Thermochemistry of Binding of α- and β-N-Acetylglucosamine to Hen Egg-White Lysozyme. Effects of Specific Oxidation of Tryptophan-62†

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ABSTRACT: The binding of the α and β anomers of N-acetylglucosamine to hen egg-white lysozyme and to the Trp-62-oxidized enzyme has been measured by sensitive microcalorimetry at 5° and at two values of pH. Binding of mutarotated mixtures of saccharide was studied at 5 and 25°. At pH 5.0 the α anomer binds somewhat more strongly to native lysozyme than the β anomer, though the binding is less exothermic. Specific oxidation of tryptophan-62 markedly reduces the binding of the β anomer, but has a lesser effect on the α form of the saccharide. At pH 3.0 differences in the binding of the two anomers are less marked, and oxidation of Trp-62 has a smaller effect on binding. It is proposed, on the basis of the apparent enthalpies and dissociation constants at pH 5.0, that α-N-acetylglucosamine might bind in two, mutually exclusive, orientations in subsite C of the lysozyme active cleft, one of which corresponds to the orientation taken up by the β anomer.

Binding of simple saccharide inhibitors to hen egg-white lysozyme has been useful in the interpretation of the interactions involved in the lysozyme-substrate complex (Imoto *et al.*, 1972), and the system is particularly valuable for the study of the thermodynamics of protein-ligand interactions since detailed structural information is available (Blake *et al.*, 1967; Beddell *et al.*, 1970). Of particular interest is the binding of N-acetylglucosamine, a monosaccharide subunit of the polysaccharide substrate, which binds in the most specific site (subsite C of Blake *et al.*, 1967) of the active cleft of the enzyme. Studies of this inhibitor are, however, complicated by the mutarotation of the saccharide in solution to a mixture of the α and β anomers (α:β ratio approximately 60:40 at equilibrium) both of which bind, competitively, to the same site on the enzyme. Crystallographic (Blake *et al.*, 1967) and nuclear magnetic resonance studies (Dahlquist and Raftery, 1968) have indicated that, at about pH 5 at least, the two anomers bind in somewhat different orientation, so that detailed interpretation of measurements with mutarotated mixtures is not straightforward. Furthermore, the difference in binding of these two closely related molecules is of potential value in evaluating the various contributions to protein-ligand interactions.

Formation of the α-GlcNAc-lysozyme complex has been studied by ultraviolet (uv) difference spectroscopy (Kowalski and Schimmel, 1969) and the binding of the α and β anomers in mutarotated solution have been differentiated by nmr (Dahlquist and Raftery, 1968) but previous calorimetric studies have been confined to the mutarotated mixture (Bjurulf and Wadsö, 1972). Experiments are reported here of the binding of the individual GlcNAc[1] anomers to hen egg-white lysozyme at pH 5.0 and 3.0, using a sensitive flow microcalorimeter. These experiments are performed at 5°, at which temperature mutarotation is slow enough that essentially pure α or β anomer exists in solution during the time of the experiment. Results are also reported for the interaction of NBS-oxidized lysozyme with α- and β-GlcNAc. Hayashi *et al.* (1965) have shown that, under the conditions used here, NBS treatment of native lysozyme results in specific oxidation of tryptophan-62, which occupies an important position in the GlcNAc binding site (Blake *et al.*, 1967). This modification turns out to be a useful probe for interpretation of the binding data for the two anomers.

Experimental Section

Materials. Hen egg-white lysozyme (three-times crystallized) and α-N-acetyl-D-glucosamine were obtained from Sigma, and used without further purification. β-GlcNAc was prepared by acetylation of β-glucosamine according to the method of Kuhn and Haber (1953). Ninhydrin analysis indicated less than 1% free glucosamine in this preparation. (See Results section for optical rotation data.) Specific oxidation of lysozyme by treatment with equimolar N-bromosuccinimide followed the procedure of Hayashi *et al.* (1965) except that their dialysis step was replaced by more rapid desalting on a G-25 Sephadex column prior to lyophilization, without any apparent effect on the extent of oxidation.

Buffers used in the calorimetric studies were 0.1 M acetate or 0.1 M piperazine (pH 5.0) and 0.1 M citrate (pH 3.0), pH measured at room temperature on a Radiometer TTT1a pH meter. Distilled or deionized water was used throughout.

Native lysozyme concentrations were determined spectrophotometrically using an E_{280nm} of 26.5 (Bjurulf and Wadsö, 1972). The extinction coefficient for NBS-oxidized lysozyme was determined from the decrease in extinction at 280 nm during the oxidation reaction. This also served to check the extent of tryptophan oxidation (Spande and Witkop, 1967). Two samples of oxidized lysozyme, estimated to contain 0.94 and 1.04 oxidized tryptophans, respectively, were used without significant differences in results. The molecular weight of lysozyme was taken to be 14,600.

Protein solutions for calorimetry, made up immediately before use, were equilibrated with the appropriate buffer on a G-25 Sephadex column. Initial protein concentrations were routinely about 1.3 mM.

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[1] Abbreviations used are: GlcNAc, N-acetyl-D-glucosamine; NBS, N-bromosuccinimide.

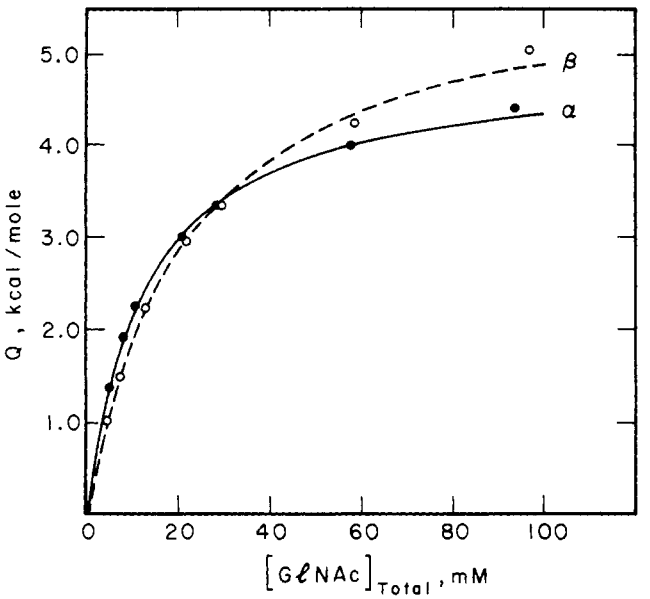

FIGURE 1: Calorimetric heats of binding of α and β anomers of *N*-acetylglucosamine to native lysozyme in 0.1 M acetate buffer (pH 5.0, 5°) as a function of total saccharide concentration. Lines are theoretical hyperbolic binding curves, calculated using the parameters given in Table II. The final enzyme concentration in this experiment was 0.546 mM.

Mutarotation of GlcNAc was followed at 589.3 nm using a Cary 60 recording spectropolarimeter fitted with 1-cm thermostatted cells. Saccharide concentrations were about 1% in 0.1 M acetate buffer (pH 5.0). Solutions for mutarotation equilibrium measurements were incubated overnight in stoppered containers at the appropriate temperature.

Calorimetric measurements utilized the flow modification of the Beckman Model 190 microcalorimeter (Sturtevant and Lyons, 1969; Sturtevant, 1969; Velick *et al.*, 1971) fitted with a LKB Perpex peristaltic pump for injection of saccharide solutions. Binding of individual anomers was measured at 5° and mutarotated solutions at 5 and 25°. For studies of α- and β-GlcNAc binding the pump and saccharide solutions were maintained at 5°, or below, to minimize mutarotation prior to reaction. GlcNAc solutions were made up immediately before use in ice-cold buffer. At the flow rates used in these experiments about 15 min are required for solutions to reach the reaction junction of the calorimeter and for the instrument to achieve a steady state. Thus the heats of reaction could be measured about 15–20 min after dissolution of the saccharide. Experiments with α and β anomers were run in parallel in order to minimize systematic variations. For the binding of mutarotated mixtures of GlcNAc the saccharide solutions were left at room temperature for at least 4 hr before use. Viscous heating and heats of dilution of protein and saccharide solutions were measured separately, under identical conditions, and the appropriate corrections were made to the observed heats of reaction. Heat of dilution of the enzyme solutions was insignificant in all cases. GlcNAc solutions were varied over at least a tenfold concentration range (between 5 and 100 mM, final concentration) to generate lysozyme–GlcNAc thermal titration curves (Figure 1, for example). The calorimeter was calibrated at 5 and 25° using the heat of protonation of Tris (National Bureau of Standards solution calorimetry standard) and heat of ionization of water (Grenthe *et al.*, 1970).

Thermal binding data were analyzed to obtain the apparent enthalpies and dissociation constants assuming simple 1:1 complex formation between saccharide and enzyme. Experimental heats were fitted by iterative least-squares procedures to the double-reciprocal expression (Bjurulf and Wadsö, 1972)

$$-1/Q = 1/\Delta H + K/\Delta H[\mathrm{I}]$$

and to the complete hyperbolic binding equation

$$Q = \frac{-\Delta H}{2C_E}(C_E + C_I + K) \times \left\{1 - \sqrt{1 - \frac{4C_E C_I}{(C_E + C_I + K)^2}}\right\}$$

where Q is the observed heat of binding per mole of enzyme; C_E and C_I are total enzyme and saccharide concentrations, respectively; [I] is the free saccharide concentration; and, ΔH and K are the apparent enthalpy and dissociation constant of the complex.

Except in the case of very weak binding (*i.e.*, β-GlcNAc to NBS-treated lysozyme) both procedures gave estimates of ΔH and K that were identical within estimated error. Double-reciprocal plots were linear over the concentration range studied.

TABLE I: Thermodynamic Data for Interaction of Mutarotated GlcNAc with Lysozyme at pH 5.0.[a]

Temp (°C)	$K_{\alpha\beta}$ (mM)	$-\Delta H_{\alpha\beta}$ (kcal/mol)	$-\Delta G^\circ_{\alpha\beta}$ (kcal/mol)	$-\Delta S^\circ_{\alpha\beta}$ (eu)
25[b]	25.1	5.81	2.18	12.1
25[c]	23.1	5.81	2.23	12.0
25[d]		5.81	2.15	12.2
5[b]	16.3	5.18	2.28	10.5
25[e]	34.5	2.99	1.99	3.35
Estimated error limits	±2	±0.2	±0.05	±1

[a] Using $\Delta G^\circ = \Delta H - T.\Delta S^\circ = RT \ln K$, 1 cal = 4.184 J. [b] Native lysozyme, 0.1 M acetate. [c] Native lysozyme, 0.1 M piperazine. [d] Results of Bjurulf and Wadsö (1972), native lysozyme, 0.1 M acetate (pH 5.0). [e] NBS-oxidized lysozyme, 0.1 M acetate.

Results

Mutarotation of GlcNAc. The specific rotation of fully mutarotated solutions of α- or β-GlcNAc, $[\alpha]_D$ +40.2° (±0.3°), was found to be the same, within experimental error, at 5, 25, and 40°, and compares well to the value of +40.4° found by Kuhn and Haber (1953). By extrapolation to zero time, the specific rotations of the individual anomers were estimated to be: α-GlcNAc, +81.0° (±1.0) at 5 and 25° (Kuhn and Haber, +82°); β-GlcNAc, −22.7° (±1.0°) at 20° (Kuhn and Haber, −21.5°). The halftime for mutarotation at 5° was found to be 125 min compared to about 30 min at 20° (Kuhn and Haber, 1953). This implies that in the calorimetric binding experiments with the pure anomers at 5° less than about 5% mutarotation takes place before the measurement is made. Furthermore, the invariance of optical rotation with temperature implies that the enthalpy of mutarotation of GlcNAc is very small ($|\Delta H_{mut}| < 50$ cal/mol) so that no significant heat effect due to mutarotation would interfere with the calorimetric binding studies; and indeed, no such heat effect was observed. This heat of mutarotation is somewhat smaller than observed for other saccharides (Sturtevant, 1937, 1941; Kabayama *et al.*, 1958).

Calorimetric Binding Studies. Thermodynamic data for the interaction of fully mutarowated mixtures of α- and β-GlcNAc with native and NBS-oxidized lysozyme are given in Table I.

TABLE II: Data for Binding of α- and β-GlcNAc to Native and NBS-Oxidized Lysozyme at 5°.[a]

	Anomer	K_{app} (mM)	$-\Delta H_{app}$ (kcal/mol)	$-\Delta G°_{app}$ (kcal/mol)	$-\Delta S°_{app}$ (eu)
pH 5.0[b]					
Native	α	12.7	4.89	2.41	8.9
	β	22.4	6.04	2.10	14.1
NBS treated	α	26.5	3.72	2.01	6.1
	β	90 (±15)	4.6 (±0.4)	1.3 (±0.1)	12 (±2)
pH 3.0[c]					
Native	α	19.6	4.58	2.17	8.6
	β	17.1	5.01	2.25	9.9
NBS treated	α	23.4	3.82	2.08	6.25
	β	36 (±5)	3.29 (±0.3)	1.8 (±0.1)	5.2 (±1.5)

[a] Except where indicated, estimated errors are as in Table I. [b] 0.1 M acetate. [c] 0.1 M citrate.

Results were superimposable regardless of whether mutarotation was from the α or the β preparation, and the values at 25° for native lysozyme are in excellent agreement with those of Bjurulf and Wadsö (1972) under the same conditions. Furthermore, the identity of the enthalpies of binding in acetate and in piperazine buffers implies that, at pH 5.0, there is no change in net protonation of the enzyme upon complex formation with mutarotated GlcNAc. This follows because the heat of protonation of acetate is essentially zero (Christensen *et al.*, 1967) whereas that of piperazine, at pH 5, is about −7.1 kcal/mol (Paoletti *et al.*, 1963), and it is consistent with the plateau found for the binding of various ligands at this pH. Nor do there appear to be any specific buffer effects.

From the apparent enthalpies of binding at 5 and 25° one can estimate $\Delta C_p° = -0.0315$ (±0.02) kcal/(deg mol), which is consistent with the value −0.053 (±0.038) kcal/(deg mol) determined by Bjurulf and Wadsö (1972) from measurements at 25 and 40°.

Oxidation of Trp-62 with NBS reduces the apparent free energy of GlcNAc binding by about 0.2 kcal/mol at 25°, but effects a somewhat greater reduction in the apparent enthalpy of the complex.

Table II lists the data for binding of the individual anomers to native and NBS-oxidized enzyme. No attempt has been made to correct for any contribution due to the small amount of mutarotation prior to the calorimetric measurements. Any such corrections would be small in this case. For the native enzyme at pH 5.0 there is a significant difference in both the dissociation constants and the enthalpies of binding of the two anomers (Figure 1). α-GlcNAc has almost double the binding affinity of the β anomer, though the binding of β-GlcNAc is the more exothermic. A similar difference in binding constants has been observed in nmr studies (Dahlquist and Raftery, 1968). Oxidation of Trp-62 decreases both binding affinity and enthalpy of both anomers, though the effect on the β anomer is the more marked. At pH 3.0 differences are less apparent, though the effects of oxidation are qualitatively the same.

As a check on the consistency of the results one may use the binding data for the individual anomers to estimate the parameters one should observe for a fully mutarotated mixture under the same conditions. For the scheme

$$E + \alpha \rightleftarrows E\alpha \qquad \Delta H_\alpha, K_\alpha = [E][\alpha]/[E\alpha]$$

$$E + \beta \rightleftarrows E\beta \qquad \Delta H_\beta, K_\beta = [E][\beta]/[E\beta]$$

$$\alpha \rightleftarrows \beta \qquad \Delta H_{mut}, K_{mut} = [\alpha]/[\beta]$$

it is straightforward to show that the apparent enthalpy ($\Delta H_{\alpha\beta}$) and dissociation constant ($K_{\alpha\beta}$) for the mutarotated mixture are given by

$$K_{\alpha\beta} = \frac{K_\beta(1 + K_{mut})}{1 + (K_\beta K_{mut}/K_\alpha)}$$

and

$$\Delta H_{\alpha\beta} = \frac{(K_{mut}K_\beta/K_\alpha)\Delta H_\alpha + \Delta H_\beta}{1 + (K_{mut}K_\beta/K_\alpha)} + \frac{K_{mut}[1 - (K_\beta/K_\alpha)]\Delta H_{mut}}{(1 + K_{mut})[1 + (K_{mut}K_\beta/K_\alpha)]}$$

The second term in the expression for $\Delta H_{\alpha\beta}$ arises from any shift in total α:β equilibrium due to differential binding by the enzyme. In the present case this term may be neglected, since ΔH_{mut} is so small. Setting $K_{mut} = 1.54$ (estimated from specific rotation data) and using the parameters for binding of individual anomers from Table II, one obtains $K_{\alpha\beta} = 15.3$ mM and $\Delta H_{\alpha\beta} = -5.20$ kcal/mol for the interaction of the mutarotated mixture at pH 5.0, 5°. These are in good agreement with the actual measured values (Table I).

Discussion

In the crystallographic studies by Blake *et al.* (1967) of the interaction of crystalline lysozyme with the anomeric mixture of GlcNAc, the difference electron density map was interpreted in terms of two distinct modes of binding for the two anomers in site C of the active cleft of the enzyme. It is consistent with this model that oxidation of tryptophan-62 affects the binding of β-GlcNAc rather more than the α anomer, since β-GlcNAc appears to be in direct contact with this residue *via* a hydrogen bond (Blake *et al.*, 1967; Beddell *et al.*, 1970), whereas the saccharide moiety of the α anomer lies somewhat lower in the cleft. Though we have no direct structural information, Takahashi *et al.* (1965) have shown from optical rotatory dispersion studies that NBS oxidation of a single tryptophan produces no apparent change in secondary or tertiary structure of the enzyme; thus, the most likely result of oxidation of Trp-62 is the reorientation of the oxindole derivative upon conversion of the γ-carbon from a planar to a tetrahedral configuration. This reorientation might partially block the active cleft or, possibly, displace the oxindole ring from the cleft so that it is no longer available for hydrogen bonding. In either case binding of β-GlcNAc should be weakened, though steric hindrance would appear to be the more reasonable alternative in view of the recent observation that the hydrogen bond between Trp-62 and β-saccharides makes no apparent contribution to the free energy of binding (Kuramitsu *et al.*, 1973).

Binding of α-GlcNAc to native lysozyme at pH 5.0 is little stronger than β-GlcNAc binding, and it is not clear from structural considerations why the α anomer should not bind in the same orientation as the β, as do the α-methyl glycosides (Blake *et al.*, 1967; Raftery and Dahlquist, 1969). It should be remembered that crystallography, thermochemistry and nmr (in the fast exchange limit) all give time-averaged observations, and it is not implausible to suggest that α-GlcNAc binds to the native enzyme in *both* of the modes described by Blake *et al.* (1967). If this were the case then the apparent binding parameters could be written as

$$K_{\alpha,app} = K_1K_2/(K_1 + K_2)$$

$$\Delta H_{\alpha,app} = (K_2\Delta H_1 + K_1\Delta H_2)/(K_1 + K_2)$$

where K_1, K_2, ΔH_1, and ΔH_2 are the intrinsic dissociation constants and enthalpies for binding of the ligand in two mutually exclusive orientations. Supposing that α-GlcNAc might bind in the β configuration with the same K and ΔH as β-GlcNAc and that, after oxidation of Trp-62, α-GlcNAc can bind only in the α mode, then one obtains, using the data of Table II, estimates for the apparent binding of the α anomer, at pH 5.0 and 5°, to native lysozyme: $K_{\alpha,app}$ = 12.1 mM, $\Delta H_{\alpha,app}$ = −4.98 kcal/mol, in good agreement with the observed values (Table II). Evidence such as this, though indirect, suggests that α-GlcNAc could bind equally well in two distinct modes, both consistent with the known structure of the enzyme.

Interpretation of the results at pH 3.0 is less clear, though the effect of change in pH on the binding constants illustrates one of the difficulties of working solely with the mutarotated mixture of saccharides, in that fairly large changes in binding of individual anomers may compensate to give relatively little change in the apparent parameters for the mixture. The differing results of Trp-62 oxidation on the binding of the two anomers at pH 3.0 seem inconsistent with the proposal of Studebaker *et al.* (1971) that both anomers bind in the same orientation at this pH.

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