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Engineering the *E. coli* β-galactosidase for the screening of antiviral protease inhibitors

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Abstract

Site-specific proteolysis is essential in many fundamental cellular and viral processes. It has been previously shown that the *Escherichia coli* β-galactosidase can be useful for the high-throughput screening of human immunodeficiency virus type 1 protease inhibitors. Here, by using crystallographic and functional data of the bacterial enzyme, we have identified a new accommodation site between amino acids 581 and 582, in a solvent-exposed and flexible β-turn of domain III. The placement of the model peptide reproducing the matrix-capsid (p17/p24) gag cleavage sequence renders a highly active and efficiently digested chimeric construct. The use of this insertion site, that increases the cleavage potential of this reporter enzyme, can improve the sensitivity and dynamic range of the antiviral drug assay. This simple and highly specific analytical test may also be extended to the screening of other specific protease inhibitors by a convenient colorimetric assay.
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Protein engineering offers the possibility to adapt enzymes to a diversity of analytical requirements. The *Escherichia coli* β-galactosidase has been one among the most used reporter enzymes for early studies on regulation of gene expression [1]. Recently, this enzyme has been engineered, either by insertional mutagenesis or end terminal fusion, for different and more sophisticated applications such as the investigation of molecular interactions [2,3], pharmacological screening [4,5], and molecular sensing in diagnosis [6]. Moreover, β-galactosidase has been proven useful for the high-throughput screening of human immunodeficiency virus type 1 (HIV-1) protease inhibitors, through the display of an accessible protease target site between the β-galactosidase amino acids 80 and 81. After cleavage by the HIV-1 protease, the reporter enzyme is inactivated and therefore, the presence of active inhibitors is positively indicated by higher activity [7]. By using this system, new inhibitors with therapeutic potential have been identified. Cheng and coworkers used, for the targeted peptide display on the enzyme surface, an accommodation site that had been previously identified by trial-and-error assessment of natural restriction sites of *lacZ* gene [8]. This site was proposed before the tridimensional structure of the encoded enzyme had been solved. In this early exploration, only one (*Sau*I) among the seven tested sites allowed peptide insertion without complete lost of activity, rendering a cleavable chimera suitable for analytical purposes. However, the resulting enzyme was poorly active when compared with the wild type β-galactosidase, being its specific activity 5.3% of the wild-type enzyme. Furthermore, the digestion

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reaction was incomplete even under optimal in vitro digestion conditions [8]. For screening purposes, especially when suspected inhibitors are expected to have moderate activities, the use of highly active and efficiently cleavable reporter enzymes would be desirable. In addition, more efficient cleavage would also allow extending this system for the analysis of other viral or cellular proteases. In this work, and by using crystallographic data of the bacterial β-galactosidase, we have selected and characterized an alternative accommodation site in which an inserted p17/p24 gag cleavage site does not dramatically disturb the enzymatic activity. Additionally, the cleavage site is processed with high efficiency by the viral protease.

Materials and methods

Structural analysis. The solvent-accessible surface area (SASA) of all N, Cα, C, and O atoms of *E. coli* β-galactosidase was calculated with surface racer [9] according to coordinates given in PDB-entry 1dp0 (van der Waals radii from Richards, probe radius of 1.4 Å). *B*-factors were taken from the same entry. Protein segments constituted by at least two consecutive residues having each a backbone SASA ⩾20 Å were initially selected. The resulting set of segments was further reduced by excluding those with an average backbone SASA < 30 Å, the average being over segment residues and chains (tetramer).

Plasmid construction and protein production. The chimeric gene was constructed by using a *Bam*HI site previously introduced at the desired position of *lacZ*, in the plasmid pJX581 [10]. Two synthetic complementary oligonucleotides including the *Bam*HI site forward 5′-GATCCCTGCATGCGTCACAGAACTATCCGATTGTGCAGG-3′ and reverse 5′-GATCCCTGCACAATCGGATAGTTCTGTGACGCATGCAGG-3′ were inserted therein by PCR as described [10] to generate the matrix-capsid (MA/CA, p17/p24) gag cleavage site plus a few flanking amino acids resulting from the cloning strategy (Fig. 1). The resulting plasmid pAV581Hp encoded the hybrid gene under the control of the strong lambda lytic promoters repressed by a constitutively expressed temperature-sensitive CI repressor. Protein production was induced by temperature shift of *E. coli* MC4100 carrying pAV581Hp, as previously described [11]. AV581Hp was purified from crude cell extracts by one-step affinity chromatography [12].

Analytical procedures. For the analysis of proteolytic activity, pure AV581Hp (at 0.15 μg/μl) was incubated with HIV-1 KIIA protease generously provided by the NHI AIDS Research and Reference Reagent Program (at 0.13 μg/μl), in Z buffer without β-mercaptoethanol [13] (pH 7.0), at 37 °C for two hours, and the digestion was monitored by Western blot and by β-galactosidase activity. β-Galactosidase and β-galactosidase fragments were immunodetected with polyclonal rabbit sera raised against the wild-type enzyme [14]. Band analysis was done by using the Quantity One software from Bio-Rad. When required, the anti-viral drug saquinavir (SQV) was added to the reaction at 313 μM. β-Galactosidase activity was measured in microtiter plates using ONPG as substrate, which rendered colored components measurable spectrophotometrically. The specific activity of the modified enzyme was measured as indicated [15]. Particular assay conditions have been given elsewhere [14]. All the experiments were performed at least by triplicate.

Results and discussion

Despite the usefulness of the antiviral inhibitor screening assay based on the *E. coli* β-galactosidase [7], the modified enzyme used in the former study is poorly hydrolyzed by the HIV protease [7,8]. This cannot be accounted for by a low intrinsic cleavability of the inserted amino acid segment since among the protease target sites within the viral polyprotein, this particular one (TF/PR, formerly named p6/PR) showed the highest rate when processed in vitro as synthetic peptide [16]. Therefore, we postulated that an improper presentation in the position 80–81 could restrict its accessibility and/or processing by the viral enzyme. An accurate selection of the insertion site for the target peptide would then result in a more cleavable enzyme and in the possibility of developing more sensitive assays. Therefore, we have explored surface accessibility, distribution, and flexibility of individual residues throughout the enzyme monomer. Twelve potential insertion sites were identified (Table 1) that satisfied the constrictions imposed in the analysis (see Materials and methods). Note that among them, the position 80–81 was absent since it is poorly exposed. A few of these sites appeared especially convenient for display of functional peptides. With the exception of 798–799 (see Table 1), they were

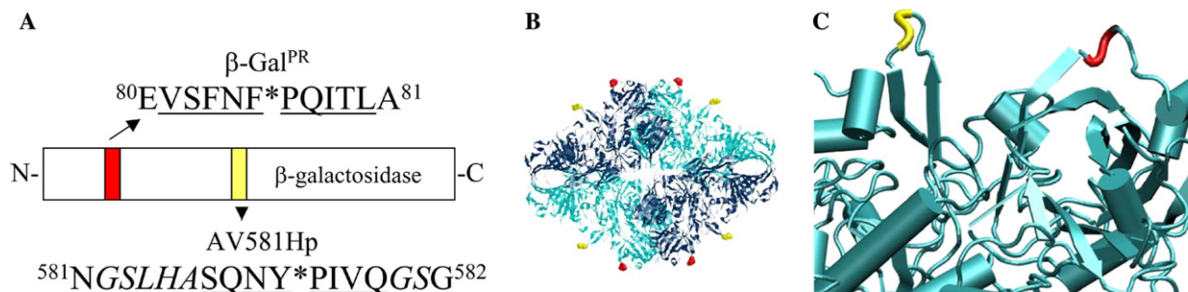

Fig. 1. (A) Sequence of the HIV peptides (underlined) accommodated in the engineered *E. coli* β-galactosidases. The precise cleavage site is indicated by asterisks, the approximate position by boxes, and the flanking residues of the enzyme by numbers. The sequence of AV581Hp has been checked by DNA sequencing and that of β-Gal^{PR} has been inferred from the given cloning details [8,7]. In AV581Hp, residues indicated in italics are those resulting from the introduction of the *Bam*HI restriction site. (B) Rasmol representation of the constructs indicating the position of the insertion sites in the whole tetrameric enzyme, by highlighting flanking residues 80 (in red) and 581 (in yellow). (C) Ribbon diagram (VMD [26]) of β-galactosidase (chain A, PDB entry 1dp0). Residues 80 and 81 are indicated in red and residues 581 and 582 in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Table 1
β-Galactosidase segments with large backbone SASA in the tetramer

Residue numbers[a]	Residue sequence	Average SASA ($Å^2$)	Average *B*-factor ($Å^2$)	Secondary structure
67–68	EA	30	22	Helix
92–95	MHGY	38	19	Helix → loop
134–135	LQ	30	20	Helix
263–264	GE	38	23	β-turn*
274–276	FGG	30	17	Loop
318–320	ADG	42	19	β-turn*
580–582	ENG	41	44	β-turn*
706–707	TA	32	18	Loop
730–735	LPAASH	30	79	Loop
798–799	AT	38	42	Loop*
845–846	QG	61	37	β-turn*
968–969	ME	33	19	Helix
80–81	EA	17	25	Loop

[a] The 12 β-galactosidase segments with large backbone SASA are shown. The last line corresponds to data on the insertion site used in the former developed analytical assays [7,8], and not revealed as a candidate in our analysis. Stretches which appear particularly adequate for peptide insertion (based on properties given in the table and the specific location in the tetramer) are indicated by an asterisk.

located in β-turns, i.e., short turns between antiparallel β-strands, a structure that may contribute to fixing the directionality of the insert. In particular, the area embraced by residues 580 and 582 exhibited a unique combination of high-solvent exposure and flexibility, it was placed in the middle of the β-turn (not siding any flanking structured area), and its four copies are homogeneously distributed on the tetramer's surface (Fig. 1). In the past, we had performed directed insertional mutagenesis in this enzyme [10,17] to generate multifunctional proteins acting as either biosensors [13,14] or non-viral, modular vehicles for gene therapy [18,19]. The accommodation between residues 581 and 582 of a peptide 27 residues long from foot-and-mouth disease virus (in the construct JX581A) resulted in a very stable enzyme and in the full exposure of functional peptides [20,10]. Therefore, we selected this precise place for HIV peptide presentation and cleavage study. For a more restrictive analysis, we chose as target peptide the matrix-capsid (p17/p24) gag cleavage site, that has been successfully used in other *E. coli*-based analytical systems for the HIV protease [21,22]. The intrinsic cleavability of the p17/p24 cleavage site is, however, only 7% of that described for TF/PR [16]. The resulting hybrid enzyme, named AV581Hp (Fig. 1), was successfully produced in *E. coli* and its specific activity represented more than 15% of that of the wild-type protein (Table 2), three times higher than that occurring when using the 80–81 site (Table 2). As observed (Fig. 2), AV581Hp was completely digested by the viral protease under standard conditions, rendering two fragments of the expected molecular weights (70.9 and 53.6 kDa for the amino- and carboxy-moieties, respectively). Such digestion was inhibited by the anti-viral drug SQV to almost completion. The high efficiency of the digestion process

Table 2
Comparative features of AV581Hp and β-Gal[PR]

	β-Gal[PR] [a]	AV581Hp
Insertion site (aa number)	80–81	581–582
Inserted residues	10	15
Specific activity (%)	5.3[b]	15.5
Relative cleavage rate of the synthetic peptide (V_{max}/K_m)[c]	1	0.07
Cleavage efficiency of the hybrid β-galactosidase (%)	55.2[d]	100

[a] β-Gal[PR] is the name given by Cheng et al. [7] to the hybrid β-galactosidase resulting from engineering of the *Sau*I restriction site, the same hybrid protein earlier constructed by Baum et al. [8] (the unnamed protein encoded by pZM-Sau). Also note that while Cheng et al. [7, p. 2441] indicate the peptide insertion as made by residue 131 of the enzyme, it actually was done by residue 80 as correctly stated by Baum and coworkers.

[b] Data from Table 1 in Baum et al. [8].

[c] Data from Table 1 in Krausslich et al. [16] obtained with monitoring the insert hydrolysis as independent, non fused peptides.

[d] Average data from Fig. 3, lanes 8 (54.05%) and 11 (56.47%) in Baum et al. [8]. The quantitative band analysis was done in this work on the published image from PubMed Central [http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=2124694].

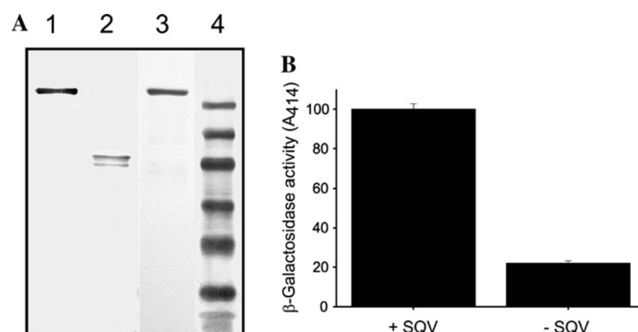

Fig. 2. (A) Western blot analysis of AV581Hp cleavage in vitro using anti-β-galactosidase antibodies as described [14]. Samples were loaded as follows: lane 1, undigested AV581Hp; lane 2, AV581Hp after two hours incubation with HIV protease; lane 3, AV581Hp after two hours incubation with HIV protease plus SQV; and lane 4, molecular markers of 198, 115, 93, 49.8, 35.8, 29.2, 21.3, and 6.4 kDa, respectively. (B) Enzymatic activity of the reaction mixtures containing AV581Hp plus the protease and with or without SQV, after two hours incubation.

represented a dramatic improvement of that described for the site 80–81, which, under optimal in vitro conditions, was around 50% (Table 2). Interestingly, two hours after adding the protease, the enzymatic activity of the mixture was largely reduced but still detectable, representing 20% of that observed in the presence of the protease inhibitor.

The emergence of viral diseases and growing concerns about re-emergence of smallpox or currently controlled viral infections prompted to urgently develop new and more efficient antiviral agents, among which protease inhibitors are specially convenient [23]. Many of these drugs have been identified for several virus species and in the case of HIV, a few efficient protease inhibitors are in clinical use. Moreover, the continuous raise of

resistance to current HIV-1 protease inhibitors is begging the development of alternative inhibitors. Recent insights into the molecular biology of this enzyme might allow the exploration of alternative mechanics of protease inhibition [24] such as impeding dimer formation [25]. This would hopefully extend the spectrum of substances available for inclusion in clinical protocols, for which high-throughput drug screening assays are highly desirable. In this context, the use of cleavable β-galactosidases displaying protease target peptides has been proven as a specially convenient principle for the identification of protease inhibitors [8]. Based on structural data, we have explored an insertion site alternative to that in current use, within a solvent-exposed, flexible β-turn of domain III in the middle of each monomer surface (Fig. 1). The evaluation of this site indicated that it is appropriate for peptide display rendering a highly active and cleavable enzyme (Fig. 2A).

Interestingly, the results presented here indicate that the proper solvent exposure of protease target sites on the carrier *E. coli* β-galactosidases may be a critical factor for cleavage, and this fact can be of relevance for further design and implementation of newer analytical tests. In this context, the insertion through the *Cla*I restriction site did not result into cleavable proteins [8]. The encoded accommodation site mapped in an unstructured loop exposed in the activating interface of the enzyme, in the vicinity of the active site. Antigenic, cell-binding viral peptides displayed there were highly immunoreactive [13,14] and efficiently interacted with receptor integrins on the cell surface [20]. This indicates more structural constraints for the proteolytic event than for specific molecular recognition involving large molecules like IgGs. These restrictions could importantly impair proper enzyme inactivation through proper digestion of exposed target peptides.

The most efficient cleavage of AV581Hp compared to that of β-Gal^{PR} (Fig. 2, Table 2) cannot be accounted for by differences in the intrinsic cleavability of the targets, since the TF/PR decapeptide used by Baum, Cheng, and coworkers was much more efficiently hydrolyzed in vitro than the p17/p24 peptide employed here (Table 2) [16]. The residual enzymatic activity observed after digestion (Fig. 2B) can be due to a minor population of intact polypeptide chains not detected by Western blot. Alternatively, and since no traces of undigested protein were observed (Fig. 2A), a percentage of cleaved molecules can be transiently active due to the high structural stability of the assembled bacterial enzyme. Irrespective of that, the use of highly cleavable enzyme derivatives based on the insertion site identified here could largely reduce the background of newly developed analytical tools, that in the current, β-galactosidase-based antiviral assay represents between 30% and 60% of the signal [7].

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