parentheses.

| Enzyme form | $\log K^\text{assoc}_{pH \text{ ref}}$ | $\Delta H^\text{assoc}_{pH \text{ ref}}$ | $pK_i$ | $\Delta H^{0.1}$ |
|-------------|---------------------------------|---------------------------------|-------|-----------------|
| E           |                                 |                                 |       |                 |
| ES (III)    | 4.06 (0.01)                     | $-10.04$ (0.90)                 | 1.90  | 4.30 (0.01)     |
|             |                                 |                                 | 1.51  | 3.42 (0.02)     |
|             |                                 |                                 | 1.57  | 3.96 (0.01)     |
| ES (II)     | 2.98 (0.01)                     |                                 | 2.2   | $-7.3$ (1.5)    |
|             |                                 |                                 | 1.57  | 3.96 (0.01)     |

Fig. 3. Log $K^\text{assoc}$ versus pH at 15° and 30° for (GlcNAc)$_a$ and at 30° for (GlcNAc)$_3$. The solid curves are calculated using parameters of Table II.

Fig. 4. Thermodynamic parameters at 25° for (GlcNAc)$_a$ association as a function of pH. $A$, $\Delta F^\text{assoc}$ (kcal); $B$, $\Delta H^\text{assoc}$ (kcal); $C$, $T\Delta S^\text{assoc}$ (kcal). The estimated error varies with pH; standard deviations, given in order for pH 0, 2.5, 5.0, and 7.5: $\Delta F^\text{assoc}$, 0.016, 0.015, 0.011, 0.012 kcal; $\Delta H^\text{assoc}$, 0.75, 0.63, 0.19, 0.48 kcal; $T\Delta S^\text{assoc}$, 2.5, 2.1, 0.6, 1.6 e.u. The curves were computed using Equations 2c and 3 and parameters from Table II.

Comparison of Temperature Dependence of Association of Monomer, Dimer, and Trimer—Fig. 5 shows van't Hoff analyses of binding data at pH 5.3 and 0.1 ionic strength, for the monomer, dimer, and trimer of N-acetylglucosamine. Least squares treatment gave the lines of the figure and the thermodynamic parameters of Table III. Measurements that differed from the least squares value by more than three standard deviations were not included in the calculations (one 60° data point for GlcNAc and the two 9° points for (GlcNAc)$_3$).

Experiments at two temperatures at pH 2 gave for (GlcNAc)$_2$, $\Delta H^{0.0} = -6.1$ kcal. The difference between

$^5$ This number, based on 15 and 30° data only, has an estimated error of 0.5 to 1 kcal. $\Delta H^\text{assoc}$ calculated in the same way for trimer at pH 2 is $-9.1$ kcal, compared with $-8.6$ determined from the complete set of data.

TABLE III

Thermodynamics of association at pH 5.3, 25°, 0.1 ionic strength

Uncertainties are maximum values, based on error in log $K^\text{assoc}$ of 0.1 for GlcNAc and 0.05 for the dimer and trimer. Standard deviations from least squares analyses are given in parentheses.

| Saccharide | $\Delta R^3$ | $\Delta H^0$ | $\Delta S^0$ |
|-----------|----------|---------|---------|
|           | kcal     | kcal    | e.u.    |
| GlcNAc    | $-2.22 \pm 0.14$ | $-6.2 \pm 0.8$ | $-13.4 \pm 2.6$ |
|           | (0.03)   | (0.5)   | (1.0)   |
| (GlcNAc)$_2$ | $-4.55 \pm 0.07$ | $-11.4 \pm 0.6$ | $-21.6 \pm 1.9$ |
|           | (0.04)   | (0.3)   | (0.8)   |
| (GlcNAc)$_3$ | $-7.23 \pm 0.07$ | $-14.3 \pm 0.6$ | $-23.8 \pm 1.9$ |
|           | (0.02)   | (0.2)   | (0.6)   |
Data. The following two factors, which are important for proton saccharide dissociates the lysozyme dimer (25). Increase in the lysozyme undergoes pH-dependent association (24). Binding of saccharide dissociates the lysozyme dimer (25). Increase in the lysozyme concentration increased $\Delta F_{\text{H}^+}$, which accords with the contribution expected from self-association. At high pH and at the high tri saccharide concentration used in these experiments, a second molecule of ligand has been shown to bind to lysozyme with proton release (26).

**DISCUSSION**

**Thermodynamics of Oligosaccharide Binding at pH 5.3**—Table III gives thermodynamic parameters for association of lysozyme at pH 5.3 with the monomer, dimer, and trimer of $N$-acetylglucosamine. The free energy values of the present work agree well with literature data (6). The vant Hoff enthalpy values are for all three saccharides about 10% higher than the calorimetric values (8). The agreement between calorimetric and vant'Hoff values is satisfying and the simple equilibrium model used to interpret the data apparently is correct.

$N$-Acetylglucosamine binds at site C with an enthalpy of association of $-6.2$ kcal. There are four hydrogen bonds between enzyme and saccharide. If one accepts the premise that nonpolar interactions contribute much less to the enthalpy change than do hydrogen bonds, then this result suggests that formation of an enzyme-saccharide hydrogen bond (actually exchange of partners between enzyme-water and saccharide-water) is on the average associated with an enthalpy change of $-1.5$ kcal. These numbers agree with the more negative of the estimates for the enthalpy of hydrogen bond formation made from model aqueous systems of several types (27-29). This agreement is comforting considering the difficulty in drawing analogies between reactions of small molecules and macromolecules. For reasons discussed below, the enthalpy of interaction at sites A and B cannot be simply related to the number of hydrogen bonds.

The differences between thermodynamic parameters for association of lysozyme with different oligosaccharides have been used to estimate the thermodynamic values for interaction of mono saccharide units at the A, B, and C regions of the active site (for a discussion, see Imoto et al. (6)). Johnson et al. (30) noted the good correlation between number of atom-to-atom contacts and $\Delta F_{\text{H}^+}$ for the several subsites. In contrast, the enthalpy changes do not correlate well. It can be seen in Table III that there is a much larger difference in enthalpy than in free energy between binding of the disaccharide at regions B and C and of the monosaccharide at region C. The estimated enthalpy of binding of a monosaccharide unit at B is $-5.2$ kcal (difference between GlcNAc and GlcNAc), compared with $-6.2$ for C. The substrate forms one hydrogen bond at the B region and four at the C region, and the number of atom-to-atom contacts are in the ratio 1:3. An anomaly that involves a charged group that interacts with substrate can explain the large enthalpy change for binding at region B (see below).

**Assignment of pK Values to Side Chain Groups**—Table IV correlates pK values of Table II with groups in the lysozyme active site. Ionizations with pK near 6 and 4 have been assigned to glutamic 35 and aspartic 101, respectively, in earlier publications (1, 8). Aspartic 101 is the most exposed of the side chain carboxyls of lysozyme and it is reasonable for it to have a normal pK. Glutamic 35 is largely buried in a nonpolar region of the cleft and it is not hydrogen bonded to other protein groups. This environment is consistent with an abnormal and high pK.

Aspartic 52 has been reported to have a pK above 4 and not to be affected by saccharide binding (31). Parsons and Raftery (31) in a titration comparison of lysozyme esterified at aspartic 52 with the native protein showed that there is ionic-strength dependent interaction between aspartic 52 and glutamic 35. They estimated the enthalpy of ionization of glutamic 35 as 2.9 kcal.
A group of pK below 2 has been seen in one previous report on (GlcNAc)₃ binding (1). These measurements are confirmed and are extended to the disaccharide. The assignment of the low pK group as aspartic 66 follows most directly from the crystallographic observation that this is the only carboxylate acid residue, other than the above three, that has environment perturbed by saccharide binding. Aspartic 66 is completely buried within the wing of the cleft that moves slightly when saccharide binds. Several points are of interest in connection with this abnormal ionization assigned to aspartic 66. This carboxyl lies near arginine 68; it participates in several hydrogen bonds; one of the hydrogen bonds involves tyrosine 53, which is of abnormally high pK (>12; Tojo et al. (32)); denaturation studies show one or more groups of pK <2 (for review, see Ikeda et al. (6)). Since aspartic 66 does not contact saccharide, the pK shift (pK₆₆ < pK₃₆ ≈ 1.9), which is expected is the same for dimer and trimer, presumably reflects small changes in conformation associated with movement of the structure about tryptophan 62. The complete withdrawal of a charged group from solvent is noteworthy. Apparently a hydrogen-bond network can adequately disperse charge without ion pair formation (arginine 68 is 7 A from aspartic 66). It is also noteworthy that the pK is shifted to a lower value. Regardless of the free energy cost of burying a charged group, the hydrogen-bond network within the protein favors carboxylate over carbonyl more than does water.

Ikeda and Hamaguchi (5) report pK 2.5 for GlcNAc binding, which was not observed with the β-methyl-glycoside (8). Vandenhoff (33) could not confirm the pK 2.5 for GlcNAc but observed a group of pK 1.5 to 2 for GlcNAc and its β-methyl-glycoside, which is in accord with the dimer and trimer results.

**Anomalous Enthalpy of Protonation of Aspartic 101 in Complex**

Formation of the trisaccharide-lysozyme complex shifts the pK of aspartic 101 down by 0.9 units, a loss through protonation of 1.2 kcal in ΔH°, assoc (Table IV). Although this number is substantial, the corresponding change in ΔH°, ≈ -7.3 kcal, is notably larger. It is balanced by an almost equivalent change of -21 e.u. in ΔS°. Fig. 4 also shows clearly the compensation between enthalpy and entropy.

Aspartic 101 forms two hydrogen bonds with (GlcNAc)₃ and one with (GlcNAc)₂. There are no adequate experimental determinations of ΔH° for formation of carbonyl-lysine or carbonyl-hydroxy hydrogen bonds in aqueous solution. Acceptable estimates would not run greater than -1 to -2 kcal (29), i.e., about -3 kcal for the pair of aspartic 101 interactions. The -7 kcal effect of protonation is thus anomalous, more strikingly so if we consider that protonation of aspartic 101 likely does not rupture but only weakens its hydrogen bonds in the complex.

Dimer perturbs the pK of aspartic 101 about half as much as does trimer, which accords with only one hydrogen bond between dimer and aspartic 101. However, change in pH from 5.3 to 2 increases ΔH°, assoc by 5.3 kcal for dimer compared to 5.9 kcal for trimer. The enthalpy anomaly found for the trimer complex exists also with dimer.

The strong compensation between changes in enthalphy and entropy focuses attention on the solvent. Aspartic 101 is the most exposed carboxylate of the lysozyme molecule; binding of trimer reduces its exposure to solvent to below that of all carboxylates except aspartate 66 (6). Aspartic 101 in the complex is nearly enclosed in a rigid matrix of protein and saccharide atoms, which unlike a water shell, cannot rearrange during protein dissociation. There is support for the suggestion that replacement of the water about an ionizable group can make a large negative contribution to the enthalpy of proton dissociation. The thermodynamic parameters for ionization of carboxylic acids typically show, for transfer from water to mixed aqueous solvent, positive values of ΔAFA, with much smaller and sometimes negative values of ΔAH° and negative values of ΔAS° (e.g. acetic acid from water to 70% dioxane, ΔAFA = 4.9 kcal, ΔAH° = -0.5 kcal, ΔAS° = -18.0 e.u. (34); to 70% ethylene glycol, 1.5, 0.3, -4.0 (35); to 50% methanol, 1.2, 0.04, -4.0 (36). This does not conform to naive expectation, which because of the presumably greater electrostatic work required to charge in the medium of lower dielectric constant, is that ΔAH° should be positive and the principal determinant of ΔAFA. The fact that this is not true has been explained, as have the negative values for ΔAS°, in terms of changes in solvent structure (37). The point made here is that acetic and other acids give unexpected behavior in a transfer like the protein process of interest. Specifically ΔAH° is more negative than ΔAFA for increased concentration of nonaqueous solvent. Also, transfer of an ion to a mixed aqueous solvent should show residual solvation effects that would be absent for transfer into the more rigid protein ligand complex, and the absence of these should magnify for the protein the negative contribution of ion transfer to ΔAH° and ΔAS°.

Since charged side chains frequently participate in protein interactions, large negative entalpies of ionization generally may be a factor in protein reactions. Unfolding of the structure would remove anomalies, and ionizable groups perhaps contribute more substantially than has been thought to enthalpies of protein denaturation.

**REFERENCES**

1. **RUPLEY, J. A., BUTLER, L., ORRINGTON, M., HARTDEGEN, F. J., AND PECORARO, K. (1967) Proc. Nat. Acad. Sci. U. S. A. 57, 1088**

2. **CHIPMAN, D. M., GREGSON, V., AND SHARON, N. (1967) J. Biol. Chem. 242, 4388**

3. **DAHLQUIST, F. W., JAO, L., AND RAPPERTY, M. (1966) Proc. Nat. Acad. Sci. U. S. A. 56, 29**

### Table IV

Assignment to specific groups of pK values and changes in thermodynamic parameters

| i   | Group   | pK_i^* | (GlcNAc)_3 | (GlcNAc)_2 | ΔpK^* | ΔΔH° | ΔΔS° | ΔΔF° |
|-----|---------|--------|------------|------------|-------|------|------|------|
| 1   | Asp 66  | 1.0    | 1.5        | 1.5        | -0.35 | -7.3 | -10.5 | -10.2 |
| 2   | Asp 101 | 4.3    | 3.4        | 4.0        | -0.23 | -4.3 | -7.6 | -7.4 |
| 3   | Glu 35  | 6.1    | 6.4        | 6.6        | +0.27 | +2.2 | +6.2 | +6.0 |

ΔΔF° etc., are the parameter changes for the free enzyme less that for the species with saccharide bound.
4. LEHRER, S. S., AND FASMAN, G. D. (1966) Biochem. Biophys. Res. Commun. 23, 133
5. IKEDA, K., AND HAMAGUCHI, K. (1969) J. Biochem. 66, 513
6. IMOTO, T., JOHNSON, L. N., NORTH, A. C. T., PHILLIPS, D. C., AND RAFTERY, J. A. (1972) in The Enzymes (Boyer, P. D., ed) 3rd Ed, Vol. 7, p. 665, Academic Press, New York
7. DAHLQUIST, F. W., AND RAFTERY, M. A. (1968) Biochemistry 7, 3269
8. DAHLQUIST, F. W., AND RAFTERY, M. A. (1968) Biochemistry 7, 3277
9. BJURULF, C., LAYNEZ, J., AND WADSÖ, I. (1970) Eur. J. Biochem. 14, 47
10. SYKES, B. D. (1969) Biochemistry 8, 1110
11. SYKES, B. D., AND PARRAVANO, C. (1969) J. Biol. Chem. 244, 3900
12. KUPLEY, J. A. (1964) Biochem. Biophys. Acta 83, 245
13. FRUGONI, J. A. C. (1957) Gazz. Chim. Ital. 87, 403
14. BATES, R. G. (1954) Electrolytic pH Determinations, p. 111, John Wiley and Sons, Inc., New York
15. HARTDEGEN, F. J. (1966) Ph.D. thesis, University of Arizona, Tucson
16. LEHRER, S. S., AND FASMAN, G. D. (1967) J. Biol. Chem. 242, 4644
17. WYMAN, J. (1964) Advan. Protein Chem. 19, 222
18. HAYASHI, K. T., IMOTO, T., AND FUNATSU, J. (1963) J. Biochem. 54, 381
19. DRAKE, C. C. F., JOHNSON, L. N., MAE, G. A., NORTH, A. C. T., PHILLIPS, D. C., AND SHARMA, V. R. (1967) Proc. Roy. Soc. London Ser. B 167, 375
20. WETTLEFFER, D. B. (1962) Advan. Protein Chem. 17, 303
21. DONOVAN, J. W., LASKOWSKI, M., AND SCHERAGA, H. A. (1961) J. Amer. Chem. Soc. 83, 2686
22. DAHLQUIST, F. W., AND RAFTERY, M. A. (1969) Biochemistry 8, 1115
23. MOORE, R. H., AND ZIEGLER, R. K. (1960) Los Alamos Report LA-2067 and Addendum by P. McWilliams
24. SOPHIANOPOULOS, A. J., AND VAN HOLDE, K. E. (1964) J. Biol. Chem. 239, 2516
25. SOPHIANOPOULOS, A. J. (1969) J. Biol. Chem. 244, 3188
26. ROSI, G. L., HOLLER, E., KUMAR, S., RAFTERY, J. A., AND HESS, G. P. (1969) Biochem. Biophys. Res. Commun. 7, 757
27. TANFORD, C. (1970) Advan. Protein Chem. 24, 1
28. BRANDTS, J. F. (1969) in Structure and Stability of Biological Macromolecules (TIMASHEFF, S. N., AND FASMAN, G. D., eds) p. 213, Marcel Dekker, Inc., New York
29. SCHERAGA, H. A. (1963) in The Proteins (Neurath, H., ed) Vol. 1, p. 477, Academic Press, New York
30. JOHNSON, L. N., PHILLIPS, D. C., AND RAFTERY, J. A. (1968) Brookhaven Symp. 21, 120
31. PARSONS, S. M., AND RAFTERY, M. A. (1972) Biochemistry 11, 1623, 1630, 1633
32. TOJO, T., HAMAGUCHI, K., IMANISHI, M., AND AMANO, T. (1966) J. Biochem. 60, 538
33. VANDENHOUFF, G. (1970) Ph.D. thesis, University of Arizona, Tucson
34. HARNED, H. S., AND OWEN, B. B. (1958) The Physical Chemistry of Electrotropic Solutions, 3rd Ed, Reinhold, New York
35. BANERJEE, S. K. (1966) Ph.D. thesis, Jadavpur University, India
36. PABA, M., ROBINSON, R. A., AND BATES, R. G. (1965) J. Amer. Chem. Soc. 87, 415
37. FEAHINS, D. (1967) in Physico-Chemical Processes in Mixed Aqueous Solvents (FRANKS, F., ed) p. 71, Heinemann, London
Temperature and pH Dependence of the Binding of Oligosaccharides to Lysozyme

Surath K. Banerjee and J. A. Rupley

J. Biol. Chem. 1973, 248:2117-2124.

Access the most updated version of this article at http://www.jbc.org/content/248/6/2117

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/6/2117.full.html#ref-list-1