THE ACTIVE SITE OF LYSOZYME : SOME PROPERTIES OF SUBSITES E AND F

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From their X-ray crystallographic studies of hen's egg white lysozyme, Phillips and his coworkers (Blake et al., 1967; Phillips, 1967) have concluded that the active site of the enzyme lies in a cleft located on its surface. The cleft can accommodate six sugar residues, designated as A, B, C, D, E, and F, in corresponding subsites A to F, of which the positions of the first three were located by X-ray studies of enzyme-saccharide complexes. The presence of subsites D, E, and F was inferred by fitting molecular models of substrates to the three dimensional model of the enzyme. It was further assumed that cleavage occurs at the glycosidic bond between sugar residues D and E.

The specificity of subsites A, B, C, D, and their contribution to the total binding energy of oligosaccharide substrates and inhibitors was determined by us from measurements of the association constants with lysozyme of saccharides containing N-acetyl-D-glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) (Chipman, Grisaro, and Sharon, 1967). The presence of at least one additional subsite corresponding to residue E could be deduced from studies of the lysozyme catalyzed transglycosylation reaction using a variety of acceptors (Pollock, Chipman, and Sharon, 1967; Rupley and Gates, 1967). We have now measured the relative efficiency of some saccharides to serve as acceptors, by competition experiments in which the cell wall tetrasaccharide, GlcNAc-MurNAc-GlcNAc-MurNAc,* was incubated with lysozyme in the presence of mixtures of acceptors.

In these experiments we compared the quantities of the products formed by transfer of the disaccharide residue, GlcNAc-MurNAc, to the corresponding acceptors. For example, when D-glucose (Glc) and cellobiose (Glc-Glc) were the acceptors used, the quantities of GlcNAc-MurNAc-Glc and of GlcNAc-MurNAc-Glc-Glc formed by transglycosylation were determined. The results obtained provide information on

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* All oligosaccharides are $\beta(1 \rightarrow 4)$ linked.

the specificity requirements of subsite E and give evidence for the existence of subsite F.

In a typical competition experiment a 7.5×10^{-3} M solution of cell wall tetrasaccharide tritiated by the Wilzbach technique (2.15×10^5 cpm/μmole) (Chipman, Pollock, and Sharon, manuscript in preparation) was incubated at 37^0 with hen's egg white lysozyme (2X crystallized, Worthington Biochemical Corporation, Freehold, New Jersey) in 0.1 M ammonium acetate adjusted to pH 5.25 with acetic acid, in the presence of equimolar concentrations of two acceptor saccharides. Samples were withdrawn at various time intervals and spotted on Whatman No. 3 paper. Electrophoresis was then carried out at a constant voltage of 50 V/cm for 90 minutes at pH 6.5 (1.2 M pyridine-acetic acid buffer). The electrophoretic strips were passed through a Vanguard gas flow strip scanner which recorded the radioactive peaks, and the paper was then cut up into sections of 1 cm or less on the basis of the proximity of the peaks. The paper sections were placed in vials with Bray's scintillation solution (Bray, 1960) and counting rates were determined with a Packard Tri-Carb-scintillation counter.

Fig. 1 shows the distribution of radioactivity in a typical electrophorogram. The identification of the products is based on comparison of their electrophoretic mobilities with those of products formed in incubation mixtures containing only one of the acceptors (Pollock, Chipman, and Sharon, 1967). Since the molar activity of all transfer products must be equal, the ratio of products of transfer is equal to the ratio of the total counts in the corresponding peaks. Some variation in this ratio over the course of the reaction was observed and the results given in Table I are the averages obtained after 2 and 5 hours of incubation.

In cases where the new compounds formed had similar electrophoretic mobilities, competition experiments were carried out by using unlabelled tetrasaccharide and two acceptor saccharides labelled with different radioisotopes. For comparison of GlcNAc and Glc, equimolar concentrations (2.5×10^{-2} M) of N-acetyl-D-glucosamine-1-^{14}C (1.99×10^4 cpm/μmole) and D-glucose-6-^{3}H (9.64×10^4 cpm/μmole) were used. In the region of the electrophorogram corresponding to GlcNAc-MurNAc- GlcNAc and GlcNAc-MurNAc-Glc, ^{3}H and ^{14}C were simultaneously determined by means of double channel counting and from the number of counts and the specific activities of the acceptors, the ratio of products was calculated. In a similar manner, D-glucose-6-^{3}H (1.59×10^4 cpm/μmole) and D-xylose-U-^{14}C (1.71×10^4 cpm/μmole) were compared. The results of these experiments are also given in Table I.

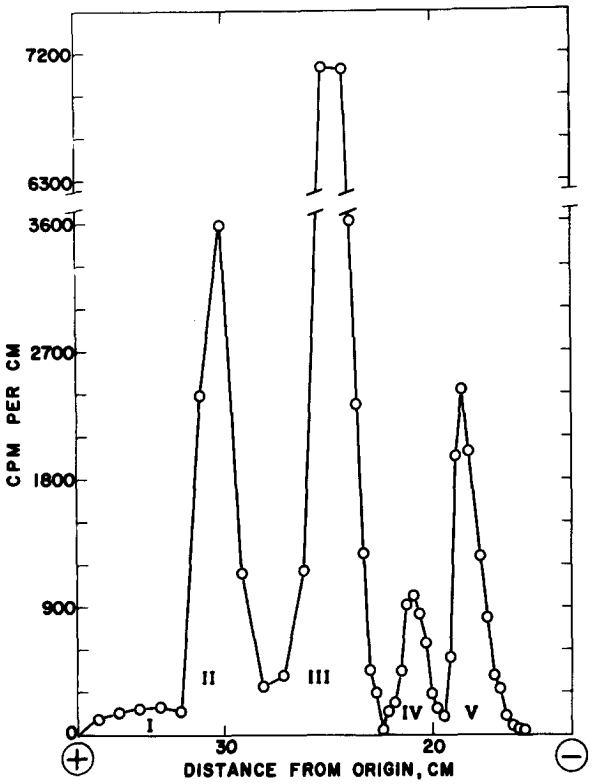

Fig. 1. Paper electrophoresis of a reaction mixture after 5 hours incubation at 37^0 consisting of tritium labelled tetrasaccharide (7.5×10^{-3} M), equimolar concentrations (7.5×10^{-2} M) of glucose and cellobiose and hen's egg white lysozyme (0.25 mg/ml) in 0.1 M ammonium acetate-acetic acid buffer pH 5.25. Sections (1 cm or less) from the electrophorogram were cut up and counted by liquid scintillation: I region of higher oligosaccharides, II GlcNAc-MurNAc-GlcNAc-MurNAc, III GlcNAc-MurNAc, IV GlcNAc-MurNAc-Glc, and V GlcNAc-MurNAc-Glc-Glc.

The ratios given in Table I are those found in experiments in which equimolar concentrations of two acceptor saccharides were used. However, the same ratios can also be obtained indirectly by comparing two sets of experiments. For example, comparison of experiments 1 and 3 gives a value of 4.5 for the ratio of GlcNAc to Glc which is identical to that determined in the double labelling experiment (No. 5 in Table I). This agreement is of added significance in view of the fact that in the experiments reported different concentrations of acceptors were used, all of which are below the saturation level of the enzyme. Although the effect of varying acceptor concentration on the ratios of the products was not studied in detail, the above results strongly suggest that these ratios are independent of acceptor concentration. Under the experimental

Table I. Ratios of oligosaccharides formed in the lysozyme catalyzed transfer reaction*

Experiment No.	Acceptor	Saccharide Concentration, M	Transfer Product Ratio
1	GlcNAc-GlcNAc : GlcNAc	1.5×10^{-3}	2 : 1
2	Glc-Glc : Glc	7.5×10^{-2}	2.8 : 1
3	GlcNAc-GlcNAc : Glc	3.75×10^{-2}	9 : 1
4	Glc-Glc : GlcNAc	3.5×10^{-2}	0.67 : 1
5	GlcNAc : Glc	2.5×10^{-2}	4.5 : 1
6	Glc : Xyl	12.5×10^{-2}	1.0 : 1

* The products are those formed by the transfer of a disaccharide moiety GlcNAc-MurNAc, from the cell wall tetrasaccharide to the corresponding acceptors.

conditions used, the ratios seem also to be unrelated to the enzyme inhibitory capacities of some of the acceptors used (Rupley and Gates, 1967; Sharon, 1967).

It has been previously suggested that the lysozyme-catalyzed transfer reaction involves a long-lived activated glycosyl intermediate of the group transferred, in the present case GlcNAc-MurNAc. This group must be bound at subsites C and D with its reducing end at D, the acceptor molecules thus interacting with the adjacent subsites. Monosaccharide acceptors will be bound at subsite E, and since GlcNAc is a much better acceptor than Glc, it may be concluded that the acetamido group has a decisive role in the binding at this subsite. Furthermore, as no difference in the amounts of trisaccharides formed was noted upon comparison of glucose with xylose, the CH_2OH group of the former sugar does not seem to interact with the enzyme. Both results are in accord with the three dimensional enzyme-substrate model (Blake et al., 1967; Phillips, 1967) which suggests interaction between lysozyme and the 2-acetamido group of N-acetylglucosamine, but not with its CH_2OH group. If one assumes that the difference in the rates of transfer to two acceptors is due only to a difference in the affinity of the acceptors for the region of the enzyme site in question (a reasonable assumption since the attacking groups are all hydroxyl groups linked to carbon atoms of pyranose rings) the differences in accepting efficiency may be used to deduce differences in free energies of interaction of the acceptors

with the acceptor sites of the enzyme. The contribution of the acetamido group to the free energy of binding at subsite E can then be calculated by the following subtraction:

$$\Delta F_{GlcNAc} - \Delta F_{Glc} = -RT \ln \frac{k_1}{k_2} = -RT \ln \frac{[GlcNAc\text{-}MurNAc\text{-}GlcNAc]}{[GlcNAc\text{-}MurNAc\text{-}Glc]} = -0.9 \text{ Kcal/mole}$$

where k_1 and k_2 are the rates of formation of the two trisaccharides, respectively.

The disaccharides tested are 2-3 times better acceptors than the corresponding monosaccharides indicating that the free energy of interaction of the former with the enzyme is greater by 0.4 - 0.5 Kcal/mole than that of the latter. This suggests the existence of an additional subsite (F), adjacent to subsite E. From Table I, it is evident that the acetamido group does not contribute significantly to the interaction at subsite F and again this is in agreement with the structure of the enzyme substrate model in which no such interaction is indicated (Blake et al., 1967; Phillips, 1967).

It is of interest to note that in an experiment with a human lysozyme (Osserman and Lawlor, 1966), kindly supplied by Dr. Osserman, the ratio of products formed from GlcNAc-GlcNAc and GlcNAc was about 2. It thus seems likely that this enzyme, which exhibits many similarities to hen's egg white lysozyme, also possesses two subsites analogous to E and F in the hen egg white enzyme.

The total contribution of the acetamido group interacting with subsite E and the sugar residue interacting with subsite F is about 1.4 Kcal/mole. In this connection, we have now measured the binding constant to lysozyme of the cell wall hexasaccharide, GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc, by the fluorescence technique (Chipman et al., 1967) and have found it to be $3.5 \times 10^4 M^{-1}$. A comparison of this value with the binding constant of the corresponding tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc ($2.1 \times 10^3 M^{-1}$, Chipman et al., 1967) shows that the free energy of association of the hexasaccharide with lysozyme is more negative by 1.7 Kcal/mole than that of the tetrasaccharide. This additional energy of interaction can only be due to binding of the two reducing terminal residues of the hexasaccharide in subsites E and F. The total contribution of subsites E and F to the binding of cell wall saccharides is thus only a little greater than the contribution due to the interaction of an acetamido group in subsite E and a hexose residue at subsite F.

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