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A Quantitative Approach to the Evaluation of 2-Acetamide Substituent Effects on the Hydrolysis by Taka-N-acetyl-β-D-glucosaminidase

Role of the Substrate 2-Acetamide Group in the N-Acyl Specificity of the Enzyme

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The activity of Taka-N-acetyl-β-D-glucosaminidase [EC 3.2.1.30] toward fifteen newly synthesized substrate analogs with different N-substituent was determined.

The contribution of the substituent constants of the substrate N-acyl group (Vw, σ^*, and π) to the relative rate (V_R) of enzymatic hydrolysis was evaluated quantitatively by regression analysis to ascertain the role of the substrate 2-acetamide group in the N-acyl specificity of this enzyme. The following equation gave the best fit for each parameter.

$$\log V_R = 2.90 - 0.208\,V_W - 0.633\,\sigma^* + 0.538\,\pi$$

A mechanism for the enzymic hydrolysis was proposed based on this relationship.

N-Acetyl-β-D-glucosaminidase [EC 3.2.1.30] has been found in several organisms and seems to differ from other glycosidases in its specific affinity for the 2-acetamide group of the substrate. Thus, to clarify the mechanism of hydrolysis, the role of the 2-acetamide group in the enzymatic reaction must be determined.

Recently, N-acetyl-β-D-glucosaminidase was obtained in a pure form from Taka-diastase (Sankyo) (*1*), making studies on the mechanism of the reaction possible. To examine the specificity of the enzyme towards N-acyl groups, *p*-nitrophenyl 2-acylamino-2-deoxy-β-D-glucopyranosides (*p*-nitrophenyl N-acyl-β-D-glucosaminides) with several kinds of N-acyl substituent were prepared and their susceptibilities to this enzyme were investigated (*3–6*). It was concluded that the N-acetyl group of the substrate was most favorable for the enzymatic reaction, but that hydrolysis also occurred when the α-hydrogen of the N-acetyl group was substituted with monohalogen, methyl, or hydroxyl groups. In the previous work, the characteristics of the substrate N-acyl group which controlled the N-

N-Acetyl-β-D-glucosaminidase from Taka-diastase (Sankyo) is called Taka-N-acetyl-β-D-glucosaminidase here.

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acyl specificity were not evaluated quantitatively because the coefficients characterizing the properties of each N-acyl substituent could not be estimated. Thus, to investigate the reaction mechanism in more detail, quantitative analysis of the structure-reactivity relationship between the N-acyl substituent of the substrate and the N-acyl specificity is required.

The present report describes quantitative studies on the factors contributing to the N-acyl specificity and on the basis of the results a possible mechanism for hydrolysis by Taka-N-acetyl-β-D-glucosaminidase is suggested.

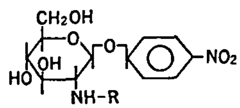
EXPERIMENTAL PROCEDURES

Fifteen *p*-nitrophenyl 2-acylamino-2-deoxy-β-D-glucopyranoside were prepared as described previously (*3–6*). The enzyme specimen, purified from Taka-diastase (Sankyo), was the same as in previous studies (*2–6*). Enzymatic reactions, with 1.00 mM of the substrate and 0.50–30 μg/ml of the enzyme, were performed in 0.1 M citrate buffer, pH 4.5, at 37°C, and reaction rates were measured very precisely using a procedure similar to that used previously (*3–6*). The reaction was followed within the range in which 1% of the substrate was hydrolysed, because the initial velocity thus obtained seems to be comparable to the maximum velocity of Michaelis-Menten equation, as noted by Dixon (*7*); each value of the initial velocity was an average of five samples. Calculations were performed with a Hewlett-Packard computer (model 10) using its program library.

TABLE I. Rates of hydrolysis of *p*-nitrophenyl 2-acylamino-2-deoxy-β-D-glucopyranosides by Taka-N-acetyl-β-D-glucosaminidase. Rates are expressed relative to that of the N-acetyl compound [02]. Reaction conditions; concentration of the substrate, 1.00 mM; temperature, 37°C; buffer, 0.1 M citrate, pH 4.5.

Comp. Nos.	N-Substituent (R)[1]	Relative rate
[02]	CH_3CO	1.00
[07]	FCH_2CO	4.96×10^{-1}
[03]	CH_3CH_2CO	4.75×10^{-2}
[13]	$HOCH_2CO$	2.31×10^{-2}
[01]	HCO	1.28×10^{-2}
[08]	$ClCH_2CO$	1.22×10^{-2}
[09]	$BrCH_2CO$	3.99×10^{-3}
[10]	F_2CHCO	6.24×10^{-3}
[14]	CH_3OCH_2CO	6.84×10^{-4}
[04]	$CH_3CH_2CH_2CO$	5.63×10^{-4}
[12]	CF_3CO	3.26×10^{-4}
[05]	$(CH_3)_2{>}CH-CO$	0.00[2]
[11]	Cl_2CHCO	0.00
[06]	C_6H_5CO	0.00
[15]	CH_3SO_2	0.00

[1] R indicates an N-substituent in the following structure;



[2] The value was nearly, but not quite zero. A very slow rate of hydrolysis was observed.

RESULTS AND DISCUSSION

The rates of hydrolysis of each substrate analog, expressed as values relative to that of the N-acetyl derivative, are shown in Table I.

The predominat contribution of the steric bulkiness of the N-substituent to the hydrolytic rate, reported previously, can also be deduced from the results in Table I. The factor corresponding to the steric bulkiness of the N-substituent is usually expressed quantitatively as Taft's Es value (*8*). The Es value was used to represent the relationship between structure and biological activity in the pioneer work of Hansch (*9*). However, it seemed to be a composite factor because it was calculated from the hydrolytic rate of aliphatic esters (*8*). Therefore, the van der Waals volume (molecular volume) (*10*) was chosen as a more direct value corresponding to the space occupied by the N-acyl substituent, because the N-acyl group of the substrate is not located in the reaction center but in its neighborhood, and because there is probably a space in the enzyme molecule into which the methyl group of the substrate 2-acetamide group fits (*3*).

To examine the relationship between the steric factor of the N-acyl substituent and the relative rate of hydrolysis, the logarithms ($\log V_R$) of the relative rates (V_R) of enzymatic hydrolysis were plotted against the van der Waals volumes (V_W), calculated by the procedure of Bondi (*10*). The results (Fig. 1) shown that the molecular volume (V_W) corresponding to the steric factor of the N-substituent contributed predominantly to the hydrolytic rate except in the case of the N-formyl compound. Moreover, linear relationships between $\log V_R$ and V_W were observed with the homologs of the n-alkyl groups (from $-CH_3$ to $-C_3H_7$) and the fluoro-substituted methyl groups (from $-CH_2F$ to $-CF_3$) respectively. However, comparison of the hydrolytic rates of N-alkyl-substituted acetyl compounds ([02], [03], [04]) and N-fluoro-substituted acetyl compounds ([07], [10], [12]) suggested that other factors besides the steric factor contributed to the hydrolytic rate since the substituents on the latter compounds are electron withdrawing while those on the former are electron donating. The exceptional behavior of the N-formyl compound with the smallest value of V_W also suggested the contribution of another factor (probably a hydrophobic factor). Hence among the other factors, electronic and hydrophobic factors seemed to be most likely to contribute, as proposed by Hansch (*9*).

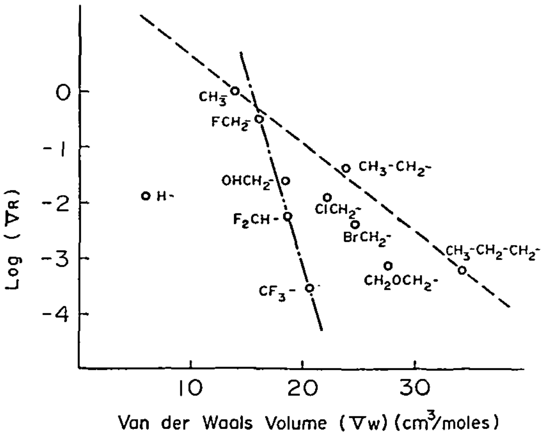


Fig. 1. Relationship between the relative rate (V_R) of enzymatic hydrolysis and the van der Waals volume (V_W) corresponding to the steric factor of each N-acyl substituent of the substrate. Values for V_W are listed in Table II. R of the N-acyl group (RCO—NH—) is shown.

The correlation between these factors (steric, electronic, and hydrophobic factors) and the rate of enzymatic hydrolysis was analysed by computerized regression analysis to ascertain the role of the substrate N-acyl group in the enzymatic hydrolysis.

TABLE II. Constants of the N-acyl group substituents of the substrate, used in derivation of the equations.

Comp. Nos.	Alkyl substituent of N-acyl group	van der Waals volume (V_W)[1]	Polar substituent constant (σ^*)[2]	Hydrophobicity (π)[3]
[01]	H	5.7	+0.490	0.00
[02]	CH_3—	13.67	0.00	0.50
[03]	CH_3CH_2—	23.90	−0.100	1.00
[04]	CH_3—CH_2—CH_2—	34.13	−0.115	1.50
[07]	FCH_2—	15.95	+1.10	0.32
[08]	$ClCH_2$—	21.85	+1.05	0.89
[09]	$BrCH_2$—	24.63	+1.00	1.10
[10]	F_2CH—	18.22	+2.05	0.16
[12]	CF_3—	20.49	+3.30	−0.01
[13]	$HOCH_2$—	18.27	+0.555	−0.66
[14]	CH_3OCH_2—	27.60	+0.520	0.03

[1] Calculated by the procedure of Bondi (*10*). [2] From Ref. *7*. [3] Calculated on the basis of the data for π_3 in the report of Hansch and Anderson (*12*).

The steric factor was expressed numerically in terms of van der Waals volume (V_W), the electronic factor in terms of Taft's polar substituent constant (σ^*) (*11*), and the hydrophobic factor in terms of hydrophobicity (π) (*12*). The set of numerical constants employed in the regression analysis are listed in Table II.

The use of regression analysis for examination of the contributions of these factors to the enzymatic hydrolysis can be illustrated by Eqs. 1–4. These equations correlate the relative rates of enzymatic hydrolysis of a set of N-acyl substituents of substrate analogs ([02], [03], [04], [07], [08], [09], [10], [12], [13], and [14]). In the equations, n is the number of compounds tested and r is the correlation coefficient.

	n	r	
$\log V_R = 1.17 - 0.143\,V_W$	10,	0.723	(1)
$\log V_R = 2.71 - 0.184\,V_W - 0.710\,\sigma^*$	10,	0.946	(2)
$\log V_R = 1.89 - 0.196\,V_W + 0.895\,\pi$	10,	0.855	(3)
$\log V_R = 2.90 - 0.208\,V_W - 0.633\,\sigma^* + 0.538\,\pi$	10,	0.955	(4)

The least important substituent effect is that of the hydrophobic factor (Eq. 3). The most meaningful correlation is that of Eq. 2, indicating that a combination of the steric factor and electronic factor contributed preferentially to the effect of the N-substituent on the enzymatic hydrolysis. Equation 4, in which all three terms (V_W, σ^* π) were combined, gave a slight improvement.

The negative correlation of the steric factor, expressed as V_W, which gave a better result than Es, indicated that the bulkiness of the N-substituent was the main factor decreasing the rate of enzymatic hydrolysis. V_W, which gave a better correlation than Es, corresponded directly to the space occupied by the N-acyl substituent. Consequently, the above results suggested that the specificity-determining site of the enzyme had a space into which the methyl group of the substrate 2-acetamide group fitted.

The negative correlation observed with the electronic factor (σ^*) indicated that an electron-withdrawing effect decreased the hydrolytic rate while an electron-donating effect increased the rate. This was best explained by considering that a neighboring 2-acetamide group participated in the enzymatic hydrolysis. It has been reported that the acid hydrolysis of 2-acetamide-2-deoxy-D-glucopyranoside was greatly enhanced by the participation of a neighboring 2-acetamide group (*13*) and that acyl migration from the 2-acylamino group to position-1 in 2-acylamino-3, 4, 6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl bromide was affected by decrease in the effect of an electron-withdrawing group in the N-acyl group (*14–16*). In the hydrolysis by Taka-N-acetyl-β-D-glucosaminidase, the electron-withdrawing effect seems to operate in a similar manner.

Hydrolysis of glycoside by Taka-N-acetyl-β-D-glucosaminidase seems to proceed by a mechanism of concerted acid-catalysis analogous to that in the reaction of lysozyme [EC 3.2.1.17]. Moreover, the sulfur isolog of *p*-nitrophenyl glycoside was found to be hydrolyzed at a very slow rate (K. Yamamoto, unpublished), just as lysozyme attacked the sulfur isolog of glycoside very slowly, and this effect could be explained in terms of similarity of the mechanism to that of non-enzymatic acid hydrolysis (*20*, *21*). From studies on the mechanism of hydrolysis by lysozyme, Vernon (*22*) and Lowe (*23*) proposed that the glycosyl carbonium ion existed as a metastable intermediate in the hydrolytic pathway and the cation was stabilized by the negative charge of the carboxylate of Asp-52 or by a nucleophile from the 2-acetamide group of the substrate itself. The results of an X-ray diffraction study (*17–19*) suggested that an Asp-52-stabilized cation intermediate was more preferable. The mechanism of hydrolysis by Taka-N-acetyl-β-D-glucosaminidase was similar to that for lysozyme, but it seemed probable that heterolysis of the glycosidic bond was as-

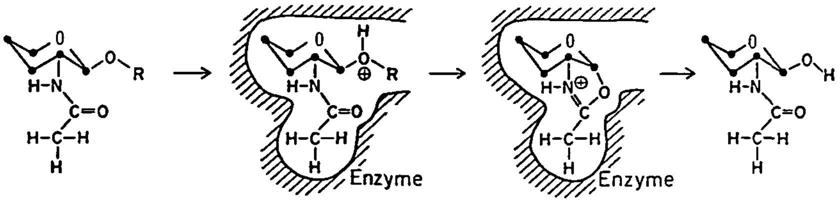


Fig. 2. Schematic representation of a mechanism for hydrolysis by Taka-N-acetyl-β-D-glucosaminidase and the contribution of the substrate 2-acetamide group to the enzymatic reaction.

sisted by participation of the 2-acetamide group, unlike the case of lysozyme. This mechanism is supported by the facts that the electronic effect of the N-acyl substituent was related to the order of its participating effect and that an N-methanesulfonylated derivative (*6*), which showed no participating effect, was not hydrolysed by this enzyme.

The hydrophobic factor contributed least to the rate of hydrolysis but it could not be excluded completely in considering the exceptional behavior of the N-formyl compound. The hydrophobic interaction was expressed as a short range interaction with a factor of (distance)$^{-6}$, and thus a smaller group (hydrogen in the N-formyl group) than methyl showed a greatly reduced interaction.

On the basis of these results, the mechanism of hydrolysis by Taka-N-acetyl-β-D-glucosaminidase was shown schematically in Fig. 2.

It was concluded that the 2-acetamide group of the substrate has the following role in the hydrolysis by Taka-N-acetyl-β-D-glucosaminidase. The methyl group of the 2-acetamide group fits into a space in the specificity-determining site of the enzyme due to hydrophobic interaction. The carbonyl group assists heterolysis of the protonated intermediate and subsequently stabilizes the resulting carbonium ion with an oxygen nucleophile.

The relative rates of hydrolysis observed in this work may be regarded as approximate ratios of apparent rate constants because the initial velocities of hydrolysis were compared as values in constant concentrations of substrate (1.00 mM) and enzyme (0.50 μg/ml). Thus it is very interesting that the logarithms of the relative rates of enzymatic hydrolysis correlated with linear combinations of the factors for the steric, electronic, and hydrophobic effects of the N-substituents; in other words, that a linear free energy relationship held in the enzymatic reaction.

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