THE DETERMINATION OF THE DISSOCIATION CONSTANTS OF PRODUCTIVE AND UNPRODUCTIVE LYSOZYME SUBSTRATE COMPLEXES

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1. Introduction

It has been established that lysozyme can accomodate up to six adjacent pyranose rings of an oligosaccharide chain [1–5]. The six sites on the enzyme are designated A through F. Only those substrates whose pyranose ring fits into sites D and E are catalytically hydrolysed [6]. Filling site D requires an energetically unfavourable distortion of the pyranose ring. These properties of the binding site of the enzyme result in productive and unproductive binding modes of substrates. Interpretations of mechanistic studies of lysozyme-catalyzed reactions depend on a knowledge of the concentration of the various enzyme complexes. In this paper we describe the direct determination of the dissociation constant for three major enzyme-chitohexose complexes and the method used in these studies.

The principle of the method and the main substrate binding modes are illustrated in fig. 1. The dye, Biebrich Scarlet, forms a 1:1 complex with the enzyme and is a reversible inhibitor of the lysozyme-catalysed hydrolysis of cell walls of *Micrococcus lysodeikticus* [7]. The absorbance maximum at 505 nm for the free dye shifts to 510 nm when the dye is bound to the enzyme [7]. Displacement of the inhibitor by substrate can, therefore, be measured quantitatively. Perturbation of the dye spectrum is also obtained by oligosaccharides which interact with the A, B, C site of the enzyme and do not displace the inhibitor (fig. 2). The formation of these unproductive complexes can, therefore, also be measured quantitatively.

2. Method

The binding of chitooligosaccharides to form various complexes with hen egg white lysozyme was measured at pH 7.5 (0.1 M potassium phosphate buffer) 25°C, by difference spectroscopy between a solution containing an equal concentration of Biebrich Scarlet, a constant concentration of enzyme and varying concentrations of a saccharide. The absorbance difference was measured at 495 nm with reference to the isosbestic point at 630 nm. Typical concentrations were 0.017 mM Biebrich Scarlet (Matheson Coleman, recrystallized from ether), 0.2 mM lysozyme (Worthington), and 0.002 mM to 20 mM saccharide prepared from chitin [8]. A Cary model 14 spectrophotometer equipped with a 0.1 absorbance slide wire was used. For each saccharide concentration absorbance was measured separately, within 30 sec after mixing of reactants.

Three different modes were measured for the binding of the saccharides to lysozyme: (a) Unproductive binding was measured at concentrations below 1 mM

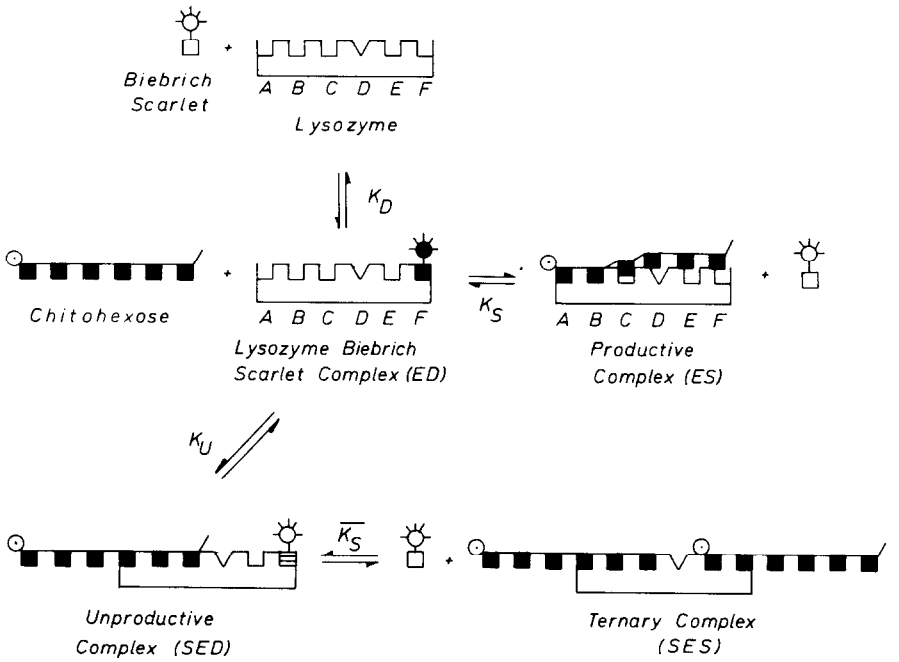

Fig. 1. The three major lysozyme-chitohexose complexes in the presence of the reporter group Biebrich Scarlet. For details see text.

saccharide (complex **SED** in fig 1). At this condition, Biebrich Scarlet functions as a reporter group. This was shown by titration of free enzyme and enzyme–chitotriose complex (in the presence of 1 mM chitotriose [3,4]) with dye. For both titrations a dissociation constant was measured which had an identical value of K_{ED} = 0.14 mM. This result provides evidence that the dye reports binding of the saccharide to sites A–C by perturbation of the absorbance difference ($\Delta\Delta\epsilon_{M(630-495)}$ = 1000 M^{-1} cm^{-1}) but not by its ability to bind. (b) Biebrich Scarlet is displaced at high saccharide concentrations (Formation of complex SES in fig. 1). Thus, the absorbance difference in fig. 2b tends to approach zero. (c) At concentrations below 1 mM, chitohexose causes a greater decrease for $\Delta A_{630-495}$ than the other saccharides. This additional decrease is ascribed to a third type of complex and displacement of dye (complex **ES**, fig. 1).

3. Results and discussion

For all chitosaccharides investigated, fig. 2b indicates biphasic binding. Evaluation of the dissociation constants for unproductive binding was accomplished by standard procedures [9] from data such as shown in fig. 2a for chitotriose. As a result, chitotriose, chitotetrose and chitopentose, all bind with the same dissociation constant K_u = 0.01 mM. This value is in agreement with the values reported from other spectroscopic measurements [4] and with the findings from crystallographic data and model building [1–3, 10]. Evaluation for chitohexose was complicated by the observed fast additional decrease of the absorbance difference; but from model building [2] unproductive binding is supposed to exist with comparable stability. We have incorporated the complex ES into the evaluation procedure for the absorbance changes at saccharide concentrations higher than 1 mM. The procedure was based on the scheme in fig. 1 and was accomplished with the use of equation (1).

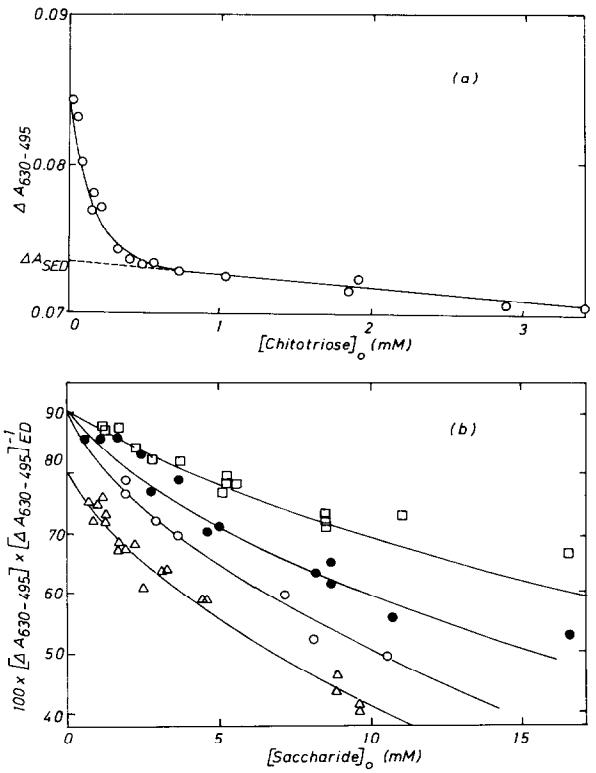

Fig. 2. The binding of chitooligosaccharides to lysozyme in the presence of Biebrich Scarlet, (a) at low concentrations of chitotriose. The initial decrease was used for evaluation (9) of the dissociation constant K_u. (b) At high concentrations of chitotriose (□), chitotetrose (•), chitopentose (○) and chitohexose (Δ). The absorbance difference is given with reference to the absorbance difference in the absence of saccharides. Initial changes for the formation of complexes SED are not shown.

$$\Delta A_{630-495} \cdot \Delta A_{SED}{}^{-1} = (E_o + K_{ED})\,\theta - \Delta A_{630-495} \cdot \Delta A_{SED}{}^{-1}\, S_o K_{ED} (K_u K_s)^{-1} \quad (1)$$

The symbols are defined and the equation derived in the Appendix. The coordinates of the lines in the fig. 2b were obtained using equation (1) and a least square computer program. An excellent fit of the data to equation (1) is observed. For chitohexose the values for K_s and $\bar{K}_s$ are 0.03 mM and 5 mM respectively. All other saccharides form SES complexes with values for $\bar{K}_s$ of approximately 10 mM, but none of them forms a complex ES as is also anticipated from the structural dimensions of the ligands and from the known crystallographic data [1, 2].

Our results are consistent with the formation of high affinity productive complexes for chitohexose (K_s = 0.03 mM) and low affinity productive complexes for chitotriose and chitotetrose ($\bar{K}_s \cong 10$ mM). High affinity productive complexes compete with unproductive complexes, but low affinity productive complexes do not. Our results are in agreement with those obtained previously with steady-state kinetics [3, 11].

4. Appendix

When S_o is the initial oligosaccharide concentration, E_o the initial lysozyme concentration, and D_o the initial Biebrich Scarlet concentration, the concentration of the lysozyme-dye complexes in the presence of saccharides is given by the equation below, providing

$S_o > E_o > D_o$:

$$ED + SED = \frac{E_o D_o}{E_o + K_{ED}\left(1 + \frac{S_o}{K_u} + \frac{S_o}{K_s} + \frac{S_o}{\bar{K}_s} + \frac{S_o{}^2}{K_s K_u}\right)\left(1 + \frac{S_o}{K_u}\right)^{-1}} \quad (2)$$

When using equation (2) to evaluate only the displacement of the dye at high saccharide concentrations, the following approximation can be made: (1) $SED \gg ED$, and the concentration of ED can be neglected. (2) The K_u value determined in these and previous experiments is about 10^{-5} M [10, 11]. In the substrate concentration range of interest, $S_o > 0.5$ mM, and therefore $S_o K_u{}^{-1} \gg 1$. The simplified equation becomes:

$$SED = \frac{E_o D_o}{E_o + K_{ED}\left(1 + \frac{K_u}{K_s} + \frac{K_u}{\bar{K}_s}\right) + \frac{K_{ED}}{K_s} S_o} \quad (3)$$

SED is expressed by $\Delta A_{630-495}$ according to the following equation:

$$SED = \frac{1}{\frac{\Delta A_{SED}}{ED}} \cdot \Delta A_{360-495} = \frac{\Delta A_{630-495}}{\Delta A_{SED}} \cdot \frac{E_o D_o}{E_o + K_{ED}} \quad (4)$$

ΔA_{SED} refers to the absorbance difference at saturation of the unproductive site by the saccharide. It is obtained by extrapolation of the $\Delta A_{630-495}$ values measured at high saccharide (> 1 mM) to zero concentration, fig. 2a. When equation (4) is integrated in equation (3), and when

$$\theta = E_o + K_{ED}\left(1 + \frac{K_u}{K_s} + \frac{K_u}{\overline{K}_s}\right), \quad (5)$$

the linear form of equation (3) is equation (1) given in the text.

References

[1] Blake, C.C.F., Johnson, L.N., Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R. (1967), Proc. Roy. Soc. Ser. B. 167, 378–388.

[2] Blake, C.C.F., Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R. (1967) Proc. Roy. Soc. Ser. B 167, 365–377.

[3] Rupley, J.A., Butler, L., Gerring, M., Hartdegen, F.Y. and Pecararo, R. (1967) Proc. Natl. Acad. Sci. U.S. 57, 1088–1095.

[4] Chipman, D.M., Grisaro, V. and Raftery (1967) J. Biol. Chem. 242, 4388–4394.

[5] Chipman, D.H., Pollock, J.J. and Sharon, N.J. (1968) J. Biol. Chem. 243, 487–496.

[6] Imoto, T., Johnson, L.N., North, A.C.T., Phillips, D.C. and Rupley, J.A. (1972) The Enzymes VII, 665–868.

[7] Rossi, G.-L., Holler, E., Kumar, S., Rupley, J.A. and Hess, G.P. (1969) Biochem. Biophys. Res. Commun. 37, 423–429.

[8] Rupley, J.A. (1964) Biochim. Biophys. Acta 83, 245–255.

[9] Benesi, H.A. and Hildebrand, J.H. (1949) J. Amer. Chem. Soc. 71, 2703–2707.

[10] Rupley, J.A. and Gates, V. (1967) Proc. Natl. Acad. Sci. U.S. 57, 496–510.

[11] Rupley, J.A. (1967) Proc. Roy. Soc. Ser. B 167, 416–428.