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 test kits to be set up for fast diagnosis of infectious diseases. To develop a quick and homogeneous assay for the detection of human immunodeficiency virus (HIV) infection, we have explored two acceptor sites of the bacterial Escherichia coli β-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 β-galactosidases. Regulable 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 provide 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.

Insertional fusion technologies are useful instruments for the analysis of membrane protein topology and structure-function relationship, as well as 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 structure of β-galactosidase (3) permits the permissiveness of solvent-exposed loops to heterologously inserted peptides to be explored. 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 the 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).

Because 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 in better understanding 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 comprising amino acids 589–618 of the env-encoded precursor gp160 of the human immunodeficiency virus type 1 (HIV-1) (9, 10), which, as in other retroviruses, contains the most immunogenic regions (11–14). The product of the env gene 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 regulable enzymes specifically...
cally 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 diagnostic situations. Both antibody binding and enzymatic modulation data were analyzed in the context of the local conformation of the displayed peptides by three-dimensional modeling of the engineered enzymes, and these are discussed in regard to structure-function relationship.

**Experimental Procedures**

**Bacterial Strains, Plasmids, and Cloning Strategy**

The *E. coli* K12 strain MC1061 (*F-, λ-, supE44Δ::Tn10 mini-Tn10Tc lacZΔM15 K12 strain MC1061 (F−, λ−, ΔaraA−araL−7687, ΔcodelacI3, araD139, galE165, galK16, hisD22, mcrA, mcrB, relA1, rpsL150 [strR], spoT1) was used for cloning and small-scale protein production. Protein NF795gpC (Fig. 1) was obtained at medium scale in the protease-deficient *E. coli* BL26 (Novagen), a lac– derivative of BL21 (hisD23 galK16 ompT*), *E. coli* FA113 (DH5α groEL22... mini-Tn10Tc trxB::Km supR). A derivative of DH8 (MC1000 3phoP(PvuI) phoR ΔmalF3 F( lacZΔM15 lacI Δ(lacZP)) with more oxidizing conditions in the cytoplasm was kindly provided by G. Georgiou (19) and used for the production of S795gp (Fig. 1). The plasmid pJLACZ (20) and its derivative pJX795 (5) encode engineered β-galactosidases under the control of the e1857-repressed lambda pL and pR 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 cloning at positions 278 and 795 of protein LacZ, Clal and BamHI restriction sites were added, respectively, at both ends of the 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 Clal site for position 278 or an engineered BamHI site for 795 (5). The resulting plasmid clones were characterized by polymerase chain reaction, restriction analysis, and further DNA sequencing by using an ABI PRISM sequencing kit (PE Applied Biosystems) in an ABI 373A DNA sequencer.

**Protein Production, Detection, and Purification**

Cell cultures were grown at 28 °C in Luria-Bertani medium plus 50 µg/ml ampicillin (for both *E. coli* strains MC1061 and FA113) and 30 µg/ml streptomycin (only for *E. coli* strain MC1061) until they reached an *A* 660 of 0.4 and were then transferred to a 42 °C water bath where recombinant gene expression was allowed for 3 h. The production of the β-galactosidase recombinant proteins from the pJLACZ-derived plasmids was driven by both α pL and pR promoters and controlled by the thermolabile e1857 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-polyacrylamide gel electrophoresis. Bands were developed with anti-galactosidase rabbit serum (1/500). Anti-rabbit antibodies coupled to protein LACZ, B-cell epitopes was achieved by using synthetic DNA fragments that encode the desired amino acid sequences. For cloning at positions 278 and 795 of protein LacZ, Clal and BamHI restriction sites were added, respectively, at both ends of the 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 Clal site for position 278 or an engineered BamHI site for 795 (5). The resulting plasmid clones were characterized by polymerase chain reaction, restriction analysis, and further DNA sequencing by using an ABI PRISM sequencing kit (PE Applied Biosystems) in an ABI 373A DNA sequencer.

**Enzymatic Assays and Determination of kcat**

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, S.L.) 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 determination of kcat, known amounts of recombinant proteins were incubated with different concentrations of the substrate (ortho-nitrophenyl β-D-galactopyranoside) 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 Vmax values were determined by using the SigmaPlot Scientific Graphing Software. The first-order rate constant k cat (turnover number) for each mutant protein was expressed as Vmax/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 S2000 (300 × 7.8 mm) column (Phenomenex, Aschaffenburg, Germany) using 0.1 M sodium phosphate (pH 7), 1 mM MgCl2 and 10 mM dithiothreitol 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 (Bio-Rad #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 peptides P1 and P2 (kindly provided by B Diagnostica GmbH, Penzberg, as research reagents) for 12 h 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 2 h 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 h 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 (Bio-Rad) and developed with 4-chloro-1-naphthol 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 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 plates (Nunc Polybio). Two pmol 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 min. Then, 40 µl of 2 mg/ml ortho-nitrophenyl β-D-galactopyranoside or 0.4 mg/ml chlorophenol red β-D-galactopyranoside was added, and the reaction was stopped when the corresponding color appeared by adding 50 µl of 1 M Na2CO3. The absorbance at 414 or 595 nm was read in a Labsystems iEMS Reader MF. Values were 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 reaction analysis described above. The automated test was performed in an AxSYM® immunoassay analyzer from Abbott Laboratories (Abbott Park, IL) 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.
Construction of three-dimensional Models for HIV Peptide-containing β-Galactosidases

A protocol to generate candidate three-dimensional structures for the engineered β-galactosidases displaying antigenic HIV peptides of various lengths inserted at position 795 (see Fig. 1) was developed. A comparative modeling method (Modeler software (24)) and also secondary structure prediction techniques (Fugue program (25)) were utilized to produce the three-dimensional modeled structures of the engineered proteins. The Modeler program builds a three-dimensional structure based on the satisfaction of spatial restraints extracted from the alignment of the target protein sequence with those of the template structures and the optimization of the energy of the built molecule. The model-building protocol involved the following 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 Fig. 1B) 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 of the amino acids of the sequences to be modeled. We therefore had 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. 40 three-dimensional models were built for each of the three peptides so as to perform a conformational analysis of these variations because this variety is large enough 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 among the HIV peptide models was evaluated on the basis of the r.m.s. 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 three-dimensional representative models generated for gpA, gpC, and gpD and from the x-ray structure of β-galactosidase (PDB code 1BGL), we built intermediate three-dimensional models in which the 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 until the protein chain folded into a well defined secondary structure, as detected in the wild-type β-galactosidase structure. The sequences to be modeled were extended therefore to Phe-784 on the N-terminal side and to Ala-805 on the C-terminal side (Fig. 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 three-dimensional models of β-galactosidase and from the different representative intermediate three-dimensional models, we generated three-dimensional dimeric models of the engineered proteins. The modeling of a dimer was required because 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 Three-dimensional Models

The three-dimensional 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 polyhedral faces that atoms of a given residue pair have in common. The polyhedral calculation was performed using a method based on radical planes implemented in the program SurVol (28). The conservation of the epitope conformations in the three-dimensional models evaluated by computing the r.m.s.d. for backbone atoms using a three-dimensional model of a gp41 peptide as a reference. This model was built using segments of the SIV and HIV gp41 ectodomains as templates because the HIV x-ray structure lacks most of the 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 three-dimensional 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 (Fig. 1A). Two overlapping HIV epitopes of the env-encoded gp41 glycoprotein, namely P1 (16) and P2 (17, 18), contained in five different sized peptides (Fig. 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 11 enzymes, carrying insertions that range from 15 to 45 amino acids, were produced in a heat-inducible expression system by thermal up-shift, rendering detectable amounts of β-galactosidase activity in the cell extracts (not shown). With the exception of NF795gpB that was seen only as a low molecular mass fragment in Western blot, all of the enzymes were produced as stable proteins with the expected molecular mass (Fig. 1C) and were purified efficiently 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, because 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 these proteins are shown in Table 1, in which LACZ and JX795A (the last one containing an FMDV antigenic peptide inserted at the same position 795 (5)) were added as controls. Both Km and kcat are strongly affected by peptide insertion in an apparently opposite fashion. Furthermore, increasing peptide size increases the value of Km (although the latter always remains below the Km value of the parental protein LACZ) and progressively impairs the catalytic constant. The specificity constant (kcat/Km) 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 true irrespective of the precise amino acid sequence, because protein JX795A, containing a 27-mer FMDV peptide (Table 1), 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 Fig. 2, kcat responds linearly to peptide size in the modified enzymes. Km values show a similar trend, although the linearity is not statistically significant. As expected, the resulting specificity constant is also dependent on peptide size but intriguingly, in this case, the nonmodified 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, because 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 streptavidin-coated plates incubated with P1-biotin peptide antigen, all proteins competed for the 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 Fig. 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 biotinylated P2 peptide was used as bound antigen in competitive ELISA, none of the proteins competed for the P2 monoclonal antibody anti-P2, whereas synthetic P2 alone did (Fig. 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 that are critical for antibody-epitope interaction (17). Because in the cytoplasm of *E. coli* proteins are in a reduced state, the disulfide bond formation in the P2 epitope-containing recombinant proteins is not favored, and this fact could prevent correct B-cell epitope presentation. In an attempt to promote an anti-gen-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 (Fig. 3B). This finding could indicate a poor oxidizing effect on the recombinant β-galactosidase in FA113 or alternatively an exhaustive nonspecific oxidation of the enzyme that could lead to an incorrect folding and therefore a nonfunctional presentation of the epitope. However, because 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_{cat} of β-galactosidase (Fig. 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 ac-
that a peptide length of around 25 residues could be critical for reactivation of enzymes modified in this position, whereas 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 of NF795gpA and NF795gpC (carrying 15 and 35 amino acid insertions, respectively). The hybrid protein SD795gp showed a reactivation factor comparable with that observed for S795gp, indicating again a poorly regulable 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 higher level of responsiveness of M278VP1 compared with NF278gpB, whereas 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 depend strongly on the antigenic peptides inserted into β-galactosidases. To gain more insight into the molecular mechanism involved in these processes, we attempted to determine the three-dimensional structure of some modified β-galactosidases. Because all attempts to crystallize these proteins were unsuccessful, we resorted to molecular modeling to construct three-dimensional structures of the engineered proteins to help interpret and rationalize the experimental data presented above. Models were generated for NF795gpA, NF795gpC, and NF795gpD proteins because they have been more extensively studied because of their higher enzymatic activity in comparison with 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 Gly-794 affect the binding of substrates (30). Hence, it is not unexpected that peptide insertions at position 795 alter the Km value of the protein. The structural analysis of the three-dimensional models indicates that some portions of the NF795gpA, NF795gpC, and NF795gpD inserts interact to a similar extent with residues Asn-102 and Trp-999, which are known to be important for substrate binding (31). This observation supports the hypothesis that a change in Km value relative to the wild-type enzyme should occur in the engineered proteins, but it does not permit us to predict the trend of the change of the different modified proteins versus the wild type. Table II displays the amino acid sequences in the neighborhood of the 795 residue for the wild-type protein and the different modified proteins, but it does not permit us to predict the trend of the change of the different modified proteins versus the wild type. Table II 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 Km 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 gain insight into the relationship between the structures of the modified enzymes and \( k_{\text{cat}} \) values, we computed the contacts made by residues of the inserts with the putative catalytic residues, contacts that of course are lacking in the wild-type enzyme and might explain changes in the protein activity (see Table III). NF795gpA, NF795gpC, and NF795gpD proteins all display an interaction between the insertions and His-418, 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 Glu-461, which, together with Glu-537, is the only residue strictly conserved in glycohydrolases and acts as an acid-base catalyst in the reaction mechanism. The NF795gpD three-dimensional models exhibit extra contacts between the inserts and residues Tyr-503 and His-540 (Fig. 5). These latter two residues are also essential for catalysis (33, 34). In particular, His-540 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. It is noteworthy that the number of catalytic residues involved in contacts with the inserted peptide increases concomitantly to a decrease of \( k_{\text{cat}} \) values.

The efficient recognition of P1 and P2 epitopes by an antibody may depend on the preservation of their structure and their solvent exposure upon insertion. To estimate these two properties, we modeled a gp41 peptide of 45 residues containing both epitopes. The P1 epitope in the NF795gpA, NF795gpC, and NF795gpD three-dimensional models adopts a structure rather close to that observed in the gp41 peptide, whereas P2 epitope displays conformations that differ more (Fig. 5). In particular, His-540 has been shown to help the stabilization of the transition state (35). Table III shows the catalytic residues affected by insertion, with the number of contacts for each residue.

Recombinant \( \beta \)-Galactosidase Proteins Displaying HIV-1 Epitopes Detect Anti-HIV-1 Antibodies in Human Sera—Irre-
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 V); only sera 60.0018 and 60.0029 rendered negative results. On the other hand, 1 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 whether 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 serum samples using a standard ELISA. Anti-P1 antibodies were not detectable in these sera but were detected in the analyzed control sera that 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.

**TABLE IV**

| Protein                        | Surface accessibility |
|--------------------------------|-----------------------|
| NF795gpA                       | 1230 ± 192            |
| NF795gpC                       | 1617 ± 27             |
| NF795gpD                       | 1562 ± 26             |
| gp41 fragment                  | 1402$^a$              |

$^a$ Data were computed on a single model.

**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 amino acids 272–803, in direct contact with residues forming the active site (3, 31). NF278-derived proteins are seen as intact bands in Western blot, whereas among the NF795 set, NF795gpB is unexpectedly detectable only in producing cells as a lower molecular mass fragment of 92 kDa. All of the stable proteins compete for the binding to the anti-P1 (but not anti-P2) specific monoclonal antibody in competitive ELISA (Fig. 3). The lack of anti-P2 reactivity may be due to a failure in the formation of disulfide bonds in the E. coli cytoplasm that could eventually be overridden using chimeric libraries with random peptides as shown recently (36). Furthermore, some stable HIV-1-β-galactosidase hybrid proteins undergo enzymatic modulation by the binding of specific monoclonal antibodies (Fig. 4) and also by exposure to sera from HIV-infected individuals (Fig. 6). The only modulable 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, which contains an FMDV peptide (about 200%) (7, 8), and higher than 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 (Fig. 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 three-dimensional structure. The consequences of the HIV inserts at position 795 on the protein activity can be explained to some extent on the basis of three-dimensional 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 to retain 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 of these properties, size and local secondary structure, seem to affect the local conformation of

![FIG. 6. Modulation of the enzymatic activity in the recombinant proteins containing HIV epitopes using diluted sera (1:40) from five different HIV-1-infected (gray bars) and one HIV-2-infected patient (white bars) compared with modulation in the presence of anti-P1 monoclonal antibody at 25 ng/μl (black bars).](image)

### TABLE V

| Serum     | HIV1/2 AxSYM | NF795gpC | Serum     | HIV1/2 AxSYM | NF795gpC |
|-----------|--------------|----------|-----------|--------------|----------|
| 60.0001   | 28.08a       | +        | 60.0002   | 0.41         | –        |
| 60.0003   | 29.05        | ++       | 60.0008   | 0.46         | –        |
| 60.0004   | 32.90        | ++       | 60.0009   | 0.64         | –        |
| 60.0005   | 26.20        | ++       | 60.0020   | 0.74         | –        |
| 60.0006   | 19.98        | ++       | 60.0044   | 0.30         | –        |
| 60.0007   | 24.85        | ++       | 161198 01 | 0.34         | –        |
| 60.0008   | 35.30        | ++       | 161198 02 | 0.55         | –        |
| 60.0009   | 27.59        | ++       | 161198 03 | 0.52         | –        |
| 60.0010   | 26.58        | ++       | 161198 04 | 0.42         | –        |
| 60.0011   | 21.47        | ++       | 161198 05 | 0.51         | –        |
| 60.0012   | 34.43        | ++       | 161198 06 | 0.49         | –        |
| 60.0013   | 24.30        | ++       | 161198 07 | 0.50         | –        |
| 60.0014   | 17.61        | +        | 161198 08 | 0.54         | –        |
| 60.0015   | 11.14        | +        | 161198 10 | 0.53         | –        |
| 60.0016   | 18.96        | –        | 161198 11 | 0.60         | –        |
| 60.0017   | 23.70        | ++       | 161198 12 | 0.30         | –        |
| 60.0018   | 30.92        | ++       | 161198 13 | 0.57         | –        |
| 60.0019   | 41.11        | +        | A8039 01  | 0.78         | +        |
| 60.0020   | 24.78        | ++       | A8039 02  | 0.35         | –        |
| 60.0021   | 24.87        | ++       | A8039 03  | 0.47         | –        |
| 60.0022   | 35.60        | ++       | A8039 04  | 0.47         | –        |
| 60.0023   | 31.13        | +        | A8039 05  | 0.50         | –        |
| 60.0024   | 36.02        | +        | A8039 06  | 0.42         | –        |
| 60.0025   | 15.07        | ++       | 6022956   | 0.33         | –        |
| 60.0026   | 14.60        | –        | 6022982   | 0.29         | –        |
| 60.0027   | 21.38        | ++       | 6023012   | 0.30         | –        |
| 60.0028   | 21.91        | ++       | 6117482   | 0.33         | –        |
| 60.0029   | 15.00        | ++       | 6117488   | 0.39         | –        |
| 60.0030   | 31.90        | +        | 6210075   | 0.30         | –        |
| 60.0031   | 31.49        | +        | 6210075   | 0.30         | –        |
| 60.0032   | 24.48        | ++       | 6210075   | 0.30         | –        |
| Anti-P1   | ND           | +        |           |              |          |

**Note:** AxSYM readings above 1 are considered positive, and readings below 1 are HIV1/2-negative.
the protein in the vicinity of the active site and impair its catalytic activity as shown by the $k_{\text{cat}}$ values. The three-dimensional models built also show that P1 compared with 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 also 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 $I_{C50}$ values for the different engineered proteins.

The results presented here confirm the potential of recombinant epitope-displaying $\beta$-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 a 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 with the HIV-1/2 test. From 32 positive sera, only two appear as negative sera under our assay conditions, but this result can be explained by the absence of anti P1 antibodies in these samples. On the other hand, 1 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 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 proper activity enhancement in the modulation assay through activating antibodies. However, this effect has not been observed in any of the $\beta$-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 $\beta$-galactosidase inactivating antibodies in HIV-immune sera cannot be excluded completely, 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). Because 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 $\beta$-galactosidase biosensor for the detection of antibodies to HIV-2 in blood could be approached in the same way as described here for HIV-1 to complement the assay. In summary, we propose the epitope-displaying $\beta$-galactosidase model as a biosensor with potential generic applications in fast and homogenous assays, which could be adopted in multiple intermolecular detection systems using easy protein engineering procedures.

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REFERENCES

1. Doi, N. & Yanagawa, H. (1999) FEBS Lett. 457, 1–4
2. Jacob, F. & Monod, J. (1961) J. Mol. Biol. 3, 318–356
3. Jacobson, R. H., Zhang, X. J., DuBose, R. F. & Matthews, B. W. (1994) Nature 369, 761–766
4. Benito, A., Feliu, J. X. & Villaverde, A. (1996) J. Biol. Chem. 271, 21251–21256
5. Feliu, J. X. & Villaverde, A. (1998) FEBS Lett. 434, 23–27
6. Benito, A. & Villaverde, A. (1994) FEBS MicrobioL Lett. 127, 103–112
7. Feliu, J. X., Ramirez, E. & Villaverde, A. (1998) FEBS Lett. 438, 267–271
8. Feliu, J. X., Ramirez, E. & Villaverde, A. (2000) FEBS Lett. 473, 123
9. Sanchez-Pescador, R., Power, M. D., Barr, P. J., Steiner, K. S., Steimpen, M. M., Brown-Shimer, S. L., Gee, W. W., Renard, A., Randolph, A. & Levy, J. A. (1985) Science 227, 484–492
10. Ratner, L., Haseltine, W., Patarca, R., Laval, K. L. J., Starich, B., Josephs, S. F. P., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Pettey Jr., S. R., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Girayeh, J., Chang, N. T., Gallo, R. C. & Wong-Staal, F. (1985) Nature 313, 277–284
11. Barin, F., McLane, M. F., Allan, J. S., Lee, T. H., Groupman, J. E. & Essex, M. (1985) Science 228, 1094–1096
12. Chanh, T. C., Dreesman, G. R., Kanda, P., Linette, G. P., Sparrow, J. T., Ho, D. D. & Kennedy, R. C. (1986) EMBO J. 5, 3065–3071
13. Kennedy, R. C., Henkel, R. D., Pauletti, D., Allan, J. S., Lee, T. H., Essex, M. & Dressman, G. R. (1986) Science 231, 1556–1559
14. Allan, J. S., Coligan, J. E., Lee, T. H., McLane, M. F., Kanki, P. J., Groupman, J. E. & Essex, M. (1985) Science 230, 810–815
15. Wang-Staal, F. & Gallo, R. C. (1985) Nature 317, 395–403
16. Wang, J. J., Steel, S., Wainsworth, R. & Wang, C. Y. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6159–6163
17. Stigler, R. D., Ruder, F., Katinger, D., Elliott, G., Hohne, W., Henklein, P., Ho, J. X., Keeling, K., Carter, D. C. & Nugel, E. (1995) Protein Eng. 8, 471–479
18. Gnann, J. W., Jr., Schwimmbeck, P. L., Nelson, J. A., Truax, A. B. & Oldstone, M. B. (1987) J. Infect. Dis. 156, 261–267
19. Bessette, P. H., Aslund, F., Beckwith, J. & Georgiou, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13703–13708
20. Benito, A., Vidal, M. & Villaverde, A. (1993) J. Biotechnol. 29, 299–306
21. Ullmann, A. (1984) Gene 29, 27–31
22. Seeger, A., Schnepp, B., McCarthy, J. E. G., Deckwer, W. D. & Rinas, U. (1995) Enzyme Microb. Technol. 17, 947–953
23. Benito, A., Valero, F., Lafuente, J., Vidal, M., Cairo, J., Sula, C. & Villaverde, A. (1993) Enzyme Microb. Technol. 15, 66–71
24. Salt, A. & Bundell, T. L. (1993) J. Mol. Biol. 234, 779–815
25. Kitman, M. J., Kocher, J.-P. A. & Wodak, S. J. (1992) Biochemistry 31, 10226–10238
26. Kelley, L. A., Gardner, S. P. & Sutcliffe, M. J. (1996) Protein Eng. 9, 1063–1065
27. Laskowski, R. A., McArthur, M. W., Moss, D. S. & Thornton, J. M. (1993) J. Appl. Crystalogr. 26, 283–291
28. Alard, P. (1990) Calculs de Surfaces et d’Énergie dans le Domaine des Macromolécules. PhD thesis, Université Libre de Bruxelles, Brussels
29. Delhaise, P., Van Belle, D., Bardiaux, M., Alard, P., Hamers, P., Van Cutsem, E. & Wodak, S. J. (1985) J. Mol. Graph. 3, 116–119
30. Martinez-Bilbao, M. & Huber, R. E. (1994) Biochem. Cell Biol. 72, 313–319
31. Jaquard, D. H., Huber, R. E. & Matthews, B. W. (1999) Protein Sci. 8, 122–136
32. Roth, N. J. & Huber, R. E. (1994) Biochem. Biophys. Res. Commun. 201, 866–870
33. Cupples, C. G. & Miller, J. H. (1988) Genetics 120, 637–644
34. Ring, M. & Huber, B. E. (1990) Arch Biochem. Biophys. 283, 342–350
35. Roth, N. J. & Huber, B. E. (1996) J. Biol. Chem. 271, 14296–14301
36. Legendre, D., Soumillon, P. & Fastrez, J. (1999) Nat. Biotechnol. 17, 67–72
37. Ferrer-Miralles, N., Feliu, J. X. & Villaverde, A. (2000) Biochem. Biophys. Res. Commun. 275, 360–364
38. Hughes, A. & Corrah, T. (1990) Blood Rev. 4, 158–164
39. Kahina, A., Otto, K., Ruther, U. & Muller-Hill, B. (1983) EMBO J. 2, 593–597