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ON THE BINDING OF *N*-ACETYLGLUCOSAMINE AND CHITOBIOSE TO HEN LYSOZYME IN THE SOLID STATE AT HIGH TEMPERATURE

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1. Introduction

We have described in hen lysozyme (EC 3.2.1.17) a temperature-dependent transition in the solid state: the classical tetragonal crystals (A form) [1] are stable up to 25°C where they gave place to orthorhombic crystals (B form) provided that they are in presence of the mother liquor. This transformation was independent of other factors such as pH, ionic strength (I) or protein concentration [2]. The possibility of a conformational transition of the enzyme molecule was thus considered which could account for the existence of the two crystalline phases.

In solution, a temperature-dependent transconformation was detected between 20°C and 30°C by ^{13}C NMR spectroscopy [3]; furthermore the finding of a sharp break in the Arrhenius plots at 25°C cast also evidence on the existence of a non-denatured new conformation of the lysozyme molecule as a function of temperature and pH [4]. *N*-Acetyl-glucosamine (GlcNAc) and its short polymers such as chitobiose are competitive inhibitors of lysozyme at low and normal temperatures. Above 25°C a decrease in the affinity of the enzyme for these compounds was noticed: at 40°C (physiological temperature for birds) and at pH 5.2–7.5 and $I = 0.1$, the inhibition constants K_i were 2–3-times higher than at 20°C [5]; the reversibility of the inhibition by GlcNAc as a function of temperature and of pH was studied in detail and an inhibitor-insensitive form was described at 40°C and pH 4–4.8 [6].

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This paper deals with the crystallization of lysozyme at 40°C and 50°C in the presence of GlcNAc or chitobiose.

2. Materials and methods

Hen egg-white lysozyme, 6 times crystallized, was purchased from Miles. GlcNAc was from IBF (Clichy) and chitobiose was isolated from acid hydrolysates of chitin [7]. All other reagents (analytical grade) were from Prolabo or Merck. Enzyme–inhibitor complexes were obtained either by diffusion or co-crystallization. Our co-crystallization experiments at 20°C, 40°C and 50°C were achieved as follows: to 65 mg lysozyme and GlcNAc (final conc. 10^{-3}, 10^{-2} or 10^{-1} M), or to 65 mg lysozyme and chitobiose (final conc. 2×10^{-2} M), dissolved in 0.5 ml water, were added 0.0625 ml 0.2 M acetate buffer (pH 4.7) and 0.1875 ml water; after centrifugation, if necessary, 0.75 ml of a 10% NaCl solution was added. The crystals were analyzed by the precession camera technique.

The methods used for the study of lysozyme in solution in the presence of GlcNAc have been described [6].

3. Results and discussion

3.1. *Crystallization in the absence of GlcNAc or chitobiose at high temperature*

At 20°C the classical tetragonal form called A was

obtained ($P4_32_12$, $a = b = 79.1$ Å, $c = 37.9$ Å) [8,9]. At 40°C and 50°C, the orthorhombic form called B appeared ($P2_12_12_1$, $a = 56.3$ Å, $b = 73.8$ Å, $c = 30.4$ Å). [1]. If pregrown A crystals were put at 40°C, they dissolved from the walls of the tube and a transformation of the A into the B form was observed [1].

3.2. *Direct crystallization in the presence of GlcNAc or chitobiose at high temperature – Comparison with the lysozyme behaviour in solution*

Since the observation in [10], it is well known that GlcNAc and its polymers are, in solution, good inhibitors of the lysozyme activity. Using the diffusion technique with these sugar molecules into preformed crystals or co-crystallization, the inhibitors were shown to bind to the enzyme molecules even in the solid state [11]. The location of the binding allowed the proposal of a scheme of the enzymatic action [11]. All these studies have been carried out at low or room temperature.

First we verified that the crystals obtained by co-crystallization or diffusion with GlcNAc at room temperature were tetragonal (modified A crystals) [11]. However, at 20–25°C some normal B crystals (devoid of sugar) already appeared: the transition point seemed to be somewhat lowered in the presence of GlcNAc.

At 40°C, no crystals containing GlcNAc were obtained by any of our crystallization techniques. The diffraction pattern of the B crystals which appeared corresponded to the classical one determined in the absence of the sugar.

The crystals obtained at 20°C by co-crystallization with chitobiose were tetragonal but their external form was modified from that obtained in the absence of the sugar (A modified): the diffraction pattern showed slight differences from the pattern of native crystals as classically described [11]. The diffusion technique lead to the same result.

In contrast to the situation encountered with GlcNAc, the presence of chitobiose prevented the appearance of B crystals above 25°C which was the transition temperature when no sugar was present. Indeed, at 40°C, the modified A form described at 20°C was formed by crystallization. At 50°C, however, the crystals were orthorhombic and apparently no longer contained chitobiose. The diffraction pattern of these B crystals was the same as that of the crystals formed without the sugar and no difference in the intensities has been detected so far.

When the diffusion technique was used, the preformed B crystals (without chitobiose) were very sensitive to the presence of the sugar even when mild experimental conditions were used. They exhibited cracks and quickly were fragmented.

These observations can be compared with our experiments performed in solution: at $\leqslant 25$°C, GlcNAc and its polymers were good inhibitors, while at >25°C they became poorer ones [5] and sometimes no longer inhibited the enzyme [6].

3.3. *Transition of pregrown A crystals obtained in the presence of GlcNAc or chitobiose (modified A crystals) at higher temperatures*

If the crystals obtained at 20°C in the presence of GlcNAc (modified A) were put at 40°C, B crystals devoid of the sugar were obtained within a day. This experiment corroborated our observation that the complex with GlcNAc was unstable. Moreover a lowering of the transition point was observed.

Quite different was the behaviour of the modified A crystals obtained in the presence of chitobiose. The sugar was more tightly bound to the molecule as the crystals remained unchanged at 40°C, even after several weeks. Only at >40°C was the complex broken: at 50°C, orthorhombic crystals appeared in a few hours which were of excellent optical quality but frequently presented a yellowish colour: they apparently no longer contained chitobiose. It seemed thus, that there was at least a shift in the transition point: chitobiose might make the A conformation more solid.

Our results were once more in favour of a temperature-induced structural rearrangement occurring near the active site [3]. They were also in accordance with discussions concerning a fluctuation widening of the substrate-binding cleft [12] as well as with remarks performed in the study of the enzyme activity as a function of temperature [13]: in order to explain the enhancement of activity, the existence of a rearrangement in the vicinity of the active site was suggested, making a better fit of the enzyme and its substrate. At 30°C we showed by ^{13}C NMR spectroscopy [3] a shift on one side of the cleft of the lysozyme molecule which included, among others, changes in the α-methyl groups and more particularly of alanine residue 107. According to [14,15] GlcNAc was

bound to subsite C and formed at room temperature with Ala 107 one of the 4 bonds with the enzyme. Rearrangements at this site of the molecule might explain the observations described here and also the decrease of affinity of GlcNAc for lysozyme in function of increasing temperature. The stronger binding of chitobiose to lysozyme in the B and C subsites was in accordance with the free energy change measured [16] corresponding presumably to hydrophobic interactions with tryptophan residue 62.

In conclusion, the molecular area of lysozyme involved in its enzymatic mechanism appears flexible [3,17] and seems to 'rigidify [18]' on binding certain substrates or inhibitors such as chitobiose.

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