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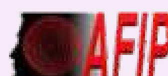

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Characterization of the interdependency between residues that bind the substrate in a β-glycosidase

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Abstract

The manner by which effects of simultaneous mutations combine to change enzymatic activity is not easily predictable because these effects are not always additive in a linear manner. Hence, the characterization of the effects of simultaneous mutations of amino acid residues that bind the substrate can make a significant contribution to the understanding of the substrate specificity of enzymes. In the β-glycosidase from *Spodoptera frugiperda* (Sfβgly), both residues Q39 and E451 interact with the substrate and this is essential for defining substrate specificity. Double mutants of Sfβgly (A451E39, S451E39 and S451N39) were prepared by site-directed mutagenesis, expressed in bacteria and purified using affinity chromatography. These enzymes were characterized using p-nitrophenyl β-galactoside and p-nitrophenyl β-fucoside as substrates. The k_{cat}/K_m ratio for single and double mutants of Sfβgly containing site-directed mutations at positions Q39 and E451 was used to demonstrate that the effect on the free energy of ES‡ (enzyme-transition state complex) of the double mutations ($\Delta\Delta G^‡_{xy}$) is not the sum of the effects resulting from the single mutations ($\Delta\Delta G^‡_x$ and $\Delta\Delta G^‡_y$). This difference in $\Delta\Delta G^‡$ indicates that the effects of the single mutations partially overlap. Hence, this common effect counts only once in $\Delta\Delta G^‡_{xy}$. Crystallographic data on β-glycosidases reveal the presence of a bidentate hydrogen bond involving residues Q39 and E451 and the same hydroxyl group of the substrate. Therefore, both thermodynamic and crystallographic data suggest that residues Q39 and E451 exert a mutual influence on their respective interactions with the substrate.

Key words: β-glycosidase; Substrate specificity; Site-directed mutagenesis; *Spodoptera frugiperda*

Introduction

The rational design of enzymes usually requires information from two or more simultaneous mutations. However, the manner in which effects of simultaneous mutations combine to change enzymatic activity is not easily predictable because these effects on enzyme activity are not always additive in a linear manner (1-4).

The interaction between the residues that bind the substrate can be detected by comparing the effect of double mutations ($\Delta\Delta G^‡_{xy}$) on the free energy of the ES‡ (enzyme-transition state complex) with that of the corresponding single mutations ($\Delta\Delta G^‡_x$ and $\Delta\Delta G^‡_y$). The difference between $\Delta\Delta G^‡_{xy}$ and the sum of $\Delta\Delta G^‡_x$ and $\Delta\Delta G^‡_y$, known as "coupling energy" ($\Delta G^‡_I$), corresponds to the extent to which the interaction between two residues (x and y) affects the stabilization of ES‡ and consequently the substrate specificity of enzymes (1).

β-glycosidases (EC 3.2.1.21) from family 1 of the glycoside hydrolases (GH1) catalyze the hydrolysis of β-glycosidic bonds, releasing monosaccharides from the non-reducing end of glycosides (5). Their active site is divided into several subsites, which are identified by positive and negative numbers relative to the scissile bond of the substrate (6). Subsite -1 binds the monosaccharide from the non-reducing end of the substrate, also called glycone. Spatial structures of complexes between β-glycosidases and substrates or inhibitors show that a network of hydrogen bonds is formed between the hydroxyls of the glycone and amino acid residues from subsite -1 (7-11). The energetic contribution of these interactions to the substrate binding has been studied for β-glucosidase from *Agrobacterium* sp (10), lactase-phlorizin hydrolase from lamb (11) and a β-glycosidase from the fall armyworm *Spodoptera fru-*

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giperda (Sfβgly) (12).

The energy of interactions of glycone hydroxyls 4 and 6 with residues Q39 (glutamine 39) and E451 (glutamate 451) in the ES‡ was evaluated in Sfβgly (12) and it was shown that these residues are essential elements in determining subsite -1 specificity for fucosides, glucosides, and galactosides. Single mutations of these residues showed that replacement of Q39 with E and N (glutamate and asparagines) causes a drastic decrease (about 70%) in the energy of the interactions with the glycone hydroxyls 4 and 6. In addition, substitution of E451 with Q did not affect the interactions with glycone hydroxyl 4, whereas substitutions with D and S (aspartate and serine) produced large reductions in the energy of these interactions (13). However, these interactions were analyzed on a single site mutant so that the effect of mutation of one residue on the interactions formed by a second was not studied. Indeed, the manner in which multiple mutations in the active site are combined and affect the substrate specificity has not been characterized for any β-glycosidase.

In the present study, in order to identify the influence of interactions formed with the substrate glycone by residues 39 and 451, three double mutants of Sfβgly (A451E39, S451E39 and S451N39) were produced and characterized using enzyme kinetic parameters (k_{cat}/K_m) and $\Delta\Delta G^‡$ was compared to single mutant (A451, S451, E39, and N39) and wild-type Sfβgly.

Material and Methods

All reagents, unless otherwise specified, were purchased from Sigma (USA) and Merck (Germany).

Site-directed mutagenesis

A pT7-7 vector coding the wild-type Sfβgly was used as a template in site-directed mutagenesis experiments employing the kit "QuikChange site directed mutagenesis" (Stratagene, USA). The primer used for mutation at position E451 was 5' GGAGTCTAATGGACAACTTT***NNN***TGG ATGGAGGGTTATATTGAGCG 3' with **TCA** and **GCC** as mutagenic codons (***NNN***) for S451 and A451, respectively. The primer used for mutation at position Q39 was 5' CGCT ACAGCCTCCTAC***NNN***ATCGAAGGTGCTTGG 3' with **GAG** and **ACC** as mutagenic codons for E39 and N39, respectively. Thus, to produce a double mutant two different pairs of primers were used in two sequential experiments. The DNA segment coding the double mutant Sfβgly was then amplified by the polymerase chain reaction and cloned in the pET46 vector (Novagen, USA) using the "Ek/LIC cloning" kit (Stratagene). The incorporation of the mutations was confirmed by DNA sequencing.

Production and purification of the Sfβgly recombinant

BL21 DE3 cells transformed with pET46 vectors coding the double mutant Sfβgly were grown in Luria-Bertani broth containing 50 µg/mL carbenicillin at 37°C and 150 rpm until A_{600} = 0.6-1.0 was obtained. The expression of the Sfβgly recombinant was then induced using 1 mM isopropyl thio-β-D-galactoside for 3 h at 25°C and 150 rpm. The cells were harvested at 7000 *g* for 20 min at 4°C and the identity of the recombinant protein was checked by SDS-PAGE (14). The induced bacteria were resuspended in 30 mL 20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl, 20 mM imidazole, 0.1% Triton X-100 (v/v) and 0.2% chicken hen egg white lysozyme (w/v). After incubation for 30 min with slow shaking (10 rpm), cells were disrupted by sonication (5 pulses of 30 s at output 4 in a Branson sonifier adapted with a microtip) and the suspension was centrifuged at 7000 *g* for 20 min at 4°C. The supernatant was collected and the Sfβgly recombinant was purified by affinity chromatography on a HisTrap FF column (GE Healthcare, UK). About 15 mL of the supernatant was passed through 0.22-µm filters (Millex, Millipore, USA) and injected into the column. It was eluted with 20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and 20 mM imidazole (flow rate: 1 mL/min). The non-retained proteins were washed out with 15 column volumes of the same buffer. The retained proteins were then eluted with 15 column volumes of 20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and 0.5 M imidazole. Sfβgly activity was detected using 7.2 mM NPβfuc (p-nitrophenyl β-D-fucopyranoside; Sigma) prepared in 100 mM citrate phosphate, pH 6.0. The purification of the Sfβgly mutant was confirmed by SDS-PAGE (14).

Protein was determined on the basis of absorbance at 280 nm. The ε_{280} of each Sfβgly double mutant was calculated according to Gill and von Hippel (15).

Enzyme kinetic and thermodynamic parameters

Steady-state kinetic parameters, k_{cat} and K_m, were determined at 30°C using at least 10 different substrate concentrations [0.2 to 20 mM for NPβfuc and 0.02 to 8 mM for NPβgal (p-nitrophenyl β-D-galactopyranoside)]. Two independent experiments were performed. NPβgal and NPβfuc were prepared in 100 mM citrate-phosphate, pH 6.0. Substrate hydrolysis was detected by the release of *p*-nitrophenolate by measuring absorbance at 420 nm. Initial rate data were fitted to the Michaelis-Menten equation using the Enzfitter software (R.J. Leatherbarrow; Elsevier-Biosoft, UK).

Changes in the free energy ($\Delta\Delta G^‡$) of the ES‡ caused by single or double mutations were calculated using Equation 1 (16):

$$\Delta\Delta G^‡ = -RT \ln (k_{cat}/K_{m\ mutant}) / (k_{cat}/K_{m\ wild\text{-}type}) \quad [1]$$

where R is the gas constant, T is the absolute temperature (303 K), and k_{cat}/K_m is the *apparent* second-order rate constant of hydrolysis of a substrate. $\Delta\Delta G^‡$ represents the difference in free energy between the ES‡ complex and the

free enzyme and substrate (E + S).

The $\Delta\Delta G^‡$ caused by a double mutation ($\Delta\Delta G^‡_{xy}$) can be related to the $\Delta\Delta G^‡$ caused by the corresponding single mutations ($\Delta\Delta G^‡_x$ and $\Delta\Delta G^‡_y$) using Equation 2 (1):

$$\Delta\Delta G^‡_{xy} = \Delta\Delta G^‡_x + \Delta\Delta G^‡_y + \Delta G^‡_I \quad [2]$$

$\Delta G^‡_I$, also known as coupling energy, corresponds to the extent to which the interaction between residues X and Y affects the stabilization of $ES^‡$ and consequently the rate of substrate hydrolysis.

Thermal inactivation

Enzyme samples (wild-type Sfβgly, A451E39 and S451E39) were incubated at 50°C for different times (0 to 4 min). Next, the remaining activity was determined using 8 mM NPβfuc prepared in 100 mM citrate-phosphate, pH 6.0. Inactivation rates were compared by plotting the log of the relative remaining activity versus incubation time.

Results and Discussion

Mutants E39, N39, A451 and S451 (replacement of Sfβgly residues Q39 or E451 by E, N, A, and S, respectively) were previously produced and purified. These single mutations caused a large reduction in the k_{cat}/K_m ratio for the hydrolysis of NPβglycosides due to a reduction of about 70% in the energy of the interactions formed by Q39 and E451 with the substrate in the $ES^‡$ complex (13).

Double mutants of Sfβgly (A451E39, S451E39 and S451N39) were prepared by site-directed mutagenesis, expressed in BL21 DE3 cells in order to determine interactions between sites of mutation.

Steady-state kinetic parameters (k_{cat} and K_m) were determined for the hydrolysis of NPβgal and NPβfuc using a purified Sfβgly double mutant (Table 1). The double mutations modify k_{cat} 10^2- to 10^3-fold, whereas K_m presents smaller changes. This pattern was also observed for the single mutations of residues Q39 and E451 (12,13). Thermal inactivation experiments showed that the structures of the A451E39 and S451E39 double mutants are similar to the wild-type Sfβgly ($t_{1/2}$ ~1.2 min at 50°C), suggesting that the mutational effects on k_{cat} did not result from large structural modifications of the protein.

On the basis of k_{cat}/K_m data of the wild-type and double mutant Sfβgly, changes in the transition state stabilization energy caused by a double mutation ($\Delta\Delta G^‡_{xy}$) and by single mutations that compose a double mutant ($\Delta\Delta G^‡_x$ and $\Delta\Delta G^‡_y$) were calculated as described in Material and Methods (1,16) (Table 2). These $\Delta\Delta G^‡_{xy}$ were compared to the corresponding $\Delta\Delta G^‡_x$ and $\Delta\Delta G^‡_y$ resulting in the coupling energy ($\Delta G^‡_I$) between residues at sites 451 and 39 of Sfβgly (Table 2).

$\Delta G^‡_I$ is different from zero for all double mutants, except for S451N39 when NPβfuc is used as substrate. Thus, the replacement of E451 and Q39 with S and N, respectively, has a simple additive effect on Sfβgly activity when NPβfuc is the substrate, whereas in all remaining double mutants a complex additive effect is observed. Complex additivity of mutational effects resulting in $\Delta G^‡_I$ different from zero has been observed for other enzymes (1-4), but it has not been described for GH1 β-glycosidases.

Table 1. Steady-state kinetic parameters for the hydrolysis of NPβglycosides by the wild-type and double mutant Sfβgly.

	NPβgal			NPβfuc		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Wild type	0.35	2.0	0.17	3.3	0.49	6.7
A451E39	0.0036	0.22	0.016	0.0236	2.7	0.0087
S451E39	0.0065	0.38	0.017	0.0342	2.3	0.014
S451N39	0.0006	0.11	0.0050	0.00040	2.2	0.00018
A451	0.000285	0.50	0.00057	0.00061	1.53	0.0039
E39	0.0377	2.8	0.0134	1.49	2.3	0.64
S451	0.00093	1.7	0.00053	0.0186	0.68	0.027
N39	0.00038	1.5	0.00025	0.0220	0.26	0.084

Experiments were carried out using at least 10 different substrate concentrations. Two independent experiments were performed. Parameters were calculated using the Enzfitter software. Standard errors were less than 12% of the mean values. Data for wild-type and single mutant A451 were taken from Ref. 12. Data for single mutants E39, S451 and N39 were taken from Ref. 13. NPβgal = p-nitrophenyl β-galactoside; NPβfuc = p-nitrophenyl β-fucoside; A = alanine; E = glutamate; N = asparagine; S = serine.

Table 2. Changes in the ES‡ complex energy resulting from double and single mutations in Sfβgly.

	NPβgal				NPβfuc			
	$\Delta\Delta G^{‡}_{xy}$(a)	$\Delta\Delta G^{‡}_{x}$(b)	$\Delta\Delta G^{‡}_{y}$(c)	$\Delta G^{‡}_{I}$(d)	$\Delta\Delta G^{‡}_{xy}$(a)	$\Delta\Delta G^{‡}_{x}$(b)	$\Delta\Delta G^{‡}_{y}$(c)	$\Delta G^{‡}_{I}$(d)
A451E39	6	14	6.4	-14	16	24	6	-14
S451E39	6	14	6.4	-14	15	14	6	-5
S451N39	9	14	16	-21	26	14	11	1

Data are reported as kJ/mol. [a]$\Delta\Delta G^{‡}_{xy}$ was calculated by comparing the double mutant and wild-type Sfβgly. [b]$\Delta\Delta G^{‡}_{x}$ was calculated by comparing a single mutant at position 451 and the wild-type Sfβgly. Data from Ref. 13. [c]$\Delta\Delta G^{‡}_{y}$ was calculated by comparing a single mutant at position 39 and the wild-type Sfβgly. Data from Ref. 13. [d]$\Delta G^{‡}_{I}$ was calculated using the equation $\Delta\Delta G^{‡}_{xy} = \Delta\Delta G^{‡}_{x} + \Delta\Delta G^{‡}_{y} + \Delta G^{‡}_{I}$ (Ref. 1) and corresponds to the coupling energy between residues at positions 451 and 39. NPβgal = p-nitrophenyl β-galactoside; NPβfuc = p-nitrophenyl β-fucoside; A = alanine; E = glutamate; N = asparagine; S = serine.

Negative $\Delta G^{‡}_{I}$ indicates that double mutations are less damaging to the enzyme activity (measured by k_{cat}/K_m) than the sum of the single mutations that compose them ($\Delta\Delta G^{‡}_{xy} < \Delta\Delta G^{‡}_{x} + \Delta\Delta G^{‡}_{y}$). This difference also indicates that the effects of the single mutations partially overlap. Hence, this common effect counts just once in the $\Delta\Delta G^{‡}_{xy}$. Moreover, this common effect could result from the presence of a mutual influence between residues at positions 451 and 39, which affects their bonds with the substrate and favors substrate hydrolysis.

In the specific case of NPβgal hydrolysis, $\Delta G^{‡}_{I}$ presents similar values for all double mutants. In addition, the effects of the double mutations ($\Delta\Delta G^{‡}_{xy}$) are less than those of the most damaging single mutations ($\Delta\Delta G^{‡}_{x}$), which involve residue 451 (Table 2). Thus, mutations at residue 39 are partially suppressing the damaging effect of the mutations at residue 451, which is compatible with a mutual influence between residues at positions 451 and 39.

On the other hand, in the case of NPβfuc hydrolysis, $\Delta G^{‡}_{I}$ changes depending on the double mutations (Table 2). Variation of $\Delta G^{‡}_{I}$ depending on the type of residue introduced in the mutant enzyme was also observed for tyrosyl-tRNA synthetase and subtilisin BPN' mutants (1). In the A451E39 mutant the observation of a negative $\Delta G^{‡}_{I}$ and a $\Delta\Delta G^{‡}_{xy}$ lower than $\Delta\Delta G^{‡}_{x}$ suggests that the introduction of E39 is reducing the damage caused by mutation A451, an effect similar to that described for the hydrolysis of NPβgal by this same double mutant. The suppressing effect completely disappears in the mutants S451E39 and S451N39 when NPβfuc is the substrate. In spite of that, the $\Delta\Delta G^{‡}_{xy}$ is still lower than $\Delta\Delta G^{‡}_{x} + \Delta\Delta G^{‡}_{y}$ for mutants S451E39 and S451N39, confirming the presence of a mutual influence between residues at positions 451 and 39.

Therefore, the present data indicate a mutual effect of residues occupying positions 39 and 451 on their interactions with the substrate. According to previous observations, negative $\Delta G^{‡}_{I}$ occurs when the mutated residues form direct or indirect interactions (1). Crystallographic data for β-glycosidases show that residues corresponding to Q39 and E451 do not interact directly because they are more than 3 Å apart. Nevertheless these residues form bidentate hydrogen bonds with the hydroxyl 4 of the glycone substrate (17-20). As the hydroxyl 4 is simultaneously bound to Q39 and E451, these interactions are not isolated. Thus, interacting with the same group within the substrate rather than with different groups may result in different interaction energies. Hence, the sharing of the same group within the substrate may be the source of the mutual influence between residues Q39 and E451 for Sfβgly.

Comparison of sequence data shows that residues forming the subsite -1 are highly conserved among the GH1 β-glycosidases (17) and several of them share a common group (glycone hydroxyl) in their interactions with the substrate (9,17-20), suggesting that a mutual influence between residues involved in substrate binding may be a common characteristic within this group of enzymes. Therefore, this property should be considered in the characterization and/or modification of the substrate specificity of GH1 β-glycosidases.

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