Difference between tortoise and hen lysozymes in the mode of association with *N*-acetyl D-glucosamine as revealed by proton magnetic resonance spectroscopy

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Tortoise lysozyme, unlike hen lysozyme, does not distinguish between the α- and β-anomeric forms of *N*-acetyl D-glucosamine. This indicates that α- and β-anomeric forms of the inhibitor have the same affinities and experience identical magnetic environments in tortoise lysozyme. The dissociation constant of tortoise lysozyme–inhibitor complex was calculated from chemical shift data and found to be 3.5×10^{-2} M. The enthalpy of dissociation was calculated to be 5.0 kcal/mol.

Lysozyme association N-Acetyl D-glucosamine PMR Chemical shift (tortoise, hen)

1. INTRODUCTION

Investigations on crystallographic and solution properties of hen lysozyme and its complexes with *N*-acetyl D-glucosamine (NAG) and its oligomers have contributed significantly to our understanding of the molecular basis of enzyme action in general [1,2]. Structural studies on lysozymes isolated from different species can play a useful role in the elucidation of the chemistry of enzyme action since a comparison of their three-dimensional structures, amino acid sequences and solution properties is expected to reveal the way in which relatively minor structural alterations are reflected in the dynamic properties of the enzyme. Tortoise egg-white lysozyme, the first to be purified from a reptilian source, was found to resemble hen lysozyme in M_r-value, amino acid composition and enzymatic properties [3] as well as in primary structure [4]. Crystallographic study [5] of tortoise lysozyme showed that while the tertiary structure is closely homologous to that of hen lysozyme, it incorporates a large proportion of liquid extending to the active site; a feature which has been stated to be suitable for low temperature studies of the enzyme–substrate complex. In view of the gross similarities and subtle differences between tortoise and hen lysozymes, the study of the interaction of tortoise lysozyme with NAG by proton magnetic resonance (PMR) spectroscopy which has the ability to detect even minor environmental changes appeared to be of much interest.

From a PMR study of the association of hen lysozyme with NAG [6], in the presence of the enzyme, the resonance corresponding to the acetamido methyl protons underwent a chemical shift to a higher field as well as splits into two separate lines corresponding to the α- and β-anomeric forms of the inhibitor. It was shown that while the α- and β-anomeric forms of NAG compete for the same site of the enzyme, they have different affinities and bind in different orientations [7]. Further, while β-NAG, α- and β-methyl *N*-acetyl glucosaminides experience identical magnetic environments in hen lysozyme, α-NAG was unique in that it binds with a different orientation. It was of interest to see whether tortoise lysozyme displayed similar features.

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2. MATERIALS AND METHODS

The lysozyme used in this study was isolated and purified from the egg whites of *Trionyx gangeticus* Cuvier as in [3]. About 3×10^{-3} M tortoise lysozyme solutions were made in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.5% acetone as an internal standard. The exact concentrations of the enzyme in the samples used for PMR measurements were determined from ultraviolet absorbance at 280 nm using an extinction coefficient of 27.00 [3].

NAG (Sigma Chemicals, St Louis MO) was dissolved in the buffer 20 h before measurements were taken to allow it to attain mutarotational equilibrium.

The measurements of PMR spectra were performed on a Varian Model EM 390 PMR spectrometer with a variable temperature unit model EM 3940. The chemical shift of the acetamido methyl protons of NAG was measured relative to the internal standard of acetone (0.5%). When working at different temperatures, the samples were allowed to attain thermal equilibrium with the probe temperature.

3. RESULTS AND DISCUSSION

Fig.1 shows the PMR spectrum at 90 MHz of the acetamido methyl protons of a mutarotated solution of NAG in the absence as well as the presence of tortoise and hen lysozymes. Both samples contained 0.5% acetone as internal standard. It can be seen that in the presence of tortoise lysozyme there is no splitting of the resonance signal as in the case of hen lysozyme although there is a slight broadening as well as an upfield shift which indicate binding of NAG to tortoise lysozyme. The splitting of the acetamido methyl proton resonance of NAG in the presence of hen lysozyme as shown in [6] was reproduced by us (fig.1). Thus, tortoise lysozyme, unlike hen lysozyme cannot distinguish between the α- and β-anomeric forms of NAG. This indicates that α- and β-anomers of NAG have the same affinities for tortoise lysozyme and they experience identical magnetic environments since a difference in either binding constants or magnetic environments would be enough to cause splitting of the resonance into two separate lines [6,7].

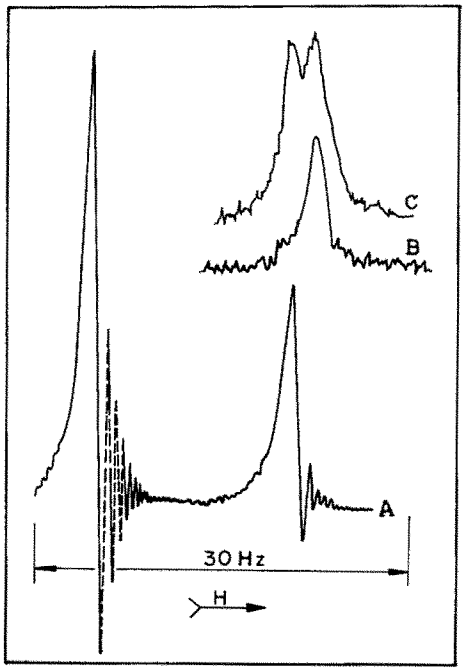

Fig.1. (A) PMR spectrum at 90 MHz of the acetamido methyl protons of NAG in 0.05 M sodium phosphate buffer (pH 7.0). Acetone (0.5%) was used as an internal standard and its resonance appears at lowest field in the spectrum. (B) PMR spectrum of a similar solution of NAG (7.0×10^{-2} M) in the presence of tortoise lysozyme (3.24×10^{-3} M). (C) PMR spectrum of a similar solution of NAG (7.0×10^{-2} M) in the presence of hen lysozyme (3.24×10^{-3} M).

In the case of a rapid exchange of NAG between free and bound environments the dissociation constant of the enzyme–inhibitor complex and the chemical shift of the bound form of the inhibitor (referred to that of the free inhibitor) can be determined by using the expression [7]:

$$S_o = \frac{E_o \Delta}{\delta} - K_s - E_o$$

where:

S_o = the total NAG concentration;
E_o = the total enzyme concentration;
K_s = the dissociation constant of the enzyme inhibitor complex;
Δ = the chemical shift of the bound form of NAG;
δ = the observed chemical shift.

If the enzyme–inhibitor binding is studied by varying the inhibitor concentration at a fixed concentration of the enzyme, a plot of S_o, against $1/\delta$ should yield a straight line with intercept $-(K_s + E_o)$ and a slope equal to $E_o\Delta$. Fig.2 shows the plot of S_o, the total concentration of NAG against $1/\delta$ at 30°C, the enzyme concentration being kept constant at 3.24×10^{-3} M. The plot is linear within experimental error. The dissociation constant, K_s and the chemical shift, Δ, of the bound form of NAG were found to be 3.5×10^{-2} M and 0.94 ppm, respectively.

The above results showed that the value of the dissociation constant of tortoise lysozyme–NAG complex is the same within experimental errors, as that of $(3.3 \pm 0.2) \times 10\ M^{-2}$ found for the hen lysozyme–β-NAG complex [7]. However, the chemical shift of 0.94 ppm, found for NAG bound to tortoise lysozyme, was considerably higher than that found for either α-NAG (0.68 ± 0.03 ppm) or β-NAG (0.51 ± 0.03 ppm) bound to hen lysozyme. In the absence of crystallographic data of tortoise lysozyme–NAG complex, it is not possible to speculate on the factors contributing to the increased shielding of the acetamido methyl protons in tortoise lysozyme.

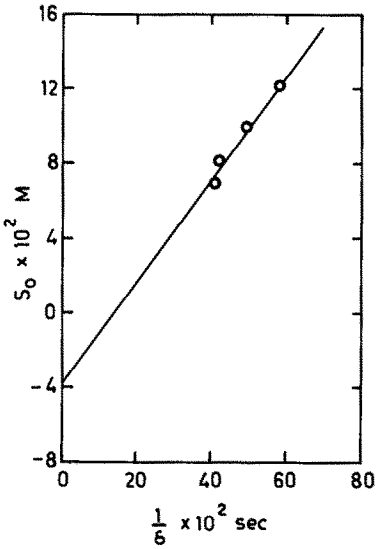

Fig.2. Plot of chemical shift (○) data for the association of NAG with tortoise lysozyme at 30°C. The ordinate, showing $S_o \times 10^2$ refers to the concentration of NAG in the presence of a constant concentration of tortoise lysozyme (3.24×10^{-3} M).

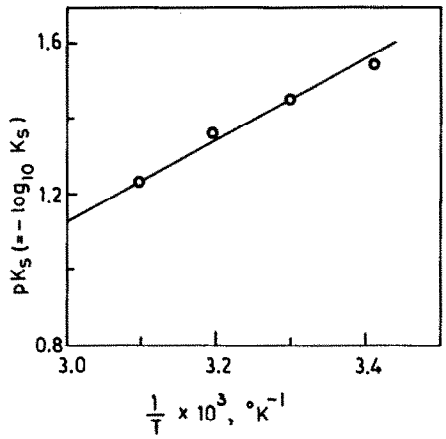

Fig.3. Plot of temperature-dependence of the dissociation constant, K_s, for NAG and tortoise lysozyme. The ordinate shows pK_s ($= -\log_{10} K_s$).

Table 1 shows the dissociation constants of tortoise lysozyme–NAG complex and the chemical shifts of the bound form of NAG determined at different temperatures. The values of Δ for the acetamido methyl protons of NAG remained constant within experimental error over the temperature range studied. Since Δ is determined by the magnetic environment, it appears that tortoise lysozyme does not undergo any conformational change at the binding site for NAG over the temperature range studied. The enthalpy of dissociation, ΔH was determined from the plot of log K_s against $1/T$, and found to be 5.0 kcal/mol. The value of ΔH is close to that of 5.5 kcal/mol

Table 1

Dissociation constants of the NAG-tortoise lysozyme complex and chemical shifts of the bound form of NAG at different temperatures[a]

Temp. (°C)	K_s ($\times 10^{-2}$ M)	Chemical shifts of the bound form of NAG [Δ (ppm)]
20	2.9	0.95
30	3.5	0.94
40	4.3	0.91
50	5.8	0.89

[a] Measurements were done in the presence of tortoise lysozyme (3.24×10^{-3} M) in 0.05 M sodium phosphate buffer (pH 7.0)

obtained for the interaction of NAG with hen lysozyme [8]. These results show that the values of K_s and ΔH of the complexes of NAG with hen and tortoise lysozymes show very slight differences.

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