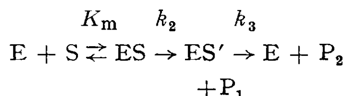
Acetamido-group Participation in Lysozyme Catalysis

By G. LOWE* and G. SHEPPARD
(*The Dyson Perrins Laboratory, Oxford University*)

THE lysozyme-catalysed hydrolysis of β-aryldi-N-acetylchitobiosides (I; R = Ar, R^1= H) into di-N-acetylchitobiose (I; R = R^1 = H) and a phenol obeys Michaelis–Menten kinetics, K_m being essentially independent of the aglycone and k_{cat} having a Hammett ρ +1·3. The products of lysozyme catalysis have the β-configuration at C(1) indicating overall retention of configuration. These results have been analysed in terms of a reaction pathway involving the formation of an enzyme–substrate complex (ES) followed by two catalytic steps, the first (k_2) which is rate-determining involving concerted nucleophilic-general acid catalysis.[1]

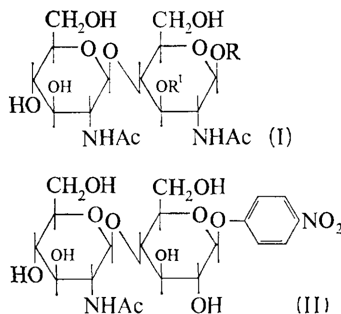


$$E + S \overset{K_m}{\rightleftarrows} ES \overset{k_2}{\rightarrow} ES' \overset{k_3}{\rightarrow} E + P_2$$
$$+P_1$$

The mechanism suggested by Blake *et al.*[2] and Vernon[3] in which glutamic acid-35 acts as a general acid catalyst, the incipient carboxonium ion being stabilised by the negative charge of the carboxylate ion of aspartic acid-52, is consistent with this evidence. An alternative mechanism in which general acid catalysis is assisted by acetamido-group participation is also consistent with the available evidence.[1,4] In order to decide between these alternative mechanisms the p-nitrophenyl glycoside (II) was synthesised. A Koenigs–Knorr reaction between N-p-methoxybenzylidene-3,4,6-tri-O-acetyl-2-amino-2-deoxy-α-D-glucopyranosyl bromide[5] and 1,2,3,6-tetra-O-acetyl-β-D-glucopyranose[6] was followed by glycoside formation with p-nitrophenol and O-deacetylation. The structures of the intermediates were confirmed by n.m.r. and mass spectrometry.

The Michaelis–Menten parameters for the p-nitrophenyl glycosides (I; R=p-$NO_2\cdot C_6H_4\cdot$, R^1= H) and (II) were determined under identical conditions and are shown in the Table. However, before the catalytic constants can be compared it is essential to know the productive binding constant (K_p) for each substrate.

X-Ray crystallographic studies on lysozyme and the lysozyme-tri-N-acetylchitotriose complex have shown that the active site of lysozyme comprises an extensive cleft in the protein which can be regarded as being made up of six subsites, labelled A to F, the reducing end of the oligosaccharide being at the subsite furthest down the cleft (*i.e.* at the subsite labelled with the highest letter).[2] It was also possible to deduce that the catalytic site was between subsites D and E, the glycosidic bond bridging these subsites being cleaved. X-Ray crystallographic studies on the complex between lysozyme and the glycoside (I; R = Ph, R′= H) showed that the two sugar residues were bound at the B and C subsites and the phenyl group over but not in the D subsite.[7] This conclusion was confirmed in solution by n.m.r. spectroscopy. The glycoside (I; R = Ph, R^1= H; 3·8 mg.) in D_2O (0·5 ml.) showed signals at τ 7·93 ($W_{h/2}$ 1·6 c./sec.) and 7·98 ($W_{h/2}$ 1·3 c./sec.) (64 spectrum accumulations) due to the acetamido-groups, the



high-field signal being assigned to the acetamido-group adjacent to the phenyl group by comparison with spectra of the methyl glycoside (I; R = Me, R^1= H), where the assignment was made with a specifically deuteriated derivative,[8] and with phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (τ 7·98). After addition of lysozyme (10 mg.) signals at τ 7·93 ($W_{h/2}$ 1·7 c./sec.) and 8·01 ($W_{h/2}$ 2·6 c./sec.) were observed (77 spectrum

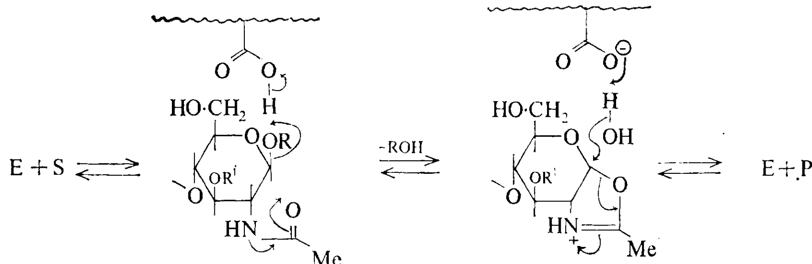
TABLE

The Michaelis–Menten parameters (K_m and k_{cat}) for lysozyme-catalysed hydrolyses at pH 5·0 *and* 35° *under conditions where* $[E_0] \gg [S_0]$. *K_p is the productive binding constant.*

Substrate	K_m (mM)	K_p (mM)	k_{cat} (10^{-7} sec.$^{-1}$)	$k_{cat}K_p/K_m$ (10^{-5} sec.$^{-1}$)	rel.$k_{cat}K_p/K_m$
(I; R = p-$NO_2\cdot C_6H_4\cdot$, R^1= H) ..	1·5	$\geqslant$25	120	$\geqslant$20	$\geqslant$100
(II)	3·4	12	5·6	0·2	1

accumulations). Similar observations have been made with the methyl glycoside (II; R = Me, R^1= H).[8] An up-field shift and marked line broadening are expected for the acetamido-group bound in the C subsite since the methyl group is firmly held close to and directly over the face of the indole ring of tryptophan-108. Since only the high field signal was markedly affected in this way it can be concluded that the predominant binding mode for this glycoside is with the sugar residues bound to the B and C subsites, that is in a non-productive binding mode. Some of the observed line broadening of the low field signal may be due to the effects of lysozyme other than binding in the C subsite (productive binding), so that it is only possible to estimate a lower limit for the productive binding constant (K_p). Using this and the observed K_m, a lower limit for the corrected catalytic constant ($k_{cat}K_p/K_m$) can be calculated (see Table).

The glycoside (II, 3·8 mg.) in D_2O (0·5 ml.) showed a signal at τ 7·94 ($W_{h/2}$ 1·7 c./sec.) (33 spectrum accumulations) due to the acetamido-group which on addition of lysozyme (10 mg.) showed a signal τ 7·94 ($W_{h/2}$ 2·0 c./sec.) (48 spectrum accumulations). The line broadening of this signal is significantly greater than that of the 7·93 signal in the glycoside (I; R = Ph, R^1= H) indicating more binding of this acetamido-group at the C subsite. The data allow the productive binding constant (K_p) for the glycoside (II) to be estimated and this together with the corrected catalytic constant ($k_{cat}K_p/K_m$) for this substrate are shown in the Table. Since the σ_I values for the hydroxyl (+0·25) and acetamido (+0·28) groups are very similar,[9] the greater rate constant for the glycoside (I; R = p-$NO_2 \cdot C_6H_4 \cdot$, R^1= H) relative to the glycoside (II), (100 : 1), can be attributed to acetamido-group participation. The mechanism of action of lysozyme is therefore as follows.



It is not possible to deduce the precise conformation of the sugar residue in the D subsite, but it is clear that it cannot be accommodated in the chair conformation.[2] Any departure from the chair conformation is bound to facilitate catalysis by assisting the ionisation of the glycosidic bond and by promoting acetamido-group participation. Three factors therefore appear to make a contribution to catalysis, (i) conformational distortion of the sugar residue in the D subsite, (ii) general acid catalysis, and (iii) acetamido-group participation. The identity of the general acid catalyst depends on the conformation of the sugar residue in the D subsite. The boat conformation would require aspartic acid-52 to be the general acid catalyst but the lactyl side chain of a *N*-acetylmuramic acid residue could not be accommodated unless some protein conformational change took place. The 'half-chair' conformation would require glutamic acid-35 to be the general acid catalyst, but this conformation is of exceptionally high energy and is incompatible with the binding and catalytic constants observed. The precise conformation of the sugar residue in the D subsite in the ES complex is probably somewhere between these two limits which would favour glutamic acid-35 as the general acid catalyst.

We thank Professor D. C. Phillips for valuable discussions and the S.R.C. for a Research Studentship (for G.S.).

(*Received, March* 19*th*, 1968; *Com.* 337.)

[1] G. Lowe, G. Sheppard, M. L. Sinnott, and A. Williams, *Biochem. J.*, 1967, **104**, 893.
[2] C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. Roy. Soc.*, 1967, *B*, **167**, 378.
[3] C. A. Vernon, *Proc. Roy. Soc.*, 1967, *B*, **167**, 389.
[4] G. Lowe, *Proc. Roy. Soc.*, 1967, *B*, **167**, 431.
[5] L. Zervas and S. Konstas, *Chem. Ber.*, 1960, **93**, 435.
[6] H. Bredereck and G. Höschele, *Chem. Ber.*, 1953, **86**, 1286.
[7] J. Moult and D. C. Phillips, private communication.
[8] E. W. Thomas, *Biochem. Biophys. Res. Comm.*, 1967, **29**, 628.
[9] M. Charton, *J. Org. Chem.*, 1964, **29**, 1222.