THE INACTIVATION OF β-GALACTOSIDASE BY *N*-BROMOACETYL-β-D-GLUCOSYLAMINE

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1. Introduction

A classical approach for mapping the active site of an enzyme is provided by chemical modifications. Correlation between the loss of activity and the modification of residues could allow the identification of the essential groups of the protein [1]. The use of 'active site directed reagents', by reducing the number of modified groups, could facilitate their identification [2–8].

Evidence that two subsites are at the active center of β-galactosidase, a galactose subsite, is presented elsewhere [9]. Therefore, an active site-directed reagent could bind to one or the other subsite, leading to a broader definition of specificity. To study such a possibility, the effect of *N*-bromoacetyl-β-D-glucosylamine on β-galactosidase has been tested, and compared to that of *N*-bromoacetyl-β-D-galactosylamine, studied by Naider et al. [7,8].

2. Materials and methods

β-Galactosidase was prepared from *E. coli* strain 2EO1 as previously described [10]. *N*-Br-Glu was prepared as described [11].

Abbreviations: *o*-Np-Gal, *o*-nitrophenyl-β-D-galactoside; *N*-Br-Gal, *N*-bromoacetyl-β-D-galactosylamine; *N*-Br-Glu, *N*-bromoacetyl-β-D-glucosylamine

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Enzymatic assays were performed in a 0.1 M sodium phosphate buffer, pH 7.0, with 2.3 mM *o*-Np-Gal and in the presence of 1 mM $MgSO_4$, at 25°C. The appearance of *o*-nitrophenol was recorded at 373 nm, in a Cary 16 spectrophotometer.

Inactivation was monitored at 25°C, in 0.1 M sodium phosphate buffer, pH 7.5, 1 mM $MgSO_4$, with different concentrations of *N*-Br-Glu. Aliquots of 20 μl were withdrawn at various time and assayed for activity in 3 ml of incubation mixture. In this dilution, no inactivation occurs during the time assay.

Reactivation by β-mercaptoethanol was studied under the following conditions: β-galactosidase inactivated by 56 mM *N*-Br-Glu as previously described was mixed with the same vol. 1 M Na_2HPO_4 (final pH 8.4) at 29°C, and 0.176 M β-mercaptoethanol was added. Aliquots were withdrawn, at various times, and the enzymatic activity was measured.

3. Results

3.1. *Inactivation of the enzyme by* N-*bromoacetyl-β-D-glucosylamine*

β-Galactosidase is inactivated by *N*-Br-Glu. The kinetics are pseudo-first order, as shown in fig.1, until at least 99% of the reaction.

The apparent rate constant seems to depend upon the reagent concentration according to a hyperbolic saturation curve (fig.2). However, it is not possible to test concentrations high enough to reach a plateau value.

These results allow to conclude that the inactivation by *N*-Br-Glu proceeds, similarly to the inactiva-

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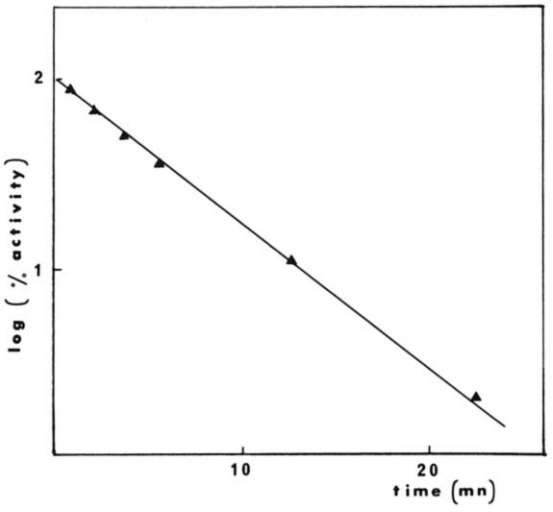

Fig.1. Inactivation of β-galactosidase by *N*-bromoacetylglucosylamine. Semi-logarithmic plot of the enzymatic activity versus time, in the presence of 5.3 mM *N*-Br-Glu.

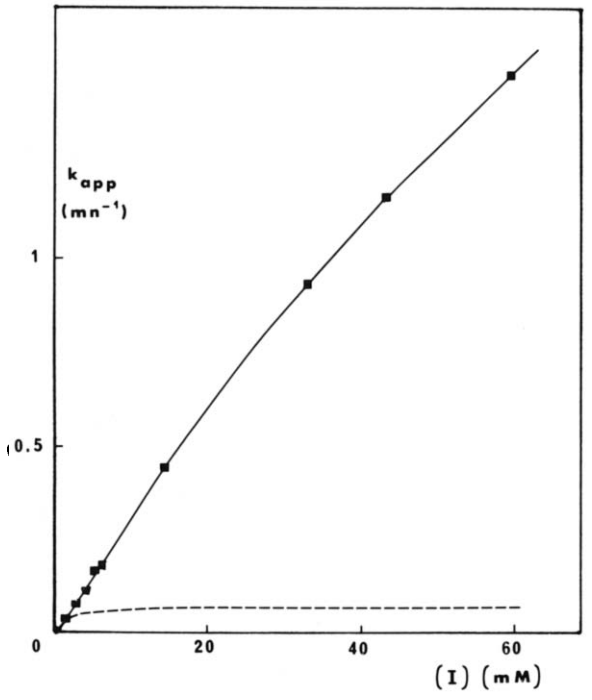

Fig.2. Dependence of the first order rate constant on the concentration of *N*-Br-Glu. The curve is calculated assuming k_i = 7 mn^{-1} and K_I = 0.2 M (see text). Dotted line: Data of Naider et al. [8] with *N*-Br-Gal.

tion by *N*-Br-Gal [8], via a first reversible binding of the reagent to the enzyme according to scheme I:

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_i} EI' \qquad \text{Scheme I}$$

The apparent first order constant is given by the equation:

$$k_{app} = \frac{k_i}{1 + K_I/I}$$

An evaluation of both constants K_I and k_i can be obtained by a statistical treatment of the data of the fig.2 according to Cleland [12]. K_I is about 0.2 M and k_i 7 mn^{-1}.

3.2. *Reversion of the inactivation by β-mercaptoethanol*

In this experiment, β-galactosidase previously inactivated by *N*-Br-Glu was incubated at alkaline pH, in the presence of 0.176 M β-mercaptoethanol. A reactivation is observed. The appearance of the activity is pseudo-first order reaction (fig.3) with a rate constant of 0.042 mn^{-1}. In the same conditions, β-galactosi-

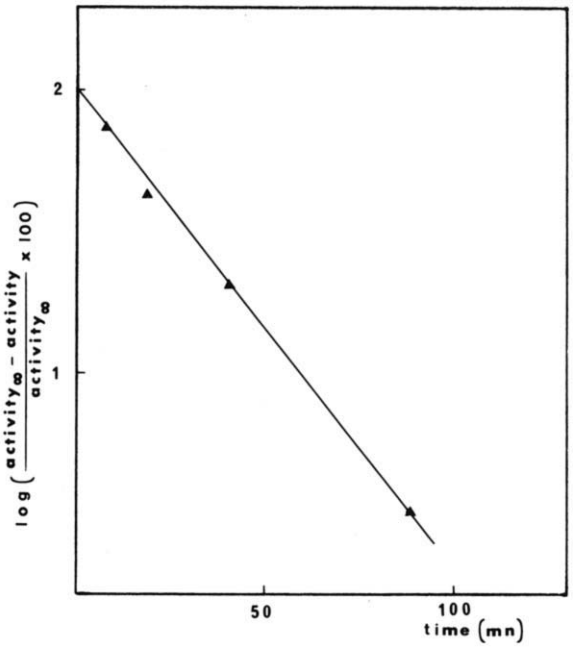

Fig.3. Reactivation of β-galactosidase alkylated with *N*-Br-Glu, on incubation with 0.176 M mercaptoethanol.

dase inactivated with *N*-Br-Gal would be reactivated with a rate constant of 0.044 mn^{-1}, as calculated from the data of Naider et al. [8].

3.3. *Inhibition of the action of* N-*Br-Glu by galactose*

Galactose inhibits the action of *N*-Br-Glu on β-galactosidase. The study of this competitive inhibition has been performed at low reagent concentration (5.05 mM), in the presence of 45.5 mM galactose. Under these conditions, the pseudo-first order constant decreases from 0.147 mn^{-1} to 0.0682 mn^{-1}. This gives a competitive inhibition constant of 40 mM, which is the inhibition constant obtained when galactose inhibits the hydrolysis of *o*-Np-Gal [9].

4. Discussion

β-Galactosidase inactivated by *N*-bromoacetyl-β-D-glucosylamine is reactivated by treatment with β-mercapthoethanol; therefore, this result allows the conclusion that, similarly to *N*-Br-Gal, *N*-Br-Glu attacks a methionin [8,13]. Furthermore, using enzyme in which methionin was partially replaced by norleucine, it was shown that the two reagents attack the same methionin (A. J. Gilboa, unpublished results and [14]).

Although the same methionin is modified, the inactivation parameters are different for the two reagents (table 1). The pseudo-first order constants at low reagent concentration, which reflect therefore the k_i/K_I ratio, are nearly the same for both compounds; however, the less specific reagent becomes more efficient for higher concentrations. This is the consequence of very large differences in the individual parameters (table 1); in spite of its very low affinity for the enzyme, indicated by the large K_I value, *N*-Br-Glu exhibits an inactivation rate constant larger by two orders of magnitude than that corresponding to the specific reagent, *N*-Br-Gal.

To account for these results, one explanation could be that *N*-Br-Gal and *N*-Br-Glu bind to the same subsite of the enzyme, and that the binding of *N*-Br-Glu favours (whereas the binding of *N*-Br-Gal does not favour) the attack of an adjacent methionin. The high inactivation rate constant for the glucosyl reagent, which has but low affinity for the enzyme, could be only caused by enhancement of the chemical reactivity of the enzyme methionin for the bound reagent. This explanation, however, does not account for the almost identical k_i/K_I ratios for the two reagents.

Any other explanation requires that two sites (or two subsites) be involved. In order to account for the observed findings, one could assume that a reagent could bind either site A or site B with K_A and K_B as dissociation constants, and that the bindings at these sites are mutually exclusive, as in scheme II:

$$ER_B \underset{}{\overset{K_B}{\rightleftharpoons}} E + R \overset{K_A}{\rightleftharpoons} ER_A \qquad \text{Scheme II}$$

Under these conditions, the apparent dissociation constant for the binding of the reagent to site A, site B or both is $K_A K_B/(K_A + K_B)$. The fractions of the reagent bound to site A and site B are given by the ratios $K_B/(K_A + K_B)$ and $K_A/(K_A + K_B)$ respectively. In this case, if it is also assumed that only the binding to site B is followed by an inactivation with a k_i constant, the observed inactivation constant, k_{app}, is given by the equation:

$$k_{app} = k_i \cdot \frac{K_A}{K_A + K_B} \cdot \frac{R}{K_A K_B/(K_A + K_B) + R}$$

At low reagent concentration, k_{app} value will be equal to $k_i \cdot R/K_B$, which is the same value obtained if the reagent binds only to site B.

As already pointed out, the bromogalactosyl and the bromoglucosyl reagents, although they have quite

Table 1
Kinetic parameters for the inactivation of β-galactosidase by *N*-Br-Gal or *N*-Br-Glu

Inhibitor	K_I (mM)	k_i (mn^{-1})	k_i/K_I (mn^{-1} mM^{-1})
N-Br-Gal [8]	1.13	0.063	0.055
N-Br-Glu	220	7	0.032

different parameters, exhibit the same k_i/K_I ratio (table 1). This can now be explained by assuming that binding of the reagent to site A (plausibly the galactose subsite), which is specific for galactosyl residue, does not lead to inactivation and that binding to site B (plausibly the glucose subsite), which is less specific and will bind *N*-Br-Glu and *N*-Br-Gal with the same affinity, does lead to inactivation.

However, it is not possible, on the strength of the available data, to discriminate between the two possibilities: binding at one site or binding at two mutually exclusive sites. The competition between galactose and *N*-Br-Glu is not sufficient evidence that the reagent binds to the active site; steric hindrance or conformational change on binding of galactose could prevent the binding of the reagent to its site when galactose is bound to the active site of the enzyme.

The very fast inactivation obtained with moderate concentrations of *N*-Br-Glu was both unexpected and surprising. 'Active site directed reagents', which operate by increasing their concentration at the active site, do not necessarily give an intrinsically rapid inactivation rate.

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