Distinct mechanisms of antibody-mediated enzymatic reactivation in β-galactosidase molecular sensors

J.X. Feliu, E. Ramírez, A. Villaverde*

Institut de Biologia Fonamental and Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

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Abstract The antibody-mediated reactivation of engineered *Escherichia coli* β-galactosidases [Benito et al. (1996) J. Biol. Chem. 271, 21251–21256] has been thoughtfully investigated in three recombinant molecular sensors. Proteins M278VP1, JX772A and JX795A display the highly antigenic G-H loop peptide segment of foot-and-mouth disease virus VP1 protein, accommodated in different solvent-exposed loops of the assembled tetramer. These chimaeric enzymes exhibit a significant increase in enzymatic activity upon binding of either monoclonal antibodies or sera directed against the inserted viral peptide. In JX772A but not in M278VP1, the Fab 3E5 antibody fragment promotes reactivation to the same extent as the complete antibody. On the other hand, M278VP1 K_m is reduced by more than 50% in the presence of activating serum, this parameter remains invariable in JX772A and it is only slightly modified in JX795A. In these last two proteins, significant k_{cat} variations can account for the increased enzymatic activity. Alternative reactivation mechanisms in the different β-galactosidase probes are discussed in the context of the bacterial enzyme structure and its tolerance to antibody-induced conformational modifications.

Key words: Molecular sensor; Recombinant protein; β-Galactosidase; Antibody; Enzymatic activity

1. Introduction

Protein engineering is an extremely dynamic field that continuously generates both theoretical background and methodological instruments for the design of multifunctional proteins. These procedures have been successfully applied to obtain modified versions of an increasing number of well-characterised enzymes [1–4]. Despite the widespread use of *Escherichia coli* β-galactosidase as a molecular marker ([5], and references therein), its 3D structure was not solved until a few years ago [6], this fact having restricted its rational engineering. However, insertional approaches have led to the identification of tolerant sites for the accommodation of foreign, functional peptides, and their presentation on solvent-exposed surfaces of the active enzyme [7–14]. Furthermore, the positioning of an antigenic viral peptide in the activating loop of β-galactosidase (amino acids 272–287) resulted in a promising molecular sensor (protein M278VP1) for the quantitative detection of specific antibodies [15]. Binding of either sera or monoclonal antibodies elicited against the inserted peptide results in a partial recovery of the enzymatic activity that had been lost upon peptide insertion [8,16]. This reactivation is easily detectable by a rapid, standard and quantitative colorimetric assay [15]. For this reason, β-galactosidase, as previously shown for alkaline phosphatase [17], could be the basis for a new generation of enzymatic molecular sensors, that would, however, require further engineering for a more confident and sensitive use in homogeneous assays.

Since the molecular basis of antibody-mediated enzymatic reactivation had not been explored, we have analysed here both the enzymatic constants of reactivated β-galactosidase sensors and the putative requirement of bivalent antibody binding for reactivation. The data obtained prompted us to propose three classes of β-galactosidase sensors, in which reactivation occurs by distinguishable molecular mechanisms. The antibody-induced enzyme reactivation is discussed in the context of β-galactosidase structure and function.

2. Materials and methods

2.1. Bacterial strains, plasmids and proteins

E. coli BL26 strain, a *Lac*$^-$ derivative of BL21 *hsdS gal*$^-$ Lon$^-$ OmpT$^-$ [18], was used for protein production. Plasmids pJLACZ, pM278VP1, pJX772A and pJX795A are pJLA602 [19] derivatives that encode engineered, enzymatically active *E. coli* β-galactosidases under the control of the CI857-repressed, lambda p_L and p_R strong promoters [8]. An antigenic peptide from VP1 capsid protein of foot-and-mouth disease virus (FMDV), spanning residues 134–156, was inserted into solvent-exposed loops of the assembled enzyme (Fig. 1A), thus being highly immunoreactive [13,14]. More details of these proteins and plasmid construction procedures are given elsewhere [8,9,14].

2.2. Production and purification of recombinant proteins

Recombinant cell cultures were grown in 200 ml Luria-Bertani (LB) medium plus 100 μg/ml ampicillin, in 1-l shaker flasks at 28°C and 250 rpm. When the OD_{550} reached about 0.3 units, the temperature was shifted to 42°C to inactivate the CI857 repressor, and cultures were incubated for a further 4 h at 250 rpm. Cells were harvested by centrifugation, resuspended in disruption buffer and sonicated as described [20]. β-Galactosidase mutant proteins were purified by an optimised affinity chromatography procedure [21]. Briefly, crude cell extracts were applied 5 times to a 10-ml *p*-aminophenyl-β-D-galactoside-Sepharose column equilibrated with 20 mM Tris-HCl, 10 mM $MgCl_2$, 1.6 M NaCl, pH 7.5. After washing with the same buffer until the OD_{280} dropped to near zero, recombinant proteins were eluted with 100 mM $Na_2B_4O_7$, 10 mM β-mercaptoethanol, pH 10, and dialysed against Z buffer [22] without β-mercaptoethanol.

2.3. Obtaining 3E5 Fab fragments

Lyophilised ascitic fluid containing mAb 3E5 [23] was generously provided by E. Brochi. After reconstitution, 3E5 was purified in a protein A-Sepharose column and dialysed against 100 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 8 for 24 h at 4°C. Papain (Sigma Chemical), at 8 mg/ml, was activated with 25 mM sodium acetate at pH 4.5 and 0.14 M β-mercaptoethanol, for 25 min at room temperature. The enzyme was then purified with a Sephadex-G25 gel filtration column (Pharmacia Biotech) equilibrated with 100 mM acetate, pH 5.5. The fraction with the highest OD_{280} was used for antibody cleavage. The limited digestion was carried out by

*Corresponding author. Fax: (34) (93) 5812011.
E-mail: a.villaverde@cc.uab.es

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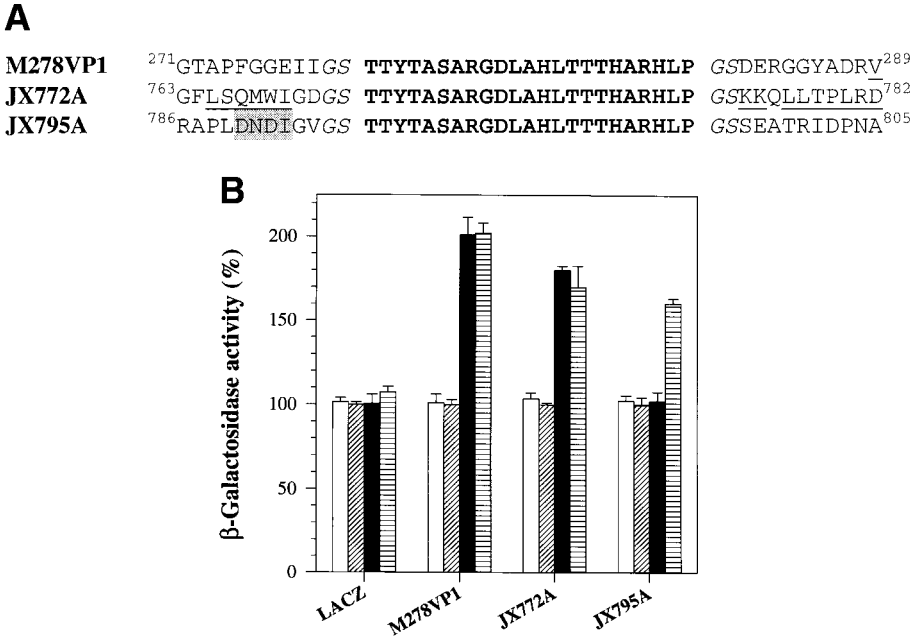

Fig. 1. A: Amino acid sequence of GH23 peptide is depicted in bold, spanning residues 134–156 of FMDV VP1 protein (clone C-S8c1). The different accommodation sites for this segment in β-galactosidase are also shown. The numbering is according to Kalnins and coauthors [29]. Light-shadowed residues represent α-helix regions and underlined segments β-strands, according to the structure of the native enzyme [6]. Positions in italics are encoded by the *Bam*HI restriction sites introduced during plasmid construction. B: Modulation of the enzymatic activity in the recombinant proteins using 3E5 anti-FMDV VP1 monoclonal antibody (black bars), immune (horizontally hatched bars) and non-immune (white bars) guinea pig sera, and in the absence of antibodies (diagonally hatched bars).

incubating the mAb and the enzyme (at 1:10 w/w ratio) at 37°C for 48 h. Proteolysis was stopped with iodoacetic acid at a final concentration of 2 mM. The reaction products were quantified by PAGE and further Coomassie blue staining or alternatively by Western blot.

2.4. Modulation assay

Antibody-mediated enzymatic modulation was determined in ELISA microtitre plates as described [15]. Two picomoles of each protein were incubated at 28°C in 100 μl of Z buffer in the presence of 1% BSA, with increasing amounts of either 3E5 mAb, 3E5 Fab, non-immune or immune anti-VP1 guinea pig sera [15] directed against the KLH-coupled, FMDV A21 peptide [24]. After 1 h, 40 μl of 2 mg/ml 2-nitrophenyl-β-D-galactopyranoside (ONPG) was added. When the yellow colour was evident (usually after 5 min), the enzyme was inactivated with 50 μl of 1 M Na_2CO_3. The intensity of the reaction was determined at OD_{405} in a multiscan spectrophotometer. Values are given as the increase in OD_{405} relative to that found in the absence of antibodies. All the assays were done at least in triplicate.

2.5. Determination of enzymatic constants

The β-galactosidase enzymatic assay was carried out as described [22] in a 1-ml reaction volume. Enzyme aliquots were incubated for 1 h at 28°C in Z buffer before adding the substrate, either in the presence or in the absence of antibodies. mAb 3E5 [23] was used for proteins M278VP1 and JX772A. An immune guinea pig serum [15] was employed for JX795A analysis, because of the poor responsiveness of this sensor to mAb 3E5 (Fig. 1B). To determine the K_m value of each protein, OD_{420} was monitored in a spectrophotometer at different concentrations of ONPG. Values of ΔOD_{420} s^{-1} were converted into rate constants and K_m and V_{max} values were determined using SigmaPlot Scientific Graphing Software. The first-order rate constant k_{cat} (turnover number) was calculated by dividing V_{max} by the total enzyme concentration. Determinations were performed three times for each protein.

2.6. Structural analysis of β-galactosidase

Rasmol software (version 2.6) was employed to determine atomic distances on the structure of native β-galactosidase [6].

3. Results and discussion

3.1. Antibody-induced reactivation in M278VP1, JX772 and JX795A

The chimaeric protein M278VP1, which displays an antigenic FMDV VP1 peptide on the activating interface, shows a titre-dependent increase in enzymatic activity upon binding to anti-VP1-specific serum [15]. Additional chimaeric β-galactosidases among those obtained in additional insertional attempts (Fig. 1A and [14]) also exhibit reactivation properties (Fig. 1B), making the identified insertion sites interesting candidates for the further development of efficient molecular sensors. Structural and functional details of these proteins are summarised in Fig. 1. For a rational design of improved β-galactosidase probes, a deep understanding of antibody-medi-

Table 1
Enzymatic properties of recombinant proteins with or without antibodies

Protein	Without antibody			With antibody		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM s^{-1})
M278VP1	0.3100 ± 0.0970[a]	1496 ± 173	4825 ± 1610	0.1384 ± 0.0165	1357 ± 54	9805 ± 1232
JX772A	0.0886 ± 0.0143	2760 ± 134	31 150 ± 5250	0.0880 ± 0.0076	5470 ± 291	62 160 ± 6305
JX795A	0.1459 ± 0.0174	3810 ± 93	26 115 ± 3180	0.1270 ± 0.0098	4825 ± 178	38 005 ± 3250

[a]Standard deviation values.

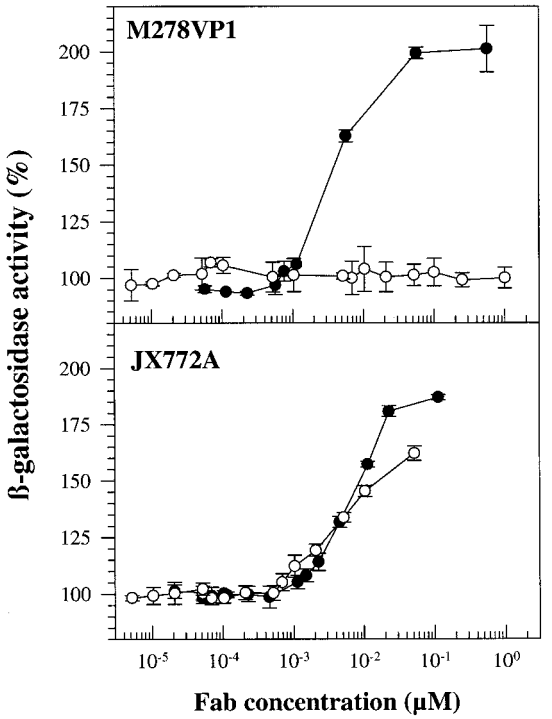

Fig. 2. Modulation of the enzymatic activity of M278VP1 and JX772A proteins at different concentrations of either 3E5 mAb (black circles) or 3E5 Fab (white circles), both depicted as molar amounts of Fab. Sensors were incubated for 1 h at 28°C with mAb or Fab prior analysis. Reactivation is depicted as the increase in OD_{405} relative to that of the protein incubated without antibodies.

ated reactivation would be required. Therefore, a functional approach has been taken to firstly investigate if a bivalent antibody binding is required to restore the activity in those sensors that are sensitive to mAb 3E5. Thus, the responses to the whole mAb and its Fab fragment were compared. Intriguingly, JX772A but not M278VP1 responds to the 3E5 Fab fragment similarly as to the whole mAb (Fig. 2). This observation indicates that in this mutant enzyme, reactivation is irrespective of the interaction of two enzyme monomers with the same IgG molecule. Moreover, it also suggests that bivalent interaction might be required for activation of M278VP1.

3.2. Enzymatic constants in antibody-activated probes

Substrate processing by M278VP1, JX772A and JX795A was monitored in the presence of activating antibodies. In Fig. 3, ONPG hydrolysis rates are plotted versus substrate concentration in the presence and absence of antibodies, and the obtained enzymatic constants are depicted in Table 1. In M278VP1-antibody complexes, K_m decreased significantly whereas k_{cat} remained unmodified. Therefore, reactivation of this protein might involve conformational changes in the active site or in its vicinity that could allow higher substrate diffusion and therefore an enhanced binding. Since in this protein the antigenic peptide is inserted in the activating interface, which is involved in the formation of the active site, its presence could cause steric impediments to the access of substrate that could be reduced by antibody-induced conformational modifications. In addition, data presented in Fig. 2 indicate that molecular tensions generated during antibody binding to monomers within the same tetramer might be required for reactivation of M278VP1.

In contrast, in JX772A, k_{cat} (and V_{max}) is doubled in the presence of antibodies, while K_m is not affected. This could be due to an increase in the actual number of active JX772A molecules promoted by the antibody, which might result from tetramer stabilisation and the consequent displacement of the monomer-tetramer equilibrium. However, the activation of this sensor by the Fab excludes this possibility, and prompts us to propose again that antibody-induced conformational changes in the assembled enzyme could be responsible for the observed reactivation. Interestingly, in activated JX795A, K_m is slightly reduced while k_{cat} increases. Therefore, the enzymatic restoration of this sensor seems to be produced by the combined effect of the molecular mechanisms that act independently in M278VP1 and JX772A. Moreover, this protein is not responsive to the mAb 3E5, although the binding of this antibody is comparable to that observed in JX772A [13]. Together, these results indicate that the three proteins examined here are members of three distinguishable β-galactosidase sensor families in which the activation proceeds by mechanisms that can be discriminated at a molecular level.

3.3. Structural and functional features of β-galactosidase molecular sensors

Variations in the activity of selected enzymes, when occurring in response to molecular interactions, can be useful for the design of molecular sensors [15,17,25]. The *E. coli* β-galactosidase is an attractive detection tag, since its activity can be determine by simple procedures [22]. This unusually complex enzyme [6] exhibits complementation between different protein fragments [5] and also modulation of its enzymatic

Table 2
Features of β-galactosidase molecular sensors

Protein	Sensor properties								
	Specific activity (U/μg)	Reactivation factor[b]	K_m variation factor[b]	k_{cat} variation factor[b]	Responsiveness to mAb 3E5	Responsiveness to Fab 3E5	Distances between insertions (Å)[c]	Distances to active sites (Å)[d]	Class
M278VP1	170 ± 43[a]	2.0	0.46	0.90	Yes	No	51/58/55	49/19	I
JX772A	515 ± 31	1.7	1.00	1.98	Yes	Yes	168/172/58	61/106	II
JX795A	675 ± 102	1.6	0.87	1.26	No	N.D.	74/91/57	15/56	III

[a]Standard deviation values.
[b]Reactivation was induced by immune guinea pig serum at dilution 1:10.
[c]Clockwise atomic distances between one insertion site and the remaining three of the assembled tetramer. The Cα of residues 278, 772 and 795 were taken as references for determination.
[d]Distances between the residues indicated in c and either the active site of the same monomer or the one sharing the activating interface. Cα of Glu^{461}, which is involved in the catalytic centre [30] was used as the reference.
N.D., not determined.

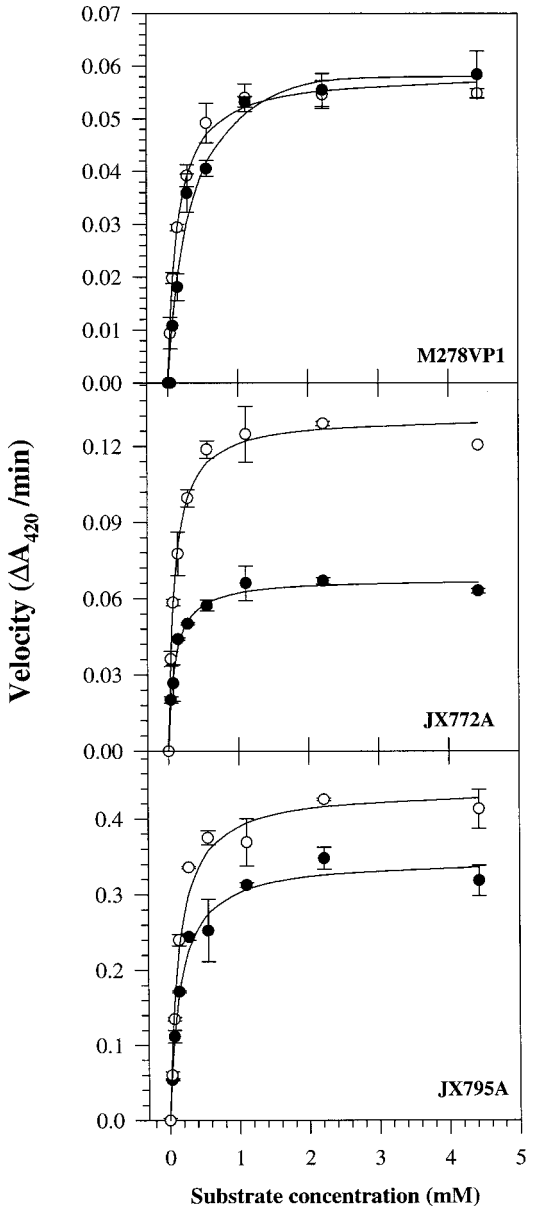

Fig. 3. Enzymatic kinetics of M278VP1, JX772A and JX795A proteins in the presence (white circles) or absence (black circles) of antibodies. Velocity is expressed as the increase of OD_{420} per minute at a given substrate concentration. Proteins M278VP1 and JX772A were activated by 3E5 mAb, and JX795A by immune guinea pig serum.

activity upon binding of antibodies elicited against complementing domains [26]. More recently, enzymatic modulation has also been observed in insertional mutants upon binding of anti-foreign peptide antibodies [15]. From the modulation parameters of three β-galactosidase molecular sensors (Figs. 2 and 3), two distinguishable reactivation mechanisms have been identified, involving significant variations in K_m and k_{cat} respectively (Table 1). In proteins M278VP1 and JX772A, substrate binding and catalysis are respectively stimulated, while in JX795A, both events occur simultaneously. According to reactivation profiles, classes I, II and III are here proposed for β-galactosidase sensors, the functional and structural properties of the representative proteins being summarised in Table 2. In both M278VP1 and JX795A, the foreign peptide has been inserted in the close vicinity of one of the four active sites of the tetramer [27]. In M278VP1, the acceptor site is a protruding loop extending towards the active site of the partner monomer, forming the activating interface between the components of the dimer [6]. In JX795A, the receiving, solvent-exposed loop embraces the Gly^{794}, an amino acid residue involved in substrate binding [28]. The atomic distances between each insertion site and both active sites in the corresponding dimer are shown in Table 2. The k_{cat} variation factors show a good linear correlation with the average atomic distances ($r^2 = 0.909$), and also with the average distances between the four insertion sites in the assembled tetramer ($r^2 = 0.990$). However, these distances correlated rather poorly with K_m variation factors ($r^2 = 0.498$ and 0.701 respectively).

Obtaining a larger number of β-galactosidase molecular sensors followed by their exhaustive characterisation would be required to finely elucidate the reactivation mechanisms. However, results presented here prompt us to suggest that the increase in catalysis rate (in class II sensors) is mediated by monovalent antibody binding to target peptides, positioned far from active sites. Therefore, reactivation of these enzymes must occur through long-distance conformational modifications. On the other hand, improved substrate binding can also result in enzymatic reactivation (in class I sensors), by bivalent binding to targets which are displayed in the vicinity of active sites. Local conformational tensions generated during bivalent binding might recover the solvent exposure of binding residues which had been occluded by the antigenic peptide, in probes in which insertions are placed at distances compatible with bivalent binding (around 60 Å). The analysis of class III JX779A sensor, in which the features mentioned for classes I and II coexist, indicates that these reactivation mechanisms are not exclusive. These findings could be of great importance for the design of improved enzymatic sensors, apart from contributing to the general understanding of protein structure-function relationships. Moreover, the existence of different classes of β-galactosidase sensors might allow the engineering of probes better adapted either to specific working conditions, such as low antibody or substrate concentrations, or to alternative substrates more advantageous for the developing of faster and more reliable tests for antibody detection.

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