Engineering regulatable *E. coli* β-galactosidases as biosensors for anti-HIV antibody detection in human sera

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\(^1\) The abbreviations used are: BSA, bovine serum albumin; CPRG, chlorophenolred-β-D-galactopyranoside; FDA, Food and Drug Administration; FMDV, foot-and-mouth disease virus; HIV, human immunodeficiency virus; ONPG, ortho-nitrophenyl β-D-galactopyranoside, PBS, phosphate buffered saline; PDB, protein data base; SIV, immunodeficiency virus, simian; 3D, three dimensional.
Abstract. The activity of engineered, peptide-displaying enzymes is modulated by binding to specific anti-peptide antibodies. This new concept of a quantitative antibody detection system allows to set up test kits for fast diagnosis of infectious diseases. To develop a quick and homogeneous assay for the detection of HIV infection, we have explored two acceptor sites of the bacterial \textit{E. coli} \(\beta\)-galactosidase for the accommodation of HIV antigenic peptides. Two overlapping epitopes (namely P1 and P2) from the gp41 envelope glycoprotein, contained in different sized peptides, were inserted in the vicinity of the enzyme active site to generate a set of hybrid, enzymatically active \(\beta\)-galactosidases. Regulatable enzymes of different responsiveness to monoclonal antibody binding were generated with both acceptor sites tested. These biosensors were also sensitive to immune sera from HIV-infected patients. Modeling data provides insight into the structural modifications in the vicinity of the active site induced by peptide insertion that strongly affect the responsiveness of the engineered proteins through different parameters of their catalytic properties.
Introduction

Insertional fusion technologies are useful instruments for the analysis of membrane protein topology, structure-function relationship and for the generation of randomized protein libraries and enzymatic biosensors (1). The β-galactosidase enzyme (EC 3.2.1.23), encoded by the Escherichia coli lacZ gene, hydrolyzes lactose into glucose and galactose (2) which are then metabolized for cell growth. In addition, this enzyme also hydrolyzes other substrates producing colored compounds useful to monitor a wide range of biological processes. The three-dimensional (3D)\(^1\) structure of β-galactosidase (3) permits to explore the permissiveness of solvent-exposed loops to heterologous inserted peptides. In this context, we previously constructed some recombinant β-galactosidases, displaying foot-and-mouth disease virus (FMDV) B-cell epitope peptides accommodated in solvent-exposed surfaces (4, 5). The peptide insertion results in hybrid β-galactosidases with reduced enzymatic activity. However, in presence of anti-peptide monoclonal antibodies or polyclonal sera, these hybrid enzymes translate the antigen-antibody interaction into an easily measurable increase of the enzymatic activity (4-6).

Since the results obtained with these FMDV-based biosensor prototypes were very promising in the context of a fast and easy diagnosis of infectious diseases in a homogeneous assay, we were prompted to design new recombinant β-galactosidases as biosensors for anti-HIV human antibodies. The development of such a homogeneous colorimetric assay could be of great impact in human health, and also be helpful to better understand the mechanism of enzymatic regulation in biosensors by testing whether β-galactosidase enzymatic modulation is restricted to particular epitopes or specific peptide sequence, length or conformation. Therefore, we have inserted in two acceptor sites of E. coli β-galactosidase different sized peptides comprised between amino acids 589-618 of the env-encoded precursor gp160 of the human immunodeficiency virus type 1 (HIV-1) (9, 10) that, as in other retroviruses, contains the most immunogenic regions (11-14). The env gene product corresponds to a polyprotein of 160 kDa that is further processed by cleavage into subunit gp120 and the transmembrane protein gp41 (15).
By a detailed characterization of the resulting engineered enzymes, we show here that some of them, containing P1 (16) and P2 (17, 18) immunodominant epitopes from gp41, are highly antigenic and responsive to binding of anti-epitope antibodies, reaching reactivation factors higher than 250%. In addition, we demonstrate that the regulatable enzymes specifically respond to anti-HIV-1 antibodies in serum of infected patients, proving the high performance and robustness of these biosensors in complex samples and their usefulness in real diagnosis situations. Both antibody binding and enzymatic modulation data were analyzed in the context of the local conformation of the displayed peptides by 3D modeling of the engineered enzymes, and discussed regarding the structure-function relationship.
EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and cloning strategy

The *E. coli* K12 strain MC1061 (F−, λ-, Δ(araA-leu)7697, Δ(cod-lacI)3, araD139, galE15, galK16, hsdR2, mcrA, mcrB, relA1, rpsL150 (strR), spoT1, was used for cloning and small-scale protein production. Protein NF795gpC (Figure 1) was obtained at medium-scale in the protease-deficient *E. coli* BL26 (Novagen), a Lac− derivative of BL21 hsdS gal Lon− OmpT−. *E. coli* FA113 (DHB4 gor522...mini-Tn10Tc trxB::Km supp), a derivative of DHB4 (MC1000 ΔphoA(PvuII) phoR ΔmalF3 F'(lac+(lacI0) pro)) with more oxidizing conditions in the cytoplasm was kindly provided by G. Georgiou (19) and used for the production of S795gp (Figure 1). The plasmid pJLACZ (20) and its derivative pJX795 (5) encode engineered β-galactosidases under the control of the cI857-repressed, lambda $p_L$ and $p_R$ strong promoters.

The construction of recombinant β-galactosidases displaying HIV B-cell epitopes was achieved by using synthetic DNA fragments that encode the desired amino acid sequences. For the cloning at positions 278 and 795 of protein LACZ, ClaI and BamHI restriction sites were added, respectively, at both ends of synthetic oligonucleotides, resulting in either one 'ID' or two 'GS' extra amino acids. These segments were inserted into a pseudo-wild type lacZ gene, either by using an existing ClaI site for 278, or an engineered BamHI site for 795 (5). The resulting plasmid clones were characterized by PCR, restriction analysis and further DNA sequencing by using an ABI PRISM sequencing kit (PE Applied Biosystems) in an ABI 373 A DNA sequencer.

Protein production, detection and purification

Cell cultures were grown at 28°C in Luria-Bertani medium plus 50 µg/ml ampicillin (MC1061 and FA113) and 30 µg/ml streptomycin (MC1061) until they reached an OD550 of 0.4, being then transferred to a 42°C water bath, where recombinant gene expression was allowed for three hours. The production of the β-galactosidase recombinant proteins from the pJLACZ derived plasmids is driven by both lambda $p_L$ and $p_R$ promoters and controlled by the thermolabile cI857 repressor encoded on the same vector. Proteins were detected in crude cell extracts by Western blot as
described (4). Essentially, small amounts of cultures (around 20 µl) were submitted to rapid centrifugation and solubilized cell sediments loaded on 7.5% SDS-PAGE. Bands were developed with anti-β-galactosidase rabbit sera (1/500). Anti-rabbit antibodies coupled to horseradish peroxidase were obtained from Sigma and used at 1/2500. For protein purification, cell pellets were resuspended in 10 ml of 20 mM Tris-HCl (pH 7.2), 10 mM MgCl₂ with protease inhibitors benzamidine and phenylmethylsulfonyl fluoride at 25 µM and 1 mM, respectively. Cells were disrupted by ultrasonication and 1.6 M NaCl was then added to the clarified supernatant before loading on an affinity chromatography column for a single-step purification (21). 10 mM β-mercaptoethanol was finally added to the elution buffer.

The medium-scale production of NF795gpC for the standard HIV detection test was done with E. coli BL26 harboring the construction pNF795gpC. Cells were grown in a 5 L bioreactor on a defined medium with glucose as carbon source and supplemented with thiamin and ampicillin. The batch culture was started with an initial OD₆₀₀ of 0.1. Recombinant gene expression was induced by temperature shift from 30ºC to 42ºC at an OD₆₀₀ of 4.0 and after 4 hours cells were harvested by centrifugation and kept at –70ºC. The composition and the preparation of the medium and the conditions for the batch culture have been described previously (22). The NF795gpC was purified by affinity chromatography as explained above except that no protease inhibitors were added and the process was done always in the presence of 10 mM DTT. Protein concentration was determined spectrophotometrically at 280 nm using the molar absorption coefficient of E. coli β-galactosidase (λ= 1.5x10⁶±1.4x10⁵ M⁻¹cm⁻¹).

Enzymatic assays and determination of kₐₕₐₜ

β-Galactosidase activity was assayed as described previously (23). The protein concentration of the purified mutant proteins was calculated from Western blot sheets, using known amounts of pure β-galactosidase (Roche Diagnostics) as standard. Taking into account that a fraction of protein could be inactive after purification, the actual concentration of active enzyme was obtained for analytical purposes by comparing the
specific activity of purified mutant proteins with that of a cell crude extract, in which the freshly produced enzyme was used as a standard. For determining $k_{\text{cat}}$, known amounts of recombinant proteins were incubated with different concentrations of the substrate (ortho-nitrophenyl β-D-galactopyranoside, ONPG) in ELISA microtiter plates. The absorbance at 414 nm was read in a Labsystems iEMS Reader MF. The values of ΔA/min were converted to rate constants and $K_m$ and $V_{\text{max}}$ values were determined by using the SigmaPlot Scientific Graphing Software. The first-order rate constant $k_{\text{cat}}$ (turnover number) for each mutant protein was expressed as $V_{\text{max}}$/active enzyme concentration (s$^{-1}$).

Size exclusion chromatography

The analysis of the multimeric state of β-galactosidase fusion proteins was carried out in a Biosep SEC S3000 (300 × 7.8 mm) column (Phenomenex, Aschaffenburg, Germany) using 0.1 M sodium phosphate (pH 7), 1 mM MgCl$_2$ and 10 mM DTT at a flow rate of 0.5 ml/min. Eluted proteins were detected with a diode array detector at a wavelength of 280 nm. For protein size estimation, a gel filtration standard (BioRad #151-1901) was used.

ELISA

Monoclonal antibodies anti-P1 and anti-P2 (kindly provided by Roche Diagnostic GmbH, Penzberg, as research reagents) elicited against the HIV epitopes P1 and P2, respectively, were used in competitive ELISA for the antigenic analysis of the displayed peptides. Streptavidin-coated 96-well MaxiSorp plates (Nunc) were incubated with Biotin-BSA-coupled synthetic peptides P1 and P2 (kindly provided by Roche Diagnostic GmbH, Penzberg, as research reagents) for 12 hours at a concentration of 50 ng/ml, at 4°C in 0.1 M carbonate (pH 9.6). ELISA microtiter plates were blocked with 100 μg/ml Biotin in PBS for at least two hours, and then washed with PBS. Different amounts of each competing antigen were mixed in 1% BSA-PBS with a pre-defined amount of monoclonal antibody. After 1.5 hours of incubation at 37°C, 95 μl of each mixture were added to the peptide-coated wells and further incubated for one additional hour. After
several washing steps with PBS plus 0.05% (v/v) Tween-20, bound antibodies were detected with a goat anti-mouse IgG horseradish peroxidase conjugate (BioRad) and developed with 4-chloro-1-naphtol and H₂O₂. The reaction was stopped by adding 50 µl of 2 M H₂SO₄, and the absorbance was read at 620 nm. Biotin-BSA-coupled synthetic peptide P2 was also used as unbound, competing antigen. Anti-P1 and anti-P2 antibodies in human sera were detected by indirect ELISA in peptide-coated plates by standard procedures.

Modulation assay for anti-HIV antibody detection

The modulation assay was performed in low protein binding ELISA microtiter plates (Nunc Polysorp). Two picomoles of purified protein were incubated with or without either anti-P1 or anti-P2 monoclonal antibodies at 25 ng/µl or incubated with or without sera from HIV-infected patients or non immune human sera in 80 µl of Z buffer [0.1 M sodium phosphate (pH 7), 10 mM KCl and 1 mM MgSO₄] with 1% (w/v) BSA at 28°C for 45-60 minutes. Then, 40 µl of 2 mg/ml ONPG or 0.4 mg/ml CPRG (chlorophenolred-β-D-galactopyranoside) were added and the reaction was stopped when the corresponding color appeared, by adding 50 µl of 1 M Na₂CO₃. The absorbance at 414 nm or 595 nm was read in a Labsystems iEMS Reader MF. Values are obtained as the relative activity between the mutant protein in contact with the antibody in relation to the activity of the same protein without antibody, expressed as a percentage. All experiments were performed in triplicate. For comparison purposes, a blinded assay was performed on a panel of human sera from different sources with both a commercial, automated HIV-1/2 test and the β-galactosidase reactivation analysis described above. The automated test was performed in an AxSYM® immunoassay analyzer from Abbott Laboratories (Abbott Park, Illinois) according to the standard protocols provided by the supplier. This is a validated routine assay for HIV-1/2 diagnosis approved by the American Food and Drug Administration (FDA).

Construction of 3D models for HIV peptide-containing β-galactosidases

A protocol to generate candidate 3D structures for the engineered β-galactosidases displaying antigenic HIV peptides of various lengths
inserted at position 795 (see Figure 1) was developed. The comparative modeling method using the Modeller software (24) and also secondary structure prediction techniques (program Fugue, ref. 25) were utilized to produce the 3D modeled structures of the engineered proteins. The Modeller program builds a 3D structure based on the satisfaction of spatial restraints, extracted from the alignment of the target protein sequence with those of the template structures, and on the optimization of the energy of the built molecule. The model building protocol involved three consecutive steps:

(i) **Modeling of the various HIV antigenic peptides to be inserted in the β-galactosidase protein sequence**

Experimental structures for the amino acid sequences of gpA, gpC and gpD corresponding to HIV antigenic peptides of increasing length (see Figure 1 B) are not known. Hence, to model possible conformations of these sequences we used two structural templates. One is the structure of a segment of the HIV gp41 ectodomain (PDB code: 1ENV) that, however, does not include all the amino acids of the sequences to be modeled. We had thus to use another template, namely the simian immunodeficiency virus (SIV) gp41 ectodomain structure (PDB code: 2EZO). The two additional 'GS' amino acids flanking the HIV peptides were modeled *ab initio*, i.e. without any spatial restraints derived from the template structures. Forty 3D models were built for each of the three peptides so as to produce enough conformational variety. Since this number is too large to proceed with the construction of engineered protein models, we used the program NMRCLUST (26) to reduce the number of HIV peptide models while keeping as much diversity as possible. This program determines clusters comprising close structures and selects one representative model per cluster. Similarity of the HIV peptide models was evaluated on the basis of the RMSD value of the backbone atomic positions, defined as the square root of the sum of the square of the distances computed between equivalent atoms divided by the number of atoms. After clustering, the number of representative models was 6, 9 and 8 for gpA, gpC and gpD peptides, respectively.
(ii) **Modeling of intermediate models**

Starting from the 3D representative models generated for gpA, gpC and gpD and from the X-ray structure of β-galactosidase (PDB code 1BGL), we built intermediate 3D models whose amino acid sequences were those of HIV peptides flanked on each side by amino acid stretches corresponding to the β-galactosidase local sequence in the vicinity of position 795. Conformational changes were allowed to potentially propagate locally along the β-galactosidase sequence till the protein chain folds into a well-defined secondary structure, as detected in the wild-type β-galactosidase structure. The sequences to be modeled extended therefore to Phe784 on the N-terminal side and to Ala805 on the C-terminal side (Figure 1A). This step was required so as to account for possible conformational changes mutually induced by the β-galactosidase and the insertions. Secondary structure predictions were performed so as to look for the local propensity of these sequences to adopt a preferred secondary structure. High propensities were taken into account in the construction of the intermediate models. After applying a clustering procedure similar to that used in step (i) we ended up with 8, 7 and 9 representative models for the sequences comprising gpA, gpC and gpD, respectively.

(iii) **Modeling of the HIV peptide-containing β-galactosidases**

Starting from the known X-ray structure of the AD dimer of β-galactosidase and from the different representative intermediate 3D models, we generated 3D dimeric models of the engineered proteins. The modeling of a dimer was required as the β-galactosidase structure shows a protruding loop (residues 272-288) of one monomer extending toward the active site of the facing monomer. The validity of the generated models was evaluated by assessing their stereochemistry using the Procheck program (27) and by visual inspection. Respectively, 7, 6 and 10 models of NF795gpA, NF795gpC and NF795gpD monomers passed the validity tests.

**Conformational analysis of the 3D models**

The 3D models were examined so as to reveal whether they could interpret the experimental data. For this purpose, the contact area between the active site residues and the rest of the structure was computed. This parameter was calculated as the sum of the areas of the polyhedra faces that
atoms of a given residue pair have in common. The polyhedra calculation was performed using a method based on radical planes implemented in the program SurVol (28). The conservation of the epitope conformations in the 3D models was evaluated by computing the RMSD for backbone atoms using a 3D model of a gp41 peptide as a reference. This model was built using segments of the SIV and HIV gp41 ectodomains as templates, since the HIV Xray structure lacks most amino acids of the epitopes. This peptide comprises 45 residues and contains P1 and P2 epitopes. Epitope solvent exposure was computed from atomic coordinates of the 3D models with a probe radius of 1.4 Å, using the analytic procedure of SurVol, implemented in the BRUGEL package (29).
RESULTS

Efficient production and purification of HIV epitope-containing β-galactosidases

The pseudo-wild-type *E. coli* β-galactosidase LACZ protein (20) was engineered to explore its potential to respond to specific anti-HIV antibodies by modulation of its enzymatic activity (Figure 1A). Two overlapping HIV epitopes of the *env*-encoded gp41 glycoprotein, namely P1 (16) and P2 (17, 18), contained in five different-sized peptides (Figure 1B), were inserted either at position 278 or position 795, both located in permissive solvent-exposed loops of the assembled enzyme tetramer. An additional protein, SD7895gp, which harbors HIV peptides at both positions in the β-galactosidase monomer, was also constructed to increase the number of displayed epitopes in the sensor molecules. The sequence of all these proteins at the insertion sites was checked by DNA sequencing of the encoding plasmid vectors after clone screening. The resulting eleven enzymes, carrying insertions that range from 15 to 45 amino acids, were produced in a heat-inducible expression system by thermal upshift, rendering detectable amounts of β-galactosidase activity in the cell extracts (not shown). With the exception of NF795gpB that was only seen as a low molecular mass fragment in Western blot, all the enzymes were produced as stable proteins with the expected molecular mass (Figure 1C) and efficiently purified by a single-step affinity chromatography procedure from disrupted cells (not shown).

Enzymatic constants of HIV epitope-containing recombinant β-galactosidases upon peptide insertion

The impact of different sized peptide insertions on β-galactosidase enzymatic parameters was studied in NF795gpA, NF795gpC and NF795gpD proteins, since their enzymatic activity seemed to be higher than the analogous NF278 series as inferred from activity of cell extracts (data not shown). The enzymatic constants for those proteins are shown in Table I, in which LACZ and JX795A (the last one containing an FMDV antigenic peptide inserted at the same position 795, ref. 5) have also been
added as controls. Both $K_m$ and $k_{cat}$ are strongly affected by peptide insertion in an apparently opposite fashion. Furthermore, increasing peptide size increases the value of $K_m$ (although the latter always remains below the $K_m$ value of the parental protein LACZ) and progressively impairs the catalytic constant. Also, the resulting specificity constant ($k_{cat}/K_m$) is reduced by peptide insertions, with the highest impact observed in NF795gpD, which accommodates the largest foreign peptide. These results suggest that peptide size has a strong but also steady influence on enzyme performance. This seems to be irrespective of the precise amino acid sequence, since protein JX795A, containing a 27mer FMDV peptide (Table I), seems to fit in the general profile observed for the HIV sensors. To confirm this presumed influence of peptide length, enzymatic constants were plotted versus peptide size. As seen in Figure 2, $k_{cat}$ responds linearly to peptide size in the modified enzymes. $K_m$ values show a similar trend though the linearity is not statistically significant. As expected, the resulting specificity constant is also depending on peptide size but intriguingly, in this case, the non-modified LACZ enzyme also fits into the correlation. The important impact of peptide insertions on the $k_{cat}$ of the bacterial enzyme was seen as a promising hint concerning the possible responsiveness of these enzymes to antibodies, since the proper performance of enzymatic biosensors depends on $k_{cat}$ modification (37).

**Binding of specific anti-HIV monoclonal antibodies to HIV epitope-displaying recombinant β-galactosidases**

The accessibility of the heterologous epitopes to specific anti-epitope antibodies is required for proper sensor responsiveness. This parameter was monitored by a competitive ELISA in which the proteins from the NF795 series were used as competitors for the binding of monoclonal antibodies to their respective antigens. In streptavidine-coated plates incubated with P1-biotin peptide antigen, all proteins competed for the anti-P1 monoclonal antibody, indicating that the epitope is surface-exposed after monomer folding and tetramer formation, although differences in the IC$_{50}$ values are seen in different proteins (for instance, compare NF795gpC and NF795gpA in Figure 3A). These differences, despite being not clearly dependent on the peptide size, might be due to slightly different structural constraints.
determined by peptide conformation that could affect either the solvent-exposure or the presentation of the B-cell epitope in recombinant P1 peptide.

When biotinilated P2 peptide was used as bound antigen in competitive ELISA, none of the protein constructs competed for the binding of the monoclonal antibody anti-P2, whereas synthetic P2 alone indeed did (Figure 3B). Noticeably, a model of the anti-HIV 3D6 Fab fragment bound to the P2 epitope predicts the cyclization of this epitope via the two cysteine residues which are critical for antibody-epitope interaction (17). Since in the cytoplasm of *E. coli* proteins are in a reduced state, the disulphide bond formation in P2-epitope containing recombinant proteins is not favored and this fact could prevent correct B-cell epitope presentation. In an attempt to promote an antigenic-prone peptide conformation, we transformed *E. coli* strain FA113, in which disulfide bond formation is more efficient in the cytoplasmic space (19), with plasmid pS795gp. Nevertheless, no binding to the recombinant P2-epitope was detected even when the protein was produced in this system (Figure 3B). This could indicate a poor oxidizing effect on the recombinant β-galactosidase in FA113 or alternatively an exhaustive unspecific oxidation of the enzyme that could lead to an incorrect folding and therefore a non-functional presentation of the epitope. However, since the β-galactosidase activity of induced FA113 cell cultures is similar to that exhibited by productive MC1061 cell cultures (not shown), this last possibility must be excluded. Also, it may be possible that the P2 epitope cannot adopt a functional conformation in this insertion site with or without the presence of the disulfide bond.

**HIV epitope-displaying recombinant β-galactosidases are modulated upon specific monoclonal antibody binding**

The impact of peptide insertion on the *k*<sub>cat</sub> of β-galactosidase (Figure 2), and the solvent accessibility of the P1 peptide in P1-displaying enzymes prompted us to explore the modulation of these proteins by specific antibodies. β-Galactosidase enzymatic activity was determined in crude cell extracts incubated with the immunoreactive anti-P1 monoclonal antibody (Figure 4). Among the 278 series, NF278gpB was the only protein
responsive to the antibody by increasing its activity 1.5 fold (Figure 4). This discriminating reactivation was not expected, since HIV peptides accommodated in both NF278gpC and NF278gpD, as in NF278gpB, contain the whole viral epitope plus additional HIV flanking sequences. Therefore, these data could indicate that a peptide length around 25 residues could be critical for reactivation of enzymes modified in this position, while the insertion of longer segments could have a high impact on the β-galactosidase enzymatic constants that could not be restored by antibody binding.

The set of 795 derived proteins showed a similar response profile. In this case, the critical peptide size could be around 35 amino acids, and a more gradual increase of reactivation level was observed with the peptide size. Protein S795gp, which accommodates a 27-mer peptide showed a reactivation factor in between those from NF795gpA and NF795gpC (carrying 15 and 35 amino acid insertions, respectively). The hybrid protein SD7895gp, showed a reactivation factor comparable with that observed for S795gp, indicating again a poor regulatable contribution of site 278.

Activity modulation of proteins M278VP1 and JX795A carrying an antigenic 27-mer FMDV peptide (4) upon monoclonal antibody 3E5 binding, is presented for comparison. It indicates a better responsiveness of M278VP1 compared to NF278gpB, while the reactivation factor of NF795gpC is much higher than the one observed in JX795A. These differences may be due to various factors such as the strength of molecular binding of the corresponding monoclonal antibodies, the solvent exposure of the epitope and the conformational changes in the epitope upon insertion or antibody binding.

Modeling the conformation of HIV peptides as accommodated in E. coli β-galactosidase

The experimental results indicate that enzymatic properties, antibody binding and reactivation factor strongly depend on the antigenic peptides inserted into β-galactosidases. To get more insight into the molecular mechanism involved in these processes we attempted to determine the three-dimensional structure of some modified β-galactosidases. Since all attempts to crystallize these proteins were unsuccessful we resorted to
molecular modeling to construct 3D structures of the engineered proteins so as to help interpreting and rationalizing the experimental data presented above. Models were generated for NF795gpA, NF795gpC and NF795gpD proteins, since they have been more extensively studied owing to their higher enzymatic activity than their analogous NF278 proteins.

The inspection of the crystal structure of wild-type β-galactosidase reveals that the 794-803 loop does not contribute directly to the shape of the active site but interacts with residues in this site. Moreover, a study has shown that substitutions for Gly794 affect the binding of substrates (30). Hence, it is not unexpected that peptide insertions at position 795 alter the $K_m$ value of the protein. The structural analysis of the 3D models indicates that some portions of the NF795gpA, NF795gpC and NF795gpD inserts interact to a similar extent with residues Asn102 and Trp999, which are known to be important for substrate binding (31). This observation supports the hypothesis that a change in $K_m$ value relative to the wild type enzyme should occur in the engineered proteins, but it does not permit to predict the trend of the change of the different modified proteins versus the wild type.

Table 2 displays the amino acid sequences in the neighborhood of the 795 residue for the wild-type protein and the different engineered proteins along with the secondary structure predictions derived from the respective amino acid sequences. Noticeably, the propensity for the inserted sequences to adopt a preferred α-helix secondary structure correlates with a stronger binding of the modified proteins for the substrate. When no or only a slight preference is shown, the $K_m$ value for the engineered proteins returns to that determined for the wild type. It is conceivable that the presence of a defined secondary structure could induce a local rigidity in this region of the protein, thereby helping in strengthening the affinity of the active site cavity for the substrate.

To get insight into the relationship between the structures of the modified enzymes and $k_{cat}$ values we computed the contacts made by residues of the inserts with the putative catalytic residues, contacts which of course are lacking in the wild-type enzyme and might explain changes in the protein activity (see Table 3). NF795gpA, NF795gpC and NF795gpD proteins all display an interaction between the insertions and His418, a residue that is important for the role of Mg$^{2+}$ on the protein activity (32).
The NF795gpC models feature additional contacts of the insertion with residue Glu461, which, together with Glu537, is the only residue strictly conserved in glycohydrolases and acts as an acid/base catalyst in the reaction mechanism. The NF795gpD 3D models exhibit extra contacts between the inserts and residues Tyr503 and His540 (Figure 5). These latter two residues are also essential for catalysis (33, 34). In particular, His540 has been shown to help the stabilization of the transition state (35). Thus, new interactions with residues important for catalysis arise in the modified enzyme models. Noteworthy, the number of catalytic residues involved in contacts with the inserted peptide increases concomitantly to a decrease of $k_{cat}$ values.

Efficient recognition of P1 and P2 epitopes by an antibody may depend on the preservation of their structure and their solvent exposure upon insertion. In order to estimate these two properties we modeled a gp41 peptide of 45 residues containing both epitopes. P1 epitope in the NF795gpA, NF795gpC and NF795gpD 3D models, adopts a structure which is rather close to that observed in the gp41 peptide whereas P2 epitope displays conformations that differ more (Figure 5). Indeed, average RMSD values computed for backbone atoms of the different NF795 models, using the gp41 peptide model as a reference, amount to about 1.8 Å and 5.6 Å for P1 and P2, respectively. As P1 displays a better structure conservation we computed the solvent accessible surface area of this epitope in the NF795gpA, NF795gpC and NF795gpD 3D models. The values are close to that evaluated in the gp41 model (see Table 4). Moreover they follow the sequence: P1(gpA) < P1(gpD) < P1(gpC), corroborating the experimental IC$_{50}$ data shown in Figure 3.

Recombinant β-galactosidase proteins displaying HIV-1 epitopes detect anti-HIV-1 antibodies in human sera

Irrespective of structural issues, we wondered if the molecular sensors sensitive to a monoclonal antibody (Figure 4) would also be responsive in more complex samples as in human sera. Note that the simplicity of such a homogeneous, colorimetric assay would represent an improvement in the detection of HIV infection, especially in the context of AIDS spread in developing areas. Five HIV-1 positive sera and one HIV-2 positive serum
previously tested by standard protocols were used at dilutions 1:40, in
modulation assays as described above, for proteins NF278gpB, NF795gpA,
NF795gpC, NF795gpD, S795gp and SD7895gp. All HIV-1/2 positive
samples induced a marked reactivation of β-galactosidase (Figure 6),
comparable with that observed with anti-P1 monoclonal antibody (Figure
4). However, the HIV-2 positive serum did not modify the enzymatic
activity of any β-galactosidase hybrid protein, proving not only a good
performance of the sensors in natural sera samples but also a high
specificity in these HIV sensors.

Since NF795gpC was proven to be the most responsive protein
(Figures 4, 6), this enzyme was selected for a large-scale comparison of the
modulation assay presented here with a FDA-approved automated standard
HIV detection method. The enzyme was produced in medium-scale and
purified in affinity columns up to 80 units per µg of protein. CPRG was
used here to reduce the background in samples of undiluted sera. More than
90% of the 32 HIV positive sera according to the standard detection test
were also detected as positive in our homogeneous assay (relative
enzymatic activity higher than 110%; Table 5), only sera 60.0018 and
60.0029 were rendering negative results. On the other hand, one among the
29 tested negative sera was revealed as positive in the modulation assay
(A8039 01), intriguingly presenting the highest value in the negative set in
the standard HIV-1/2 test. Even with these exceptions, row data from both
methods revealed a good correlation ($r^2=0.630$, $p<0.0001$).

To determine if the 60.0018 and 60.0029 sera were not activating the
sensor because of a failure of the enzyme to detect anti-P1 antibodies, we
searched for both anti-P1 and anti-P2 antibodies in the above sera samples
by standard ELISA. Anti-P1 antibodies were not detectable in these sera
but were detected in the analyzed control sera that were tested positive in
the modulation assay presented here (60.0013 and 60.0024). All four
samples exhibited anti-P2 antibodies in high titer (not shown).

**Quaternary structure and stability of recombinant β-galactosidase
NF795gpC**

Analysis of the quaternary structure of NF795gpC using size
exclusion chromatography revealed a tetrameric organization of the
chimeric protein as is observed for *E. coli* β-galactosidase (data not shown). Monomers or dimers were not observed. In addition, repetitive freeze thawing (up to 14 cycles) or prolonged incubation at temperatures above 37°C did not result in the disassembly of the tetramer into subunits but caused the formation of aggregates. Measurements of the activity and the modulation of the activity of NF795gpC by monoclonal antibodies revealed that repetitive freeze thawing resulted in decreasing absolute values of activity, but the activation ratio remained unchanged. These results show that the antibody-mediated reactivation of NF795gpC occurs on the biologically active tetramer. The above results clearly exclude the possibility that reactivation occurs by forced tetramerization through antibody binding. Moreover, they show that antibody detection can also be accomplished in the presence of denatured NF795gpC.
DISCUSSION

Two acceptor sites in solvent-exposed loops of *E. coli* β-galactosidase, previously described as tolerant to FMDV epitope-containing peptides (5, 6), have been explored for the insertion of HIV-1 specific antigenic peptides and the generation of molecular sensors for a homogeneous, HIV-infection detection assay. Several peptide segments including B-cell epitopes from the envelope protein of HIV-1 (ranging from 15 to 45 amino acids in length) have been introduced in the loop comprising amino acids 272-288 that is part of the activating interface and/or in the loop 794-803, in direct contact with residues forming the active site (3, 31). NF278-derived proteins are seen as intact bands in Western Blot, while among the NF795 set, NF795gpB is unexpectedly only detectable in producing cells as a lower molecular mass fragment of 92 kDa. All the stable proteins compete for the binding to the anti-P1 (but not anti-P2) specific monoclonal antibody in competitive ELISA (Figure 3). The lack of anti-P2 reactivity may be due to a failure in the formation of disulphide bonds in the *E. coli* cytoplasm that could eventually be overridden using chimeric libraries with random peptides as recently shown (36). Furthermore, some stable HIV-1-β-galactosidase hybrid proteins undergo enzymatic modulation by the binding of specific monoclonal antibodies (Figure 4), and also by exposition to sera from HIV-infected individuals (Figure 6). The only modulatable enzyme derived from insertions at the activating interface corresponds to NF278gpB, with a reactivation factor of 150%. Within the alternative set of constructs, NF795gpC shows the highest reactivation factor upon monoclonal antibody binding (more than 250%), higher than that previously obtained with the equivalent protein JX795A that contains an FMDV peptide (about 200%) (7, 8), and those observed in any of the enzymatic sensors constructed up to now (37). Protein SD7895, which displays a 27 amino acid peptide at positions 278 and 795, shows a moderate reactivation factor (140%), indistinguishable from that of protein S795gp. Moreover the activity of protein S278gp is not altered upon antibody binding (Figure 4). These observations indicate the poor effect of this insertion at position 278 in the reactivation mechanism for HIV sensors.
The function of a protein critically depends on the maintenance of its 3D structure. The consequences of the HIV inserts at position 795 on the protein activity can be explained to some extent on the basis of 3D models built by comparative modeling. The effect on binding of the substrate seems to be not only a function of the inserted peptide size but also on its capacity of retaining its local secondary structure as in the HIV gp41 protein. This capacity, which might induce a higher local rigidity, depends on the amino acid sequence. Furthermore, both properties, size and local secondary structure, seem to affect the local conformation of the protein in the vicinity of the active site and impair its catalytic activity as shown by the $k_{cat}$ values. The built 3D models also show that P1 compared to P2, when inserted in a protein sequence that differs drastically from that of the gp41 protein, is more prone to preserve its native conformation and then bind to anti-HIV antibodies. Not only the conformation of the epitope but its solvent exposure as well, are likely to be important for antibody recognition. With respect to the latter property our built models indicate a nice correlation between the P1 exposure to the solvent and the IC$_{50}$ values for the different engineered proteins.

The results presented here confirm the potential of recombinant epitope-displaying β-galactosidases as new generation molecular sensors for the detection of specific antibodies in human sera, discriminating between HIV-1 and HIV-2 seroreactive samples by sequence differences in respective envelope proteins (38). The comparison of the standardized method for detection of HIV-1/2 immunoreactive sera with the modulation assay using NF795gpC as biosensor shows a good linear correlation between both methods. Also, the modulation assay presents a sensitivity of 94% and a specificity of 96.5% compared to the HIV-1/2 test. From 32 positive sera, only two appear as negative sera under our assay conditions, but this result can be explains by the absence of anti P1 antibodies in these samples. On the other hand, 1 out of 29 negative sera appeared as positive. Although this sample should be considered as a false positive it shows the highest value among the negative sera using the standard protocol (0.78), indicating that the enzymatic modulation assay is at least as sensitive as the standard analysis at least under our working conditions.
Interestingly, for other enzyme-based biosensors, inactivating antibodies have been detected that reduce the specific activity, probably by reducing the substrate diffusion to the active site (36). If present in HIV-immunoreactive sera, this could be a problem for a proper activity enhancement in the modulation assay through activating antibodies. However, this effect has not been observed in any of the β-galactosidase sensors obtained so far (37). This could be due to different molecular distances between the antibody-binding site and the active site in different modified enzymes, or to different conformational constraints induced on the active site by the presence of the foreign peptide. Although the presence of β-galactosidase inactivating antibodies in HIV-immune sera cannot be completely excluded, their presence at significant levels would result in a poor correlation between classic tests (based only on molecular binding) and the modulation assay (based on enzyme activation). Since we have found an excellent correlation ($p<0.0001$), inactivating antibodies, if present, seem to be irrelevant in the context of a quantitative assay.

Furthermore, the design of a β-galactosidase biosensor for the detection of antibodies to HIV-2 in blood could be approached in the same way than the one described here for HIV-1 to complement the assay. In summary, we propose here the epitope-displaying β-galactosidase model as a biosensor with potential generic applications in fast and homogenous assays, which could be used in multiple intermolecular detection systems by easy protein engineering procedures.

Acknowledgements: J. Cabrera-Crespo kindly acknowledges a post-doctoral fellowship from FAPESP (The State of São Paulo Research Foundation FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DE SÃO PAULO (Proc. FAPESP; No 99/05554-5) and Fundação-Instituto Butantan (São Paulo, Brazil). M. Prévost is a Chercheur Qualifié at the FNRS (Belgium). We are also grateful to K. Kuerzinger for helpful discussions, to Roche Diagnostic GmbH, Penzberg for the generous gift of peptides and monoclonal anti-P1 and anti-P2 antibodies and to G. Georgiou for providing *E. coli* strain FA113.
Legends for the figures

Figure 1. A: Amino acid sequence at the selected insertion sites for the construction of recombinant β-galactosidases containing HIV epitopes P1 and P2. Residues of the LACZ protein are numbered according to (39). Underlined residues correspond to the ClaI and BamHI restriction sites, respectively, where peptide insertions have been introduced. Note that despite the used 278 notation, insertions in LACZ protein have been accommodated between residues 279 and 280. B: Amino acid sequence of the HIV epitope-containing peptides inserted in proteins encoded by pJLACZ (rendering the NF278 series) and pJX795 (rendering the NF795 series) parental plasmids. Residues are numbered according to (9, 10). Amino acids in bold correspond to P1 epitope and underlined amino acids correspond to P2 epitope. Apart from the specified amino acids, ClaI and BamHI encoded peptides at each end were added during the cloning process at positions 278 and 795, respectively. C: Western blot analysis of recombinant HIV-β-galactosidases in 7.5% SDS-PAGE. Crude solubilized cell extracts from producing cultures were loaded and bands detected with anti-β-galactosidase antibodies. The lane labeled as M corresponds to SeeBlue Pre-stained Standards from Novex and the band shown here corresponds to 98 kDa.

Figure 2. Values of parameters $K_m$, $k_{cat}$ and the $K_m/k_{cat}$ ratio were plotted versus the size of the inserted peptide for the proteins shown in Table I. For $K_m$ and $k_{cat}$, the coordinate for LACZ protein was not included in the regression but it is shown as a white square. Regressions including this point rendered the following statistics: $r^2 = 0.003$, $p > 0.1$, and $r^2 = 0.712$, $p > 0.1$, respectively. For the $K_m/k_{cat}$ ratio, LACZ protein was included in the regression analysis.

Figure 3. A: Competitive ELISA of recombinant β-galactosidases produced in E. coli MC1061 for monoclonal antibody anti-P1 with the Biotin-BSA-coupled synthetic peptide P1 as competitive antigen. B: Competitive ELISA of recombinant β-galactosidases for monoclonal...
antibody anti-P2 with the Biotin-BSA-coupled synthetic peptide P2 as competitive antigen. The same peptide P2 has been added as a control of competing soluble antigen. NF795gpA (▲), NF795gpC (●), NF795gpD (■), SD7895gp (◆), S795gp (●), S795gp produced in *E. coli* FA113 (○) and P2 (▼).

**Figure 4.** Modulation of the enzymatic activity in the recombinant proteins containing HIV epitopes using anti-P1 monoclonal antibody at 25 ng/μl (black bars for NF278 series and gray bars for NF795 series). Note that SD7895gp accommodates viral peptides in both insertion sites. As a comparison, it is also shown the modulation of recombinant proteins containing the FMDV immunodominant epitope A at the same insertion sites, when using 3E5 anti-FMDV VP1 monoclonal antibody (hatched bars) according to (7).

**Figure 5.** Left: 3D model of the NF795gpD β-galactosidase carrying the gpD HIV antigenic peptide inserted at position 795. Only domain 3 (residues 334-627) and the segment comprising the insertion are shown. Right: Structure of the HIV gp41 peptide whose sequence corresponds to that inserted in gpD. The protein trace is represented as a red line. A ribbon is drawn for the gpD insertion. The P1 epitope, P2 epitope and their overlapping portion are colored in blue, green and yellow respectively. The side chains of the active site residues which make contact with the gpD insertion are depicted as balls and sticks.

**Figure 6.** Modulation of the enzymatic activity in the recombinant proteins containing HIV epitopes using diluted sera (1:40) from five different HIV-1 (grey bars) and one HIV-2 infected patients (white bars) in comparison to modulation in the presence of anti-P1 monoclonal antibody at 25 ng/μl (black bars).
REFERENCES


Table 1 Enzymatic properties of β-galactosidase chimeric proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LACZ</td>
<td>0.188±0.033$^a$</td>
<td>18300±779</td>
<td>97340±1757</td>
</tr>
<tr>
<td>NF795gpA</td>
<td>0.049±0.010</td>
<td>3013±102</td>
<td>60114±12426</td>
</tr>
<tr>
<td>NF795gpC</td>
<td>0.085±0.012</td>
<td>1777±51</td>
<td>20907±3013</td>
</tr>
<tr>
<td>NF795gpD</td>
<td>0.171±0.016</td>
<td>813±17</td>
<td>4740±457</td>
</tr>
<tr>
<td>JX795A$^b$</td>
<td>0.107±0.026</td>
<td>2640±158</td>
<td>24800±4215</td>
</tr>
</tbody>
</table>

$^a$Standard deviation  
$^b$Data obtained from (32).

Table 2 Sequence in the vicinity of residue 795 and the corresponding secondary structure prediction along with the $K_m$ values. "H" stands for helix and "B" for β-structure.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence and Secondary Structure Prediction</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LACZ</td>
<td>$^{793}$IGVSEATRIDPN</td>
<td>0.188</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-----------</td>
</tr>
<tr>
<td>NF795gpA</td>
<td>$^{793}$IGVGSXHLYL</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-----------</td>
</tr>
<tr>
<td>NF795gpC</td>
<td>$^{793}$IGVSGIKQLQA</td>
<td>0.085</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-----------</td>
</tr>
<tr>
<td>NF795gpD</td>
<td>$^{793}$IGVGSQTVWGI</td>
<td>0.171</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-----------</td>
</tr>
<tr>
<td>JX795A</td>
<td>$^{793}$IGVGSTTYTASA</td>
<td>0.107</td>
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### Table 3. Catalytic residues affected by insertion

<table>
<thead>
<tr>
<th>Protein</th>
<th>Residues</th>
<th>$k_{cat} , (s^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LACZ (no insertion)</td>
<td></td>
<td>18300</td>
</tr>
<tr>
<td>NF795gpA</td>
<td>His418</td>
<td>3013</td>
</tr>
<tr>
<td>NF795gpC</td>
<td>His418, Glu461</td>
<td>1777</td>
</tr>
<tr>
<td>NF795gpD</td>
<td>His418, Glu461, Tyr503, His540</td>
<td>813</td>
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</table>

### Table 4. P1 surface accessibility in NF795 series

<table>
<thead>
<tr>
<th>Protein</th>
<th>Surface accessibility ($\AA^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF795gpA</td>
<td>1230 ± 192</td>
</tr>
<tr>
<td>NF795gpC</td>
<td>1617 ± 27</td>
</tr>
<tr>
<td>NF795gpD</td>
<td>1562 ± 26</td>
</tr>
<tr>
<td>gp41 fragment</td>
<td>1402 $^a$</td>
</tr>
</tbody>
</table>

$^a$ data computed on a single model
Table 5 HIV1/2 AxSYM method compared to NF795gpC enzymatic modulation in a panel of 61 human sera.

<table>
<thead>
<tr>
<th>Serum</th>
<th>HIV1/2 AxSYM</th>
<th>NF795gpC</th>
<th>Serum</th>
<th>HIV1/2 AxSYM</th>
<th>NF795gpC</th>
</tr>
</thead>
<tbody>
<tr>
<td>60.0001</td>
<td>28.08±</td>
<td>+</td>
<td>60.0002</td>
<td>0.41</td>
<td>-</td>
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<tr>
<td>60.0003</td>
<td>29.05</td>
<td>+++</td>
<td>60.0008</td>
<td>0.46</td>
<td>-</td>
</tr>
<tr>
<td>60.0004</td>
<td>32.90</td>
<td>+</td>
<td>60.0009</td>
<td>0.64</td>
<td>-</td>
</tr>
<tr>
<td>60.0005</td>
<td>26.20</td>
<td>+++</td>
<td>60.0020</td>
<td>0.74</td>
<td>-</td>
</tr>
<tr>
<td>60.0006</td>
<td>19.98</td>
<td>+++</td>
<td>60.0044</td>
<td>0.30</td>
<td>-</td>
</tr>
<tr>
<td>60.0007</td>
<td>24.85</td>
<td>+</td>
<td>161198 01</td>
<td>0.34</td>
<td>-</td>
</tr>
<tr>
<td>60.0001</td>
<td>35.30</td>
<td>+++</td>
<td>161198 02</td>
<td>0.55</td>
<td>-</td>
</tr>
<tr>
<td>60.0011</td>
<td>27.59</td>
<td>+++</td>
<td>161198 03</td>
<td>0.52</td>
<td>-</td>
</tr>
<tr>
<td>60.0012</td>
<td>26.58</td>
<td>+++</td>
<td>161198 04</td>
<td>0.42</td>
<td>-</td>
</tr>
<tr>
<td>60.0013</td>
<td>21.47</td>
<td>+++</td>
<td>161198 05</td>
<td>0.51</td>
<td>-</td>
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<tr>
<td>60.0014</td>
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<td>161198 06</td>
<td>0.49</td>
<td>-</td>
</tr>
<tr>
<td>60.0015</td>
<td>24.30</td>
<td>+++</td>
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<td>0.50</td>
<td>-</td>
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<tr>
<td>60.0016</td>
<td>17.61</td>
<td>+</td>
<td>161198 08</td>
<td>0.54</td>
<td>-</td>
</tr>
<tr>
<td>60.0017</td>
<td>11.14</td>
<td>+</td>
<td>161198 10</td>
<td>0.53</td>
<td>-</td>
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<tr>
<td>60.0018</td>
<td>18.96</td>
<td>-</td>
<td>161198 11</td>
<td>0.60</td>
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<tr>
<td>60.0019</td>
<td>23.70</td>
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<td>161198 12</td>
<td>0.30</td>
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<tr>
<td>60.0021</td>
<td>30.92</td>
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<tr>
<td>60.0022</td>
<td>41.11</td>
<td>++</td>
<td>A8039 01</td>
<td>0.78</td>
<td>+</td>
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<tr>
<td>60.0023</td>
<td>24.78</td>
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<td>A8039 02</td>
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<td>A8039 03</td>
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<td>60.0025</td>
<td>35.60</td>
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<td>A8039 04</td>
<td>0.47</td>
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<td>60.0026</td>
<td>31.13</td>
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<td>A8039 05</td>
<td>0.50</td>
<td>-</td>
</tr>
<tr>
<td>60.0027</td>
<td>14.81</td>
<td>+</td>
<td>A8039 06</td>
<td>0.42</td>
<td>-</td>
</tr>
<tr>
<td>60.0028</td>
<td>15.07</td>
<td>+</td>
<td>6022956</td>
<td>0.33</td>
<td>-</td>
</tr>
<tr>
<td>60.0029</td>
<td>14.60</td>
<td>-</td>
<td>6022982</td>
<td>0.29</td>
<td>-</td>
</tr>
<tr>
<td>60.003</td>
<td>24.53</td>
<td>+++</td>
<td>6023012</td>
<td>0.30</td>
<td>-</td>
</tr>
<tr>
<td>60.0038</td>
<td>21.38</td>
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<td>6117482</td>
<td>0.33</td>
<td>-</td>
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<td>60.0039</td>
<td>21.91</td>
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<td>6117488</td>
<td>0.39</td>
<td>-</td>
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<tr>
<td>60.004</td>
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<td>+</td>
<td>6210075</td>
<td>0.30</td>
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<tr>
<td>60.0041</td>
<td>31.90</td>
<td>+++</td>
<td>+ +</td>
<td>6210075</td>
<td>0.30</td>
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<tr>
<td>60.0042</td>
<td>31.49</td>
<td>+</td>
<td>+ +</td>
<td>(130≤relative activity&lt;150)</td>
<td>-</td>
</tr>
<tr>
<td>60.0043</td>
<td>24.48</td>
<td>+++</td>
<td>+</td>
<td>(110≤relative activity&lt;130)</td>
<td>-</td>
</tr>
<tr>
<td>anti-P1</td>
<td>nd</td>
<td>+++</td>
<td>-</td>
<td>(relative activity&lt;110)</td>
<td>-</td>
</tr>
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</table>

AxSYM readings above 1 are considered as positive while readings below are HIV1/2 negative. nd. not done.
Figure 1

A

<table>
<thead>
<tr>
<th>Parental plasmid</th>
<th>Insertion site</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJLACZ</td>
<td>&amp;textbf{LWQGETQVASGTAPFGGEII}DERGGYADRVTLRLNVENPK^{299}</td>
</tr>
<tr>
<td>pJX795</td>
<td>&amp;textbf{LLTPLRDQFTRAPLNDIGVGCSEATRDPNAWVERWKAA^ {615}}</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Fragment name</th>
<th>Inserted peptide</th>
<th>Amino acids inserted</th>
<th>Proteins</th>
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</thead>
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<tr>
<td>gpA</td>
<td>&amp;textbf{AVERYLKDQQLGIW}^{603}</td>
<td>15</td>
<td>NF278gpA, NF795gpA</td>
</tr>
<tr>
<td>gpB</td>
<td>&amp;textbf{QARILAVERYLKDQQLGIWCGSGK}^{608}</td>
<td>25</td>
<td>NF278gpB, NF795gpB</td>
</tr>
<tr>
<td>gpC</td>
<td>&amp;textbf{GKQLQARILAVERYLKDQQLGIWCGSKLICTT}^{613}</td>
<td>35</td>
<td>NF278gpC, NF795gpC</td>
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<tr>
<td>gpD</td>
<td>&amp;textbf{QLTVWGIKQLQARILAVERYLKDQQLGIWCGSKLICTTA}^{618}</td>
<td>45</td>
<td>NF278gpD, NF795gpD</td>
</tr>
<tr>
<td>S</td>
<td>&amp;textbf{AVERYLKDQQLGIWCGSKLICTTAV}^{615}</td>
<td>27</td>
<td>S278GP, S795GP, SD7895gp</td>
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</table>

C

[Image of gel electrophoresis]
Figure 2

[Graphs showing Km, Kcat, and Kcat/Km values versus peptide size. Each graph includes a regression line and correlation coefficient (r²) with p-values.]

- **Km**: 
  - Parameter value versus peptide size
  - r² = 0.810
  - p > 0.1

- **Kcat**: 
  - Parameter value versus peptide size
  - r² = 0.939
  - p < 0.05

- **Kcat/Km**: 
  - Parameter value versus peptide size
  - r² = 0.964
  - p < 0.01
Figure 3

![Graph showing antibody binding as a function of competitive antigen concentration.](image-url)
Figure 4
Figure 5
Figure 6

![Bar chart showing relative enzymatic activity for different samples.](image-url)
Engineering regulatable E. coli beta-galactosidases as biosensors for anti-HIV antibody detection in human sera

Neus Ferrer-Miralles, Jordi X Feliu, Stéphane Vandevuer, Annette Müller, Joaquin Cabrera-Crespo, Isabelle Ortmans, Frank Hoffmann, Daniel Cazorla, Ursula Rinas, Martine Prévost and Antonio Villaverde

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