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CHROMATOGRAPHIC APPROACH TO THE STUDY OF THE BINDING OF LYSOZYME TO SUBSTRATE

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SUMMARY

Column chromatography of hen egg-white lysozyme (mucopeptide *N*-acetylmueramylhydrolase, EC 3.2.1.17) on an insoluble polymeric substrate, chitin, has been studied with media having various compositions. Two modes of binding were observed, depending on pH, which probably correspond to two types of enzyme–substrate complex. The apparent binding constants as well as the free energies, the heats and the entropies of binding have been determined for both types of complex by a chromatographic technique. The results obtained are discussed in terms of the mechanism of lysozyme binding to substrate.

INTRODUCTION

A number of methods has been employed hitherto for studies on the binding of lysozyme (mucopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17) to substrate. The structure of the lysozyme–tri-*N*-acetylchitotriose in the crystalline state has been determined in an X-ray study by BLAKE *et al.*[1] The binding of lysozyme with substrates or substrate-like inhibitors in solution has been studied by ultraviolet spectroscopy[2,3], by fluorescence[4,5] and by NMR technique[6,7]. Equilibrium dialysis has also been used[5,8]. Recently, an evaluation has been reported of the binding parameters from a kinetic study on hydrolysis of soluble carboxymethylated chitin by lysozyme[9].

Our work deals with the chromatographic study of the binding of lysozyme to the insoluble polymeric substrate, chitin. In previous studies[10] we found that lysozyme can bind to chitin in two different modes depending on the pH of the medium, and it was concluded that they correspond to two types of enzyme–substrate complex. One of these complexes, tentatively named LC-1, prevails at relatively low pH (4–5); it is stable only in the presence of salts in solution and decomposes in water. The other complex, LC-2, prevails at higher pH (8–9); it is stable in water and decomposes in slightly acidic media, *e. g.* in 0.1 M acetic acid. The reversible interconversion of these complexes correlates with the ionization state of a lysozyme functional group of ap-

parent pK 6.3 (ref. 11). Recently, IMOTO *et al.*[12] have also found two types of lysozyme–substrate complex by means of chromatography of lysozyme on a column of insoluble carboxymethylated chitin.

The present paper reports the use of a chromatographic technique for the evaluation of the equilibrium binding parameters for both types of lysozyme–chitin complex.

EXPERIMENTAL AND RESULTS

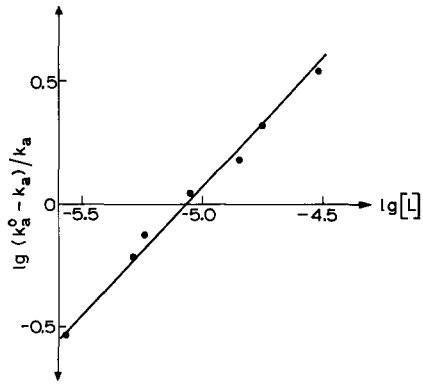
Lysozyme was obtained from hen egg-white according to the procedure of ALDERTON AND FEVOLD[13]; 3 times recrystallized enzyme was desalted on a Sephadex G-25 column and lyophilized. The preparation of chitin for chromatographic use was described earlier[14]. In some experiments chitin was deaminated with dil. HNO_2 for elimination of ion-exchange properties[15]. Powdered chitin with particles of average size 50 and 75 μ was employed. All other reagents were recrystallized or distilled.

A standard chromatographic procedure was carried out as follows. A chitin column was buffered and thermostated under the conditions used. A sample of lysozyme in buffer was applied to the column and then eluted with the same buffer (flow rate about of 15–20 ml/h); fractions of 3 ml were collected, and their absorbance was measured at 275 nm. The retention volume, V_r, was determined from the elution curve by the expression: $V_r = V' - V_0$, where V' is the elution volume of the maximum of the lysozyme peak and V_0 is a void volume of a chitin column. However, for calculation of the binding parameters a direct function of V_r, the so-called affinity coefficient, $k_a = V_r/V_0$, was of advantage. The lysozyme concentration in solution was determined spectrophotometrically assuming a molar extinction coefficient of 35 000 $M^{-1} \cdot cm^{-1}$ at 275 nm.

The experiments were carried out at two pH's: 4.7 and 8.4, corresponding to the formation of the LC-1 and LC-2 types of complex, respectively [10,11].Chromatography at pH 4.7 was carried out in 0.2 M sodium acetate buffer with the addition of NaCl up to 0.1 M. At pH 8.4 a solution of 0.15 M $NaHCO_3$ was used. Since the adsorption at pH 8.4 is very strong, urea was added to the buffer as inhibitor of binding[16]; two urea concentrations, 1 M and 2 M, were tested in view of possible extrapolation of the experimental results to zero urea concentration.

For evaluation of the binding constant, K_b, the concentration dependence of k_a was measured. Various amounts of lysozyme (2–30 mg) were applied to the column in 1 ml of the appropriate buffer solution at 25°. The binding constant was determined from the plot of lg $(k_a^0 - k_a)/k_a$ against lg [L] according to the general principle for one-to-one complex[17]. Here, [L] is the molar concentration of lysozyme at the peak maximum of the elution curve and k_a^0 is the apparent value of the affinity coefficient corresponding to zero lysozyme concentration. The value of k_a^0 was extrapolated from the experimental data by plotting k_a against [L]. The one-to-one stoichiometry of complexing resulted from the X-ray study of BLAKE *et al.*[1] and was confirmed by other methods[5–8].

For evaluation of the heat of binding the temperature dependence of k_a was examined. Samples of lysozyme (5 mg) were applied to the column in 1 ml of the appropriate buffer; series of runs were carried out at the various temperatures of the column (25–50°). The heat of adsorption, Q_a, was determined from the Van 't Hoff



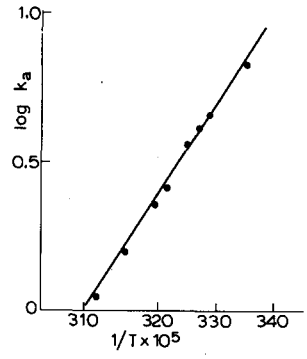


Fig. 1. Sample plot for determination of the binding constant of lysozyme to chitin. Chitin column, 0.9 cm × 19 cm; average size of chitin particles about 50 μ. Samples of lysozyme applied to the column, 2, 4, 5, 6, 8, 10 and 15 mg. $V^0 = 18$ ml; $k_a^0 = 4$; pH 4.7; temp., 25°. For details see text.

Fig. 2. Sample plot for determination of the heat of lysozyme binding to chitin at pH 4.7. Temperatures, 25, 31, 33, 35, 38, 40, 45 and 50°. For details see legend to Fig. 1 and text.

plot of lg k_a against $1/T$ according to the method of GREENE AND PUST[18]. Since the adsorption may account essentially for the specific enzyme–substrate affinity forces, the heat of binding, ΔH^0, was assumed to be equal $-Q_a$.

The values of the free energy and the entropy of binding were calculated from the expressions $\Delta F^0 = -RT\ln K_b$ and $\Delta S^0 = (\Delta H^0 - \Delta F^0)/T$, where K_b is the experimental binding constant related to 1 mole of lysozyme.

Fig. 1 shows a typical plot for the effect of lysozyme concentration on the affinity coefficient function at pH 4.7. The slope of the plot is unity, confirming the formation of a one-to-one complex. The pK_b value can be evaluated from the plot as being equal to lg [L] at $\lg(k_a^0 - k_a)/k_a = 0$. The value of K_b at pH 4.7 was found to be $1.15 \cdot 10^5$ M^{-1}. The value of K_b at pH 8.4 was determined in the same manner. It was $4.90 \cdot 10^5$ M^{-1} at 1 M and $8.51 \cdot 10^4$ M^{-1} at 2 M urea concentration. A linear extrapolation to zero urea concentration gives the approximate value of K_b $8.95 \cdot 10^5$ M^{-1} in the absence of urea. The accuracy of determination of K_b values is about ± 0.01 on the logarithmic scale.

Fig. 2 represents a typical Van 't Hoff plot for the effect of temperature on the

TABLE I

THE APPARENT THERMODYNAMIC PARAMETERS FOR ENZYME–SUBSTRATE COMPLEX FORMATION BETWEEN LYSOZYME AND CHITIN AT 25°

The values are calculated per mole of lysozyme on the assumption of one-to-one complex formation, *i.e.* binding of lysozyme molecules to the single chains of chitin particles. For discussion see text.

pH	*Complex*	K_b (M^{-1})	ΔF^0 (kcal/mole)	ΔH^0 (kcal/mole)	ΔS^0 (cal/degree per mole)
4.7	LC-1	$1.15 \cdot 10^5$	−6.9	−14.0	−23.8
8.4	LC-2	$8.95 \cdot 10^5$	−8.1	−9.6	−5.0

affinity coefficient function at pH 4.7. The ΔH^0 value determined from the plot was -14.0 ± 0.5 kcal/mole. At pH 8.4 it was -9.6 ± 0.5 kcal/mole for both 1 M and 2 M urea, indicating that urea at the concentrations taken is probably a competitive inhibitor of the lysozyme–substrate association. Hence, it may be assumed that, in the absence of urea, $\Delta H^0 = -9.6 \pm 0.5$ kcal/mole as well.

The values of the free energy and the entropy of binding were calculated from the expressions mentioned above. A complete set of the equilibrium binding parameters for the reaction lysozyme + chitin $\rightleftharpoons$ complex is given in Table I.

DISCUSSION

The chromatographic method described herein provides a direct and relatively simple tool for studying the binding of lysozyme to substrate; under certain conditions the method makes it possible to evaluate the equilibrium binding parameters for the enzyme–substrate complex formation. However, the binding parameters determined are the apparent parameters because of the strong dependence of the affinity forces on the media conditions (*e.g.* no complex of the LC-I type forms at all in saltless media[10,11]).

The binding parameters obtained by chromatographic technique at pH 4.7 are in substantial agreement with those determined by other methods at a pH near 5 for chitin-like saccharides (trimer and higher)[2,3,5]. DAHLQUIST *et al.*[2] have demonstrated that the values of binding constants for chitin-like saccharides increase with the chain length up to trimer, tri-*N*-acetylchitotriose, and remain about constant thereafter. RUPLEY *et al.*[3] concluded that the binding parameters at pH near 5 are related to the so-called "nonproductive" complex; in this complex the sugar rings of the substrate molecule occupy only three binding sites, A, B and C with the reducing end at site C, and the rest of the substrate molecule makes no contact with the enzyme. Binding measurements with occupation of all six binding sites A–F are not possible because of the high hydrolytic activity of lysozyme at pH near 5 and the cleavage of the substrate molecule between sites D and E[1]. In this regard, the agreement of the binding data for the polymeric substrate, chitin, with that for chitin-like oligomers (trimer and higher) at pH near 5 seems to be quite reasonable.

On the other hand, the results in Table I indicate that the lysozyme–chitin complex at pH 8.4 is more stable than that at pH 4.7, contrary to the data available for trisaccharide binding at this pH[3–5] (the data for binding to polymeric substrate at this pH are lacking). It is noteworthy that hydrolytic activity of lysozyme against chitin-like substrates at pH 8–9 is very low perhaps as the result of ionization of the carboxylic group of Glu 35 acting as a proton donor in the hydrolytic process[1]. Hence, it may be suggested that at pH 8.4 the binding constant corresponds to the so-called "pseudoproductive" complex in which the substrate molecule (with sufficient chain length) can occupy all six binding sites, A–F, without hydrolysis taking place. The apparent dependence of the mode of binding upon the group of pK 6.3, which seems to be the carboxylic group of Glu 35 (refs. 1, 3, 4, 6, 7), provides some support for such a suggestion (see INTRODUCTION). This can also give a reasonable explanation to the apparent discrepancies between the binding data for the polymeric substrate, chitin, and those for trisaccharide at pH 8–9.

Insofar as the other binding parameters are concerned (see Table I), the large

negative value of the entropy of binding at pH 4.7 (−23.8) as compared with that at pH 8.4 (−5.0 cal/degree per mole) probably indicates the higher extent of the conformational change by enzyme–substrate complex formation at the former pH. Perhaps the difference in the heat of binding (−14.0 kcal/mole at pH 4.7 against −9.6 kcal/mole at pH 8.4) reflects the same difference in the extent of the conformational change of the enzyme molecule on substrate binding at the pH used. Although an alternative interpretation of the results obtained is not excluded there is some information in the literature that gives support to such a proposition. HAYASHI *et al.*[19] first observed the change in the ultraviolet absorption spectrum of lysozyme on substrate binding. According to these authors, the difference spectrum observed on substrate binding is due to a change of lysozyme conformation as a whole but not to a contact of the substrate with chromophore residues as such[20]. Since an increase in pH above 5.5 results in a decrease in intensity of the difference spectrum, it may be concluded that the extent of the conformational change of lysozyme at higher pH is less than at lower.

Furthermore, CHIPMAN *et al.*[5] have concluded that the shift of the lysozyme fluorescence maximum to shorter wavelengths ('blue shift') observed on substrate binding is caused by a conformational change of the enzyme. According to the data of LEHRER AND FASMAN[4], the blue shift at pH 7.5 is less than that at pH 5.5 which may also indicate a smaller extent of the conformational change at the former pH.

Furthermore, the increase of pH up to 8–9 results in a change in the ultraviolet absorption spectrum of free lysozyme[21]; the spectral pattern observed resembles that observed on substrate binding under optimal conditions (pH 5.5)[19]. Hence, it seems likely that a change of lysozyme conformation both on substrate binding and on increase of pH in the absence of substrate proceeds in outline in a related way; this suggestion is consistent with the smaller contribution of substrate binding to the change in absorption spectrum (*i.e.* to the conformational change) of lysozyme at higher pH than at lower.

On the whole it appears likely that at pH 4.7 lysozyme possesses a "flexible" conformation which changes readily on substrate binding, the interaction being compatible with the induced fit hypothesis of KOSHLAND[22]. On the other hand, at pH 8.4 lysozyme probably has a preformed complementary conformation which does not change substantially on substrate binding, the situation that rather pertains to the well-known key and lock principle of Fisher. However, a key mechanism controlling the mode of binding still remains unknown, and the suggestions considered herein need further experimental evidence.

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