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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.

Keywords: Antiviral drug; HIV-1; Protease; Protein engineering; Screening

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 $\geqslant 20$ 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′-GATC CCTGCATGCGTCACAGAACTATCCGATTGTGCAGG-3′ and reverse 5′-GATCCCTGCACAATCGGATAGTTCTGTGACGCAT GCAGG-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

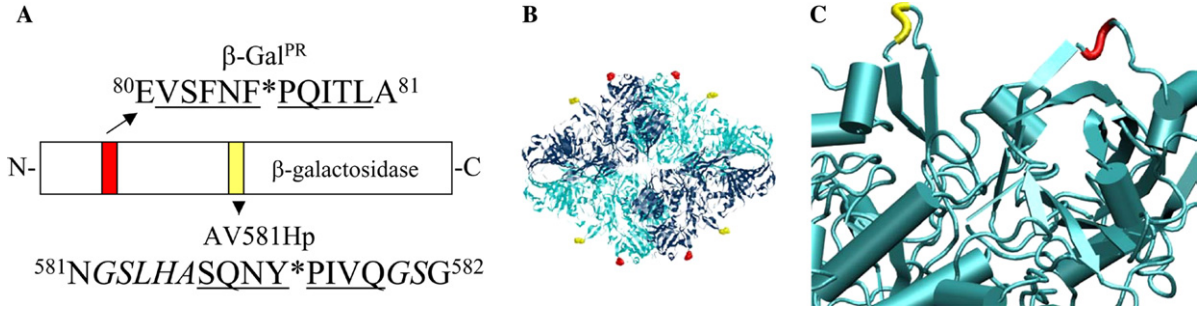

Fig. 1. (A) Sequence of the HIV peptides (underlined) accommodated in the engineered *E. coli* β-galactosidases. The precise cleavage site is indicated by asterisks, the approximate position by boxes, and the flanking residues of the enzyme by numbers. The sequence of AV581Hp has been checked by DNA 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.)