ACETAMIDO GROUP IN THE BINDING AND CATALYSIS OF SYNTHETIC SUBSTANCES BY LYSOZYME [+]

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Received April 20, 1970

Summary

Three substrates, p-chlorophenyl di-N-acetylchitobioside, p-chlorophenyl chitobioside, p-chlorophenyl di-N-ethylchitobioside, have been synthesized and their abilities to bind lysozyme and their rates of enzyme-catalyzed hydrolysis studied. Results are discussed in terms of the acetamido structure. The acetamido oxygen is essential for the formation of the nonproductive complex, whereas the acetamido group is not essential for the catalytic process of lysozyme.

Various experiments (Blake et al., 1967; Rupley et al., 1967; Neuberger and Wilson, 1967) have implicated the requirement for the acetamido group in the binding and catalysis of substrates by hen's egg-white lysozyme (N-acetylmuramide glycanohydrolase, EC 3.2.1.17). Although the participation of the acetamido group in the formation of nonproductive complex between N-acetylchitooligoses and lysozyme has been well documented (Blake et al., 1967; Raftery et al., 1969), a general agreement concerning the role of the acetamido group in the catalytic process is lacking (Chipman and Sharon, 1969). Cellubiose was reported to be hydrolyzed by lysozyme to a limited extent (Rupley and Gates, 1967), however, Piszkiewicz and Bruice (1969) were not able to demonstrate the formation of productive complexes between cellooligoses and lysozyme. An anchimeric participation of the acetamido group in the catalytic process was considered by Lowe and Sheppard (1968),

[+] This work was supported by grants from the National Research Council of Canada and the President of Carleton University.

but this factor did not explain the observation that lysozyme also cleaved glycosidic bonds at glucose and 2-deoxyglucose (Rand-Meir, Dahlquist and Raftery, 1968). To gain more information about the role of the acetamido group, the present work was undertaken to compare the effect of acetamido, amino and N-ethyl groups on the binding and catalysis of synthetic substrates by lysozyme.

Materials and Methods

Twice crystallized, salt-free lysozyme from hen's egg-white was purchased from Worthington Biochemical Corp. p-Chlorophenyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside(p-chlorophenyl di-N-acetylchitobioside, CPAC*) was synthesized by the method described previously (Tsai, Tang and Subbarao, 1969). Hydrazenolysis (Fujinaga and Matsushima, 1966) of CPAC yielded p-chlorophenyl 2-amino-4-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside(p-chlorophenyl chitobioside, CPC) which was recrystallized from aqueous methanol to give white needles, m.p. 212-214°C (decomp.), $[\alpha]_D$ = -70.0 (c 0.35 in water). The IR spectrum (KBr) showed the disappearance of amide absorptions at 1640 and 1545 cm^{-1}. The treatment of CPC with acetaldehyde followed by $NaBH_4$ reduction gave p-chlorophenyl 2-ethylamino-4-O-(2ethylamino-2-deoxy-β-D-glucophranosyl)-2-deoxy-β-D-glucopyranoside(p-chlorophenyl di-N-ethyl chitobioside, CPEC) which had m.p. 190 - 193°C (decomp.), $[\alpha]_D$=-45.3 (c 0.14 in 70% methanol) The NMR spectrum (in CD_3OD using sodium 3-(trimethylsilyl)-1-propane sulfonate as the internal standard) showed a pair of quartets at δ = 6.64 and 6.66 ppm (CH_2) and a pair of triplets at δ = 8.85 and 8.87 ppm (CH_3). Difference UV spectra were taken in a Cary 14 spectrophotometer using a 0 - 0.1 absorbance slide wire assembly (Dahlquist, Jao and

* The following abbreviations are used: CPAC, p-chlorophenyl di-N-acetyl-chitobioside; CPC, p-chlorophenyl chitobioside; and CPEC, p-chlorophenyl di-N-ethylchitobioside.

Raftery, 1966) at room temperature. The rates of hydrolysis by lysozyme were followed by the method previously described (Tsai, Tang and Subbarao, 1969).

Results and Discussion

The crystallographic study of N-acetyl aminosugar-lysozyme complexes indicates that amide nitrogen and carbonyl oxygen of the acetamido group form hydrogen bonds with the enzyme (Blake et al., 1967). The binding inhibition (Neuberger and Wilson, 1967) and NMR (Thomas, 1966) studies implicate the hydrophobic interaction between the acetamido methyl group and lysozyme. A red shift in UV spectrum of lysozyme on interaction with N-acetyl chitooligoses (Hayoshi, Imoto and Funatsu, 1963; Dahlquist, Jao and Raftery, 1966) has been interpreted in terms of conformational changes of the enzyme (Hayashi, Imoto and Funatsu, 1964; Lehrer and Fasman, 1967) accompanying the formation of nonproductive complexes. To assess contributing elements of the acetamido group in the formation of nonproductive complex and its relationship with the spectral shift, CPAC, CPC and CPEC which differ in the N-substitution, were synthesized and their effects on the lysozyme spectrum were compared.

Fig. 1A shows the difference UV spectrum of lysozyme in the presence of CPAC with four peaks in the region between 270 and 295 nm. The maximum difference at 293.5 nm is attributed to the hydrophobic perturbation of tryophane residue of the enzyme by the substrate (Hayashi, Imoto and Funatsu, 1964). The peak at 280 nm, which is not observed in N-acetyl-chitooligoses is presumably due to the interaction of the aryl group of the synthetic substrate with lysozyme. In the presence of CPC and CPEC, the positive difference spectra and, in particular, the maximum difference at 293.5 nm are not observed (Figs, 1B and C). Since CPC at a concentration of 1.5×10^{-3} M does not inhibit lysozyme (2.5×10^{-5} M) from forming the nonproductive complex with CPAC (5.0×10^{-4} M), it would appear that

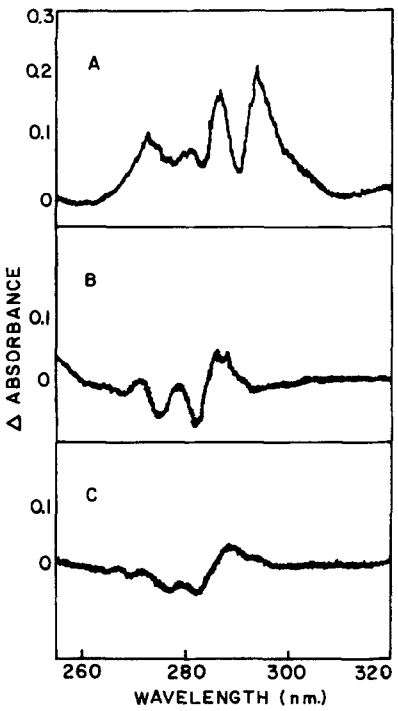

Fig. 1 Difference UV spectra of lysozyme (2.5×10^{-5} M) in the presence of 2.5×10^{-3} M of CPAC (A), CPC (B) and CPEC (C) at pH 5.1.

CPC (and CPEC likewise) did not form the nonproductive complex or formed only a very weak complex with lysozyme insufficient to generate spectral changes. The result suggests that the amide nitrogen alone or in combination with the acetamido methyl group is not sufficient to form a stable complex, and the acetamido oxygen is needed in combination with the amide nitrogen presumably to interact with both sides of the cleft of lysozyme (Blake et al., 1967).

Fig. 2 shows that all three synthetic compounds, CPAC, CPC and CPEC in that order of reactivities, are hydrolyzed by lysozyme suggesting that the acetamido group is not indispensable, though preferred for the catalytic process.

In nonenzymic systems (Piszkiewicz and Bruice, 1967), the acetamido group is shown to facilitate the hydrolysis of glycosidic linkage

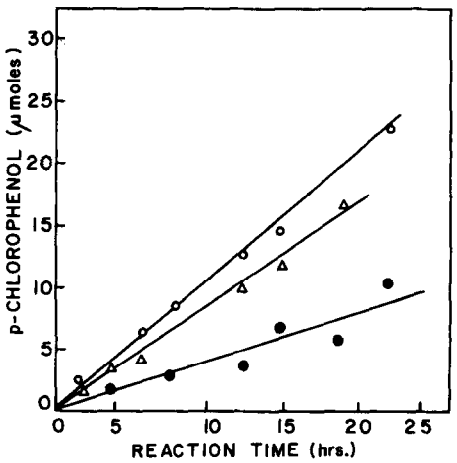

Fig. 2. Rates of lysozyme-catalyzed hydrolysis of CPAC (—o—o—), CPC (—Δ—Δ—) and CPEC (—●—●). Reaction mixtures containing 8.0 x 10^{-3} M of lysozyme and 1.0 x 10^{-4} M of substrates in 0.1 M citrate buffer (pH 5.1) were incubated at 45°C. Samples were withdrawn at suitable time intervals for the determination of p-chlorophenol.

via an anchimeric assistance which is considered by Lowe and Sheppard (1968) for lysozyme catalysis. Since lysozyme is known to cleave glycosidic linkages at glucose, 2-deoxyglucose (Rand-Meir, Dahlquist and Raftery, 1969) and 2-amino-2-deoxyglucose as shown in this experiment (Fig. 2), the anchimeric participation of the acetamido group in the catalytic process of lysozyme cannot be important. The hydrophobic interaction between the acetamido methyl group and lysozyme to provide the productive complex can be disregarded because CPEC is the poorest substrate of the three synthetic compounds tested.

The crystallographic study (Johnson and Phillips, 1965) shows that N-acetylaminosugars bind to lysozyme in such a way that the acetamido group of the reducing end interacts with site C of the cleft in the nonproductive complex. The binding of N-acetyl chitohexaose in the productive complex is inferred by fitting molecular models of N-acetyl glucosamine as an extention of the observed complex along the cleft. Aryl chitobioside forms a similar nonproductive complex (Raftery et al., 1969), however, the exact binding mode of the productive

complex which must involve sites D and E is unknown. Because of a difference in the requirement for the acetamido group of synthetic substrates in the formation of nonproductive and productive complexes, it is concluded that synthetic substrates form the productive complex (1) without interacting with site C or (2) by interacting with site C in a different mode than as suggested for the nonproductive complex.

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