KINETIC EVIDENCE FOR TWO TYPES OF ACTIVE SITE IN THE *N*-ACETYL-β-D-HEXOSAMINIDASE OF BOVINE KIDNEY

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1. Introduction

N-Acetylhexosaminidases have been reported to hydrolyse the terminal *N*-acetylglucosamine and *N*-acetylgalactosamine from β-glycosidic linkages at comparable rates. In general, it has proved impossible to separate the *N*-acetyl-β-D-glucosaminidase activity (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) from the *N*-acetyl-β-D-galactosaminidase activity (2-acetamido-2-deoxy-β-D-galactoside acetamidodeoxygalactohydrolase, EC 3.2.1.53) by conventional protein fractionation procedures, and this fact, together with the close similarity between their pH and thermal inactivation profiles, has established the view that the two activities are carried by one enzyme that has been designated *N*-acetylhexosaminidase.

Previous kinetic investigations, some rather limited, have concluded that the same active sites are responsible for the two activities [1–7]. However, a detailed kinetic study from this laboratory on the enzymes of rat colonic tissues strongly suggested the presence of two separate active sites, each able to be inhibited by the substrate for the other site [8]. In view of this apparent difference between the rat colonic enzymes and those reported from other sources, it seemed to us desirable to carry out a similar detailed kinetic investigation on a readily available preparation of *N*-acetylhexosaminidase.

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2. Experimental

N-Acetyl-β-D-glucosaminidase from bovine kidney was a gift from the Boehringer Corp. (London) as a 5 mg/ml suspension and was diluted in 50 mM citrate buffer, pH 4.2 [9], as required.

p-Nitrophenyl *N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide, phenyl *N*-acetyl-β-D-glucosaminide and *N*-acetyl-D-glucosamine were obtained from Sigma (London) Chemical Co., and *N*-acetyl-D-galactosamine and *N*-acetylgalactosaminolactone (2-acetamido-2-deoxy-D-galactonolactone) from Koch-Light Labs. Solutions of the lactone inhibitor were prepared as in [10]. All other chemicals were of analytical reagent grade or the best possible available.

The *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities were measured as in [8], using either the *p*-nitrophenolate $A_{400\ nm}$ or the determination of phenol with Fast Red B Salt (Sigma), adapted from the procedure in [11]. When both activities were to be measured in the same reaction mixture (1.0 ml), the reaction was stopped by adding 2 ml alcoholic sodium borate solution [11] instead of glycine buffer [8]. The $A_{400\ nm}$ was then measured before proceeding with the phenol estimation. It was verified that neither product interfered with the determination of the other. Kinetic studies were carried out with each run duplicated to obtain values for Michaelis and inhibition constants, and the primary data analysed statistically as in [10]. Weighted means of the values from several experiments, and their standard errors, were calculated according to [12].

Student's t-test was applied to decide whether two values were significantly different.

3. Results and discussion

The K_m and V values for both N-acetylhexosaminidase activities are shown in table 1. The dissimilarity in the K_m values for the two p-nitrophenyl substrates resembles that found for the corresponding activities in rat kidney [2], rat colon [8], ram testis [1], and bovine spleen [5]. The relative values of both kinetic parameters are in good agreement with those reported for enzyme purified from preparations of bovine serum albumin [13].

When two substrates are acting as alternatives for an enzyme, it can be shown theoretically that one will inhibit the reaction of the other competitively, with a K_i equal to its own Michaelis constant. By following p-nitrophenol and phenol production separately in the same reaction mixture, it was possible to measure such inhibition constants. Thus with the substrates phenyl N-acetylglycosaminide and p-nitrophenyl N-acetylgalactosaminide, present together, the K_i for phenyl N-acetylglucosaminide with respect to the galactosaminide was 4.7 ± 2.7 mM (2 obs.) to be compared with its K_m of 1.02 (table 1); and for p-nitrophenyl N-acetylgalactosaminide with respect to the glucosaminide, the K_i was 0.159 ± 0.010 mM (2 obs.) to be compared with 0.204 (table 1). These K_i values are significantly different ($P < 0.08$ and 0.001, respectively) from the corresponding K_m values, suggesting that there are separate active sites for the two activities, each necessarily able to be inhibited by the substrate for the other. Because of the relatively high standard error of K_i for phenyl N-acetylglucosaminide, the probability that one may be wrong in assuming the value to be different from the K_m value is somewhat larger.

Table 1
Michaelis constants and maximum velocities for the N-acetylhexosaminidase activities of bovine kidney

Substrate	No. expt.	K_m (mM)	V (nmol/h/μg protein)
p-Nitrophenyl N-acetylglucosaminide	11	1.87 ± 0.10	532 ± 24
p-Nitrophenyl N-acetylgalactosaminide	13	0.204 ± 0.008	70.2 ± 1.0
Phenyl N-acetylglucosaminide	3	1.02 ± 0.26	188 ± 31

Values given are the weighted means ± SE of the results from the number of experiments stated

A modification of the common active site mechanism that allows the values of K_m and K_i for a particular substrate to be different has been described [2]. However certain ratios, as given in eq. (1) should be equal:

$$\frac{(K_m)_a}{(K_i)_b} = \frac{(K_i)_a}{(K_m)_b} \quad (1)$$

The competing reactants are denoted by subscripts 'a' (phenyl N-acetylglucosaminide) and 'b' (p-nitrophenyl N-acetylgalactosaminide). Data reported [2] for the

Table 2
Inhibition constants for the N-acetylhexosaminidase activities of bovine kidney

Inhibitor	Substrates	
	p-Nitrophenyl N-acetylglucosaminide	p-Nitrophenyl N-acetylgalactosaminide
N-acetylglucosamine	0.384 ± 0.063 (3)	0.61 ± 0.13 (2)
N-acetylgalactosamine	1.59 ± 0.16 (2)	0.576 ± 0.069 (3)
N-acetylgalactosaminolactone	0.0293 ± 0.0024 (3)	0.00213 ± 0.00008 (2)

Values given are the weighted means ± SE of the results from the number of experiments given in parentheses, in mM units

rat kidney enzyme fitted this equation. Our data give a value of 6.4 for the left-hand side and 23.0 for the right-hand side of this equation, not consistent with the mechanism in [2].

For two of the competitive inhibitors in table 2, in which the K_i values are reported, the values for each with respect to the two substrates have been shown to be significantly different (*N*-acetylgalactosamine, $P < 0.002$; *N*-acetylgalactosaminolactone, $P < 0.001$). The values for *N*-acetylglucosamine were not significantly different ($P < 0.05$). A common active site for both activities would require that any inhibitor should have the same K_i values with respect to the two substrates; the fact that at least two inhibitors have been shown to have different K_i values is consistent with the hypothesis of separate active sites.

We have found similar evidence for the *N*-acetylhexosaminidase activities of rat colonic mucosa [8] and colonic tumours (N.M. et al., unpublished). Furthermore, kinetic data for the rat colonic enzyme [8], obtained using *p*-nitrophenyl *N*-acetylglucosaminide (substrate a) and *p*-nitrophenyl *N*-acetylgalactosaminide (substrate b) as mixed substrates in varying proportions, α, were inconsistent with the relationship [5] in eq. (2), where V_{a+b} represents the observed maximum velocity when the rate is followed by measuring the common product, *p*-nitrophenol:

$$\frac{(K_m)_a}{a(K_m)_b} = \frac{V_a - V_{a+b}}{V_{a+b} - V_b} \qquad (2)$$

This relationship assumes a common active site catalysing the two reactions. Table 3 shows the results obtained with the bovine kidney enzyme. It is clear that the data are not in agreement with eq. (2); indeed, in some cases the observed combined maximum velocity is less than the lower of the two individual values. On the other hand, the mixed substrates kinetic data obtained for the enzymes from human liver [4], bovine spleen [5], jack bean meal [3], fenugreek seeds [7] and *Trichomonas foetus* [6] were apparently consistent with eq. (2) and used as evidence of a common active site for the two activities.

In summary, our results show that K_m and K_i values for a particular compound are not equal, suggesting that when it is used as a substrate we are seeing it binding to a different site compared with when it is used as an inhibitor; a modified one-site mechanism is eliminated by the results not fitting eq. (1). The conclusion is supported by the result that some inhibitors have different K_i values for the two activities, and that mixed substrates experiments do not agree with eq. (2).

Table 3
Calculated and observed maximum velocities in the joint reaction of *p*-nitrophenyl *N*-acetylglucosaminide (a) and *p*-nitrophenyl *N*-acetylgalactosaminide (b) as substrates

$(K_m)_a$	V_a	$(K_m)_b$	V_b	α	V_{a+b}	
					Observed	Calc. according to eq. (2)
1.9	550	0.2	80	0.2	42.0 ± 1.2 (3)	90
2.2	570	0.18	70	0.5	43.0 ± 1.5 (3)	90
				1.0	52.3 ± 1.5 (3)	108
1.7	585	0.22	75	2.0	83.3 ± 6.0 (3)	180
				5.0	128.3 ± 4.4 (3)	275

Units are as in table 1, and the values for K_m and V were determined by experiments not included in the data in table 1. The observed values of V_{a+b} are arithmetic means ± SEM from the number of experiments given in parentheses

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