Conformational Adaptability of the Active Site of \(\beta\)-Galactosidase

INTERACTION OF THE ENZYME WITH SOME SUBSTRATE ANALOGOUS EFFECTORS*

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Although a great amount of data has been accumulated on \(\beta\)-galactosidase during these last years (1-16), little is known about the active site. In order to identify the essential residues, active site-directed reagents have been used (10-12). Until now, this approach has not been successful. The only amino acid blocked which has led to a loss of activity of the enzyme was shown to be a methionine, but Naider et al. demonstrated that it is not an essential group (12).

The action of different effectors, glycosides, and alcohols on the reactions catalyzed by \(\beta\)-galactosidase is analyzed in this paper. Effectors as large as tri- and tetrasaccharides have no effect on the enzyme activity, suggesting that the binding site has rather small size. Most of the \(\beta\)-galactosides produce a competitive inhibition. The other compounds assayed behave either as noncompetitive inhibitors, and they are deadened inhibitors, or as uncompetitive inhibitors which exhibit a better affinity for the chemical intermediate than for free enzyme; nearly all of them give transfer products.

The analysis of the data indicates that the active center of \(\beta\)-galactosidase is made up of two subsites: a galactose and a glucose subsite. This latter site is in a more favorable conformation in the galactosylenzyme than in free enzyme; possibly it might even be generated by the galactose binding. Conformational rearrangements of the active center deduced from the inhibition data have been directly observed by differential spectroscopy. The conformational adaptability of the enzyme and its consequence for the functional properties of \(\beta\)-galactosidase are discussed.

The active site of an enzyme is not limited to the few amino acid residues involved as catalytic groups. It extends over a larger part of the molecule, including many amino acids able to interact with substrate in the binding process. The use of various inhibitors can be fruitful to study such interactions. For instance, for mapping the active site of proteases, Berger et al. used oligopeptides of different length and of various amino acid compositions. They conclude that the papain active site extends over 25 \(\AA\) and is divided into seven subsites (17-19). Such an approach implicitly supposes a rigid structure for an enzyme. However, even if located at the active site level, the flexibility of proteins could lead to more dynamic situations, the structure of the site being different in free enzyme and in the complexes. This induced fit, as proposed for the first time by Koshland (20), could be very important for the enzymatic reaction. In such a situation the interaction between different compounds and the enzyme could not allow a suitable interpretation for the mapping of the active site of the enzyme, but it could reveal the conformational states of the enzyme in the different complexes.

The present paper, the effect of different alcohols and glycosides on \(\beta\)-galactosidase is reported. This work was initiated as an approach for mapping the rigid active site structure of the enzyme, but the results reported must be discussed in terms of conformational adaptability of the enzyme.

THEORY

It was previously shown that \(\beta\)-galactosidase-catalyzed reactions proceed through a chemical intermediate (ES') which occurs simultaneously with the liberation of the first product of the reaction (5, 21). An analogous substrate could bind to the free enzyme or to the ES' complex, but not to the Michaelis complex in which all the binding substrates are occupied (see "Discussion" below). Furthermore, the substrate itself, when it binds to the ES' complex, could be either a dead-end inhibitor or an acceptor initiating transfer product.

In this scheme, ES and ES' are, respectively, the Michaelis complex and the chemical intermediate, EI and EIS' the complexes originating from the binding of the effector on free...
enzyme and on the chemical intermediate, $K_i$ and $K''_i$, are the corresponding dissociation constants. $P_i$ could be either a transfer product or galactose itself. The specific rate constants are indicated on the arrows.

In the presence of the effector, the kinetic parameters for the appearance of the first product of the reaction are given by Equations 1 and 2:

$$k_{cat} = \frac{k_2 (k_{cat} + k_4)}{k_2 + k_{cat} + (k + k_{cat}) \frac{I}{K}}$$

(1)

$$K_{m, i} = K_i \left( \frac{1 + I}{K_i} \right) \frac{k_{cat} + k_i}{k_{cat} + k_i + k_i}$$

(2)

An interesting relationship is given by the $K_{m, i} / k_{cat, i}$ ratio, which gives a linear relationship as a function of $I$, allowing one to determine $K_i$:

$$\frac{K_{m, i}}{k_{cat, i}} = \frac{K_i}{k_{cat}} \left( \frac{1 + I}{K_i} \right)$$

(3)

The plot of $1/k_{cat, i}$ versus $I$ gives a hyperbole, and $k_{cat, i}$ varies from $k_{cat, o}$ (i.e. $k_{cat, o}/(k_2 + k_{cat} + k_i)$) in the absence of effector to $k_{cat, h}/(k_2 + k_{cat})$ in the presence of saturating concentration of effector. However, a linearization of Equation 1 can be obtained:

$$k_{cat, i} = \frac{k_{cat} - k_{cat, o}}{I} \frac{k_{cat} + k_2 + k_{cat} + k_i}{k_2 + k_i + k_{cat}}$$

(4)

When there is no transfer product formation, that is when $k_i$ is negligible or null, Equation 1 simplifies into Equation 5:

$$k_{cat, i} = \frac{k_{cat} \cdot k_{cat, o}}{k_{cat} + k_2 + k_{cat}}$$

(5)

The reverse of Equation 5 gives a linear relationship between $1/k_{cat, i}$ and $I$:

$$\frac{1}{k_{cat, i}} = \frac{1}{k_{cat, o}} + I \frac{1}{k_{cat} + k_2 + k_{cat}}$$

(6)

Thus, when the plot of $1/k_{cat, i}$ versus $I$ is linear, one can conclude that $k_i$ is negligible.

The different parameters $k_i$ and $K_i$ can be calculated from Equations 4 and 6, using the values of $k_2$ and $k_{cat}$ previously determined (5, 6, 21).

**Materials and Methods**

**Enzyme — $\beta$-Galactosidase** — Enzyme assays were performed with Escherichia coli strain E01 as previously described (2).

**Substrates** — $\alpha$-NP-Gal and $\beta$-NP-Gal were purchased from Fluka.

**Effectors** — Effectors of various structure were assayed: monosaccharides: D-(-)-lyxose (Fluka), D-(+)-arabitol (Sigma), o-(+)-arabitol (Sigma), dulcitol (Sigma), Hydroxyethyl-$\beta$-thiogalactoside was a generous gift from Dr. Gero; disaccharides: $\beta$-D-galactosidase: lactose (Sigma), $\alpha$-D-galactosidase: melibiose (Sigma), $\beta$-D-glucosidase: celllobiose (Sigma), $\alpha$-D-glucosides: trehalose (Prolabo), maltose (Prolabo), saccharose (Analar); tri saccharides: raffinose (Prolabo), melezitose (Fluka); tetrasaccharides: stachyose (Prolabo) alcohols: linear, mesoerythritol (Fluka), L(-)-arabitol (Sigma), d-(+)-arabitol (Sigma), dulcitol (Fluka); cyclic, meso inositol (Fluka).

**Compounds without effect on $\beta$-Galactosidase** — In the presence of trehalose, stachyose, raffinose, and D-(+)-melezitose, neither $V_m$ nor $K_m$ is modified. For stachyose and raffinose, which are oligosaccharides, and $\alpha$-galactosides the size and

| Compounds                  | $K_1$ | $K_2$ | $k_i$ |
|----------------------------|-------|-------|-------|
| Competitive effectors      | mM    | mM    | $s^{-1}$ |
| Galactose                  | 40    |       |       |
| Deoxyglycosylate           | 160   |       |       |
| Isopropyl-$\beta$-D-thiogalactoside | 0.085 |       |       |
| Phenyl-$\beta$-D-thiogalactoside | 0.19  |       |       |
| $\alpha$-N-Aminophenyl-$\beta$-D-thiogalactoside | 1 |       |       |
| $\alpha$-N-Nitrophenyl-$\beta$-D-thiogalactoside | 0.3 |       |       |
| Lactose                    | 1     |       |       |
| Mesoerythritol             | 9.6   |       |       |
| Dulcitol                   | 155   |       |       |
| Noncompetitive effectors   |       |       |       |
| Arabinose                  | 400   | 180   | 0     |
| Lactose                    | 105   | 70    | 0     |
| Cellobiose                 | 200   | 130   | 0     |
| Maltose                    | 290   | 135   | 0     |
| L(-)-Arabitol              | 105   | 150   | 0     |
| D-(+)-Arabitol             | 70    | 20    | 2200  |
| Uncompetitive effectors    |       |       |       |
| Fucose                     | 300   | 21    | 110   |
| Glucose                    | 630   | 34    | 330   |
| Maltoheose                 | 170   | 27    | 0     |
| Saccharose                 | 950   | 48    | 186   |
| Meso inositol              | 1090  | 50    | 420   |

1 The abbreviations used are: $\alpha$-NP-Gal, $\alpha$-nitrophenyl-$\beta$-D-galactoside; $\beta$-NP-Gal, $\beta$-nitrophenyl-$\beta$-D-galactoside; T62, [2-hydroxy-1,1-bis(2-hydroxymethyl)ethyl]amino)ethanesulfonic acid.
the structure of the molecule could be sufficient to explain
their failure to bind to the enzyme. The same explanation
could account for the behavior of d-(-)-melezitose, which is
an a-glucoside. Trehalose, which is an a-glucoside, but only a
disaccharide, could not bind to the enzyme however (Table I).

Competitive Inhibitors of β-Galactosidase - Most of the β-
galactosides behave as competitive inhibitors of β-galactosid-
ase. However, an exception has to be noted. The hydroxyethyl-
β-d-thiogalactoside is able to bind on the ES' intermediate
but with a weaker affinity than on free enzyme. Monosacchar-
ides such as galactose and deoxygalactose, and linear alcohols
such as mesoerythritol and dulcitol, inhibit the enzyme com-
petitively. Fig. 1 gives a typical plot obtained for lactose.

Noncompetitive Effectors of β-Galactosidase - The following
compounds are very poor but noncompetitive inhibitors: mal-
tose, d-(-)-lyxose, cellobiose, l-(-)-arabinose, and l-(-)-ara-
bitol. K_i and K'_i are similar and equal to 0.1 M or more
(Table I). In Fig. 2, as an example, the effect of maltose on o-
Np-Gal hydrolysis is presented. Both plots K_m/K_cat and 1/k_cat,
versus I give straight lines, which allow the

FIG. 1. The competitive inhibition by lactose of the o-Np-Gal
hydrolysis by β-galactosidase. Upper, 1/k_cat, versus lactose concen-
tration; lower, K_m/K_cat, versus lactose concentration. Experimental
conditions are described under "Materials and Methods."

FIG. 2. The noncompetitive inhibition by maltose of the o-Np-
Gal hydrolysis by β-galactosidase. Left, 1/k_cat, versus maltose concen-
tration; right, K_m/K_cat, versus maltose concentration. Experimental
conditions are described under "Materials and Methods."

FIG. 3. The "uncompetitive" inhibition by glucose of hydrolysis
of o-Np-Gal by β-galactosidase. Upper, 1/k_cat, versus glucose concen-
tration; lower, K_m/K_cat, versus glucose concentration. Experimental
conditions are described under "Materials and Methods."
Interaction of Effectors with \( \beta \)-Galactosidase

determination of \( K_r \) and \( K'_{\text{g}} \), respectively. All the other compounds exhibit the same behavior; they are dead-end inhibitors without formation of transfer products.

D-\((\pm)\)-Arabitol also has similar affinity for both free enzyme and chemical intermediates; however, when it is present, the rate of \( ES' \) decomposition increases. This compound activates the reaction (Fig. 3) as a nucleophile reagent does (5, 21).

**Uncompetitive Effectors of \( \beta \)-Galactosidase**—The other compounds (melibiose, saccharose, fucose, glucose, mesoinositol) exhibit significantly better affinity for the chemical intermediate than for free enzyme. Among them, only melibiose is unable to give transfer products. For the other effectors, the transfer products are observed, the effector or acceptor exhibits better affinity for ES' than for free enzyme.

The results corresponding to the effect of glucose on the hydrolysis of \( \alpha \)-Np-Gal by the enzyme are shown in Fig. 4.

It seems important to point out that in all the cases where transfer products are observed, the acceptor or acceptor exhibits better affinity for ES' than for free enzyme.

**DISCUSSION**

**Two Binding Subsites of Enzyme**—The major part of the glycosides can bind to the free enzyme, as is shown by the increase of \( K_m / [K_{\text{cat},1}] \). Some of them bind to the enzyme-substrate complexes, since they give a decrease of the \( k_{\text{cat},1} \) value. Such binding might be nonspecific. However, the liberation of the first product of the reaction restores an available subsite in the ES' complex, and the inhibitor effect could be ascribed to the specific binding of inhibitors to this subsite. This hypothesis is confirmed by a comparison of glucose effect on \( \alpha \)-Np-Gal and \( \beta \)-Np-Gal hydrolysis. If the inhibitory effect of glucose is related to a decrease of the ES' hydrolysis step, this effect must be more important on \( \alpha \)-Np-Gal hydrolysis, where \( k_2 \) and \( k'_3 \) are of the same order of magnitude, than on \( \beta \)-Np-Gal hydrolysis, where the \( k_2 \) step is the limiting one in the absence of effector (5). Such is the case (Figs. 4 and 5). The difference of behavior of glucose on the \( k_2 \) and \( k'_3 \) steps reveals the appearance, in the ES' complex, of a new subsite which in the ES complex is occupied by the aglycon part of the substrate. Since lactose is the physiological substrate of \( \beta \)-galactosidase, the subsite could be designated as the "glucose subsite," which exists in addition to the "galactose subsite."

**Binding of Effectors to Free Enzyme**—The data seem to indicate that there are very definite requirements in the binding of the effectors at the active center of \( \beta \)-galactosidase. The binding site is probably rather small since effectors as large as tri- and tetrasaccharides are not able to interact with enzyme. Variations as large as \( 10^4 \) are observed in the \( K_r \) values. \( \beta \)-Galactosides exhibit the best affinity for free enzyme, better than galactose itself, indicating that the aglycon part in the glucose subsite participates significantly in binding. Among the monosaccharides, galactose is the best inhibitor. In D-\((\pm)\)-fucose and L-\((\pm)\)-arabinose, the replacement of the CH$_2$OH group in position 5 by smaller groups (CH$_2$ and H, respectively) increases the \( K_r \) value, underlining the importance of CH$_2$OH group in this position. For the binding of disaccharides, the enzyme does not seem to distinguish between \( \alpha \)- and \( \beta \)-glucosides on the one hand, and \( \beta \)-galactosides on the other hand. The \( K_r \) values are of the same order of magnitude for maltose, cellobiose, and melibiose. Among the linear alcohols, the behavior of mesoerythritol is noteworthy. It exhibits a better affinity than other alcohols in C$_3$ and C$_4$, or than glycerol as studied by Van der Groen et al. (9).

**Binding of Effectors to Galactosylenzyme**—Among the various effectors tested, the best inhibitors of free enzyme, \( \beta \)-galactosides, are unable to bind significantly to the ES' intermediate, compared to their binding of free enzyme, except for hydroxyethyl-\( \beta \)-D-thiogalactoside; but, in this case, the affinity is about 10 times weaker for ES' than for \( E \). As \( \beta \)-\( \alpha \)-galactosides, galactose binds only to free enzyme with significant affinity. Other monosaccharides such as glucose and fucose, as well as disaccharides such as saccharose and melibiose, have a higher affinity for the galactosylenzyme than for free enzyme. The \( K'_r \) values for the different compounds differ maximally by two orders of magnitude. No definite specificity can be deduced from the various values.

In some cases, binding of the inhibitors leads to the formation of transfer product. The compounds which exhibit a higher affinity for the ES' complex than for free enzyme induce such reaction (melibiose is however an exception). Formation of transfer products has already been demonstrated (1, 22). In these last cases as in our work, this formation occurs through intermolecular reaction mechanisms. But it is not the only way to obtain transfer products; with lactose for instance, Huber et al. (13) described a direct intramolecular transfer for the formation of allolactose.

**Induced Conformation**—The results clearly indicate the existence of two binding subsites. The \( K'_r \) values can be easily associated with a binding to the glucose subsite. However, the \( K_r \) values are more ambiguous, since a binding on one or the other subsite could provide competitive behavior. Although some effectors are able to bind both to free enzyme and to the intermediate, it is not possible to know if they bind to the same subsite in the two species of the enzyme. The \( K_r \) values of galactose, fucose, arabinose, and glucose exactly reflect the \( K'_r \) values determined for the corresponding substrates, \( \alpha \)-Np-Gal, \( \alpha \)-nitrophenylarabinoside, \( \alpha \)-nitrophenylfucoside, and \( \alpha \)-nitrophenylglucoside, which are, respectively, 0.3 mm, 3 mm, 4.3 mm (2), and 6 mm. This result suggests that the same subsite is implicated in the binding of the glycosidic part of the substrates and of the monosaccharides.
More favorable values of $K''_e$, compared to the $K_e$ of the same compounds suggest the occurrence of conformational changes from free enzyme to the complex. The glucose subsite could be generated even by such conformational rearrangements induced by galactose binding. Such an argument could be provided by considering the energy of interaction of lactose to the enzyme. Binding of lactose involves a free energy variation of $-4$ kcal/mol. Interaction of galactose with free enzyme ($\Delta G = -1.9$ kcal/mol) and interaction of glucose on galactosyl-enzyme ($\Delta G = -2$ kcal/mol) could account for this value. The additive binding energies of galactose plus glucose ($\Delta G = -0.3$ kcal/mol) to the free enzyme do not account for free energy of lactose binding. An alternative explanation could also account for free energy of lactose binding; it involves synergistic interactions between ligands without any conformational change of the protein.

Conformational changes strongly suggested by the action of effectors on enzyme kinetics were directly investigated. As shown in Fig. 6a, it was possible to detect a differential spectrum between free enzyme and the complex obtained by saturating the enzyme with isopropyl-$\beta$-d-thiogalactoside. The spectral change is characterized by a very small amplitude, which prevents quantitative analysis. A differential spectrum was also observed between free enzyme and the intermediate obtained in the presence of $p$-galact (Fig. 6b). These results are strongly in favor of conformational changes of the enzyme upon ligand binding, and allow to discard any explanation requiring a rigid enzyme.

Probably each step of the reaction catalyzed by $\beta$-galactosidase involves very discrete conformational changes localized at the active center. These changes are very important, since their occurrence limits the rate of the overall reaction (5, 7) and further effector binding. The interaction of the galactosylmoiety at its specific subsite induces the formation of a second subsite capable of binding sugars and alcohols of appropriate configuration.

The results presented here have shown that the structure of the active site of the enzyme has a rather dynamic conformation, which changes with the various steps of the reaction pathway. In the galactosyl-enzyme, the glucose subsite either appears, or adopts a more favorable conformation for binding the corresponding effectors. Does this conformational adaptability provide a suitable situation for transgalactosylation?

A question which can be asked is the following: are those conformational effects interpretable in terms of induced fit (20) or in terms of a priori conformational states of the protein (each stabilized by ligand binding)? On the basis of these experiments it is not possible to conclude. It is even quite difficult to imagine some conclusive experiments allowing to choose one theory over the other. Nevertheless, the concept of “dynamic specificity” developed elsewhere (24) could also apply to such a dynamic occurrence of a second binding site following the first steps of the reaction which it catalyzes.

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Fig. 6. Difference spectra induced by ligand binding in $\beta$-galactosidase (a) with 1.58 mm isopropyl-$\beta$-d-thiogalactoside or (b) with 9 mm $p$-galact. Experimental conditions, spectra were determined with double compartment cells, each compartment with a 0.435-cm path length. Enzyme concentration was 1.85 mg/ml, in 10 mm Tris, pH 7, 0.145 m NaCl, and 1 mm MgSO$_4$ at 25°.
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