Contribution of Arginine Residues in the RP135 Peptide Derived from the V3 Loop of gp120 to Its Interaction with the Fv Fragment of the 0.5β HIV-1 Neutralizing Antibody*

(Received for publication, January 19, 1996, and in revised form, March 8, 1996)

Gabriel A. Faiman, Rina Levy, Jacob Anglister, and Amnon Horovitz†
From the Department of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel

The construction, expression, and purification of an active Fv fragment of the 0.5β monoclonal human immunodeficiency virus type 1 (HIV-1) neutralizing antibody is reported. The interaction between the Fv fragment and the RP135 peptide derived from the V3 loop of gp120 from HIV-1MN was studied by varying the salt concentration and by mutating arginine residues in the peptide. The mutations R4A, R8A and R11A (which correspond to residues 311, 315, and 318 in gp120 of HIV-1MN) reduce the binding free energy by 0.22 (± 0.20), 4.32 (± 0.16), and 1.58 (± 0.17) kcal mol⁻¹, respectively. The salt-dependent components of their contributions to binding are 0.02 (± 0.22), −6.55 (± 0.18), and −6.97 (± 0.19) kcal mol⁻¹, respectively. The magnitudes of the mutational effects and the extent of shielding by 1 M NaCl suggest that Arg-8 is involved in a buried salt bridge in the peptide-Fv fragment complex, whereas Arg-11 is involved in a more solvent-exposed electrostatic interaction.

The 0.5β monoclonal antibody was raised (1) against the envelope glycoprotein gp120 (from the strain HIV-1MN) which is found on the surface of human immunodeficiency virus type 1 (HIV-1) and HIV-1-infected cells. Infection of healthy T-cells is facilitated by the binding of gp120 to the CD4 protein which is present on the surface of helper T-cells. A chimeric monoclonal antibody which contains the variable region of the 0.5β antibody and human constant regions was found to protect chimpanzees from HIV-1 infection after passive immunization (2). The 0.5β antibody binds to a sequential epitope of gp120 which corresponds to its principal neutralizing determinant. This determinant is within a disulfide-bridged loop in the third hypervariable region (V3) of gp120 (3, 4). A 24-amino acid-long peptide, NNTRKRISIQGRPGAVFTIGKIG, derived from the principal neutralizing determinant of gp120 of HIV-1MN and designated RP135, is immunogenic by itself and was shown to correspond to the binding site of gp120 to the 0.5β antibody (4).

Nuclear magnetic resonance (NMR) studies on the interaction of the RP135 peptide with the Fab fragment of the 0.5β antibody have defined a 16-residue epitope from Lys-5 to Ile-20 (5). The recently solved crystal structure of the Fab fragment of a different HIV-1 neutralizing antibody, 50.1, in complex with a 16-residue peptide derived from the V3 loop of gp120 (HIV-1MN strain) shows that it interacts only with a sequential 7-residue epitope (6). The interaction between the Fab fragment of yet another HIV-1 neutralizing antibody, 59.1, with a 24-residue peptide is similar to that of the 50.1 Fab fragment with respect to the conformation of overlapping residues in the two peptides and the size of the epitope (7).

Recent advances in antibody technology (see, for review, Ref. 8) have made possible direct cloning of antibody genes from hybridomas or lymphocytes into plasmid vectors and their expression in bacteria (see, for review, Ref. 9). In particular, there has been much recent interest in smaller antibody fragments that still retain antigen binding activity. These include Fv fragments (10, 11), single-chain Fv fragments (12, 13) and Fab fragments (14). Owing to the relatively small size of Fv and single-chain Fv fragments, their structures can be determined by multidimensional NMR techniques (15), and they are expected to have improved pharmacokinetic properties (8). A single-chain Fv of the anti-HIV-1 gp120 antibody, F105, has been constructed previously (16). Here, we report the construction, expression, and purification of an active Fv fragment of the 0.5β monoclonal HIV-1 neutralizing antibody (1, 17). An accurate assay for measuring the binding of the RP135 peptide to the 0.5β Fv fragment was established and used to determine the contributions to binding of arginine residues in the peptide and their salt dependence. We have focused on arginine residues in the peptide since amino acid sequence information and model building (5) suggested that electrostatic interactions are of special importance in this system. Measurement of binding constants in the presence of high salt, which masks electrostatic interactions, facilitates partitioning of the binding energy into ionic and nonionic components. Our long-term goal is to analyze in detail the energetics of this interaction by kinetic and protein engineering methods and to determine the solution and crystal structures of the 0.5β Fv fragment-RP135 peptide complex.

**EXPERIMENTAL PROCEDURES**

Materials—Molecular biology reagents were from New England Biolabs or Promega unless stated otherwise. All Fmoc amino acid derivatives used for peptide synthesis were purchased from NovaBiochem, Switzerland. Molecular weight prestained markers were from Bio-Rad. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was obtained from Chembridge Corp. All other analytical grade reagents were purchased from Sigma.

Cloning of Amplified DNA and Construction of the 0.5β Fv Fragment Expression Vector—The 54CB1 hybridoma cell line producing the 0.5β monoclonal antibody was provided by Dr. S. Matsushita (Kumamoto University). Total RNA of 54CB1 cells was purified using an Amer-
interaction between 0.5β Fv fragment and the RP135 peptide

sham kit. The design of the PCR primers and the PCR amplification reactions were as before (18). The amplified PCR products were digested using PstI and BstEII for the V<sub>c</sub> gene and SacI and BglII for the V<sub>h</sub> gene. The digested PCR products were then subcloned into a plasmid expression vector that previously contained the genes of the VH and V<sub>k</sub> domains of the D1.3 anti-lysozyme antibody, each fused to the pEB signal sequence. In this plasmid, generously donated to us by Dr. G. Winter (Medical Research Council, Cambridge), the V<sub>k</sub> domain is fused at its C terminus to the myc tag peptide. The D1.3 pUC19 vector was digested with PstI and BstEII, purified to remove the DNA coding for the V<sub>c</sub> domain of the D1.3 antibody, and then religated with the PCR product of the 0.5β antibody V<sub>c</sub> gene. The product of this ligation reaction was digested with SacI and BglII, purified, and used for the DNA coding for the D1.3 V<sub>k</sub> domain, and then religated with the PCR product of the 0.5β antibody V<sub>c</sub> gene. A 0.9-kb EcoRI-HindIII restriction fragment of this plasmid was then subcloned into the pT219U vector (19) previously digested with the same enzymes. This vector is designated pTβ. The pT219U vector contains an <i>E. coli</i> origin of replication that allows production of single-stranded DNA upon infection with helper phage.

Site-directed Mutagenesis—Single-stranded DNA of the plasmid pTβ, harbored in the <i>E. coli</i> strain TG2, was obtained by infecting these cells with helper-phage M13K07 (Pharmacia Biotech Inc.). Site-directed mutagenesis was carried out using the method of Eckstein (20) and the Amersham kit. The following oligonucleotides were used to correct mutations owing to the PCR oligonucleotides (18) and to create an additional mutation Ser<sup>25</sup>-&gt; Phe that occurred for reasons not known. Glu<sup>-6</sup>(H) for Glu<sup>-6</sup>(B) using the oligonucleotide: 5'-CGGGGATCCGTAATATCTCCTT-3', Thr<sup>-114</sup>(H) for Thr<sup>-114</sup>(B) using the oligonucleotide: 5'-CTTATGCGACCGAATTCTTT-3', Phe<sup>-25</sup>(H) for Ser<sup>-25</sup>(B) using the oligonucleotide: 5'-CTTTGAA CCGAATCGTCC-3', Glu<sup>-3</sup>(k) for Gly<sup>-3</sup>(k) using the oligonucleotide: 5'-GGGT-CACGCGAGTTGAA-3', where H and k indicate mutations in the heavy and light chains, respectively. An asterisk follows the mismatched bases. The myc tag peptide was removed by replacing the sequence coding for the first two N-terminal residues of the tag with stop codons using the oligonucleotide: 5'-CGT GAGT GATAAGTGTG-3'. The vector with these five changes is designated pTβ11.

Fv Fragment Expression and Purification—A 5-ml starter culture of Escherichia coli TG2 cells harboring the pTβ11 plasmid was grown overnight at 37°C in 2 x T7 medium containing 50 μg/ml ampicillin and 0.1% glucose. The cells were grown until their density reached A<sub>600</sub> = 0.6, and then protein expression was induced by the addition of 1 ml IPTG. The cells were grown at 30°C, centrifuged (1000 rpm, 1 min), and the supernatant was collected (fraction B). The cell pellets were twice resuspended in 30 mM Tris-HCl, centrifuged (4°C, 2000 rpm), and pellets were resuspended in 30 mM Tris-HCl containing 0.05% Tween 20 and 1 M NaCl (when appropriate) before starting the expression. The titrations were carried out by adding a Hamilton syringe 2-μl samples of the peptide to the 0.5β Fv fragment of the antibody, with constant stirring in a thermostatted cuvette at 25.0 ± 0.1°C. Fluorescence measurements were made 4 min after adding the peptide sample.

Data Analysis—Determination of dissociation constants was achieved by directly fitting fluorescence measurements of the Fv fragment at different peptide concentrations, using Kaleidagraph (version 2.1 Synergy Software (PCS Inc.)), to the following equation for tight binding:

\[
F = F_0 + (F_s - F_0)[(F_v + [P]) + K - ([F_v][P] + [P]^2)]
\]

where \(F\) is the observed fluorescence, \(F_0\) is the fluorescence in the absence of peptide, \(F_s\) is the fluorescence in the presence of saturating concentrations of peptide, \([F_v]\) and \([P]\) are the total Fv and peptide concentrations, and \(K\) is the dissociation constant. Estimates (± S.E.) of the parameters \(F_s, [F_v],\) and \([P]\) were obtained from the fits which were carried out using a fixed value of \(F_0\). In the case of weak binding, Equation 1 is reduced to:

\[
F = F_0 + (F_s - F_0)[[P]/(1 + ([P]/K)]
\]

where \([P]\) is the concentration of free peptide and all other notations are as before. Determination of dissociation constants in the case of weak binding was achieved by directly fitting fluorescence measurements at different peptide concentrations to Equation 1 using a fixed value for \([F_v]\). Identical estimates were obtained by fitting the data to Equation 2. Standard free energies of binding were calculated from dissociation constants, as follows:

\[
\Delta G = RT \ln K
\]

where \(R\) is the gas constant and \(T\) is the absolute temperature. The coupling energies between the effects on binding of the mutations and the addition of salt were calculated, as follows:

\[
\Delta G_{\text{mut}} = \Delta G_{\text{wt}, 0 \text{ M NaCl}} - \Delta G_{\text{wt}, 1 \text{ M NaCl}} - \Delta G_{\text{mut, 0 \text{ M NaCl}}}
\]

The free energies on the right-hand side of Equation 4 are for binding of wild-type or mutant peptide to the 0.5β Fv fragment in the presence of 0 M or 1 M NaCl, as indicated.

RESULTS

Purification of the 0.5β Fv Fragment—In our pTβ11 plasmid construct, both the heavy and light chains of the 0.5β Fv fragment are fused to the pEB leader sequence and are, therefore, sequestered into the periplasmic space. The 0.5β Fv was purified from the periplasmic space by osmotic shock. It may be seen in Fig. 1 that most of the purified 0.5β Fv was released from the cells upon addition of the sucrose buffer and before the
The 0.5β Fv fragment was purified to homogeneity by affinity chromatography using a Sepharose 4B column to which a gp120-derived peptide antigen had been cross-linked. The final yield of the purified 0.5β Fv fragment is typically about 1 mg/liter using the expression and purification procedure described here. Correct processing and purification were confirmed by gel electrophoresis and amino acid analysis. The purified Fv fragment is stable as judged by two different assays. A linear relationship was observed between the activity of the Fv fragment and its concentration indicating that dissociation of the heavy and light chains is minimal (data not shown). In addition, repeated freeze and thaw cycles of the Fv fragment did not cause denaturation or dissociation as judged by nondenaturing gel electrophoresis (not shown).

Fluorescence Emission Spectra of the 0.5β Fv Fragment and Its Complex with the Peptide Antigen—Fluorescence emission spectra of the 0.5β Fv fragment were measured in the absence and in the presence of an excess amount of the RP135 peptide antigen (Fig. 2). As is evident from the spectra in Fig. 2, in the presence of excess antigen there is a blue-shift in λ \text{max} from 337 nm to 333 nm, and there is an enhancement in the fluorescence intensity at wavelengths below 349 nm and quenching above this wavelength. These changes in fluorescence were exploited in order to establish a binding assay for the peptide antigen to the Fv fragment, as described under "Experimental Procedures." Previous model building (5) showed that the framework residue Trp-47(H) is part of the potential antigen-binding site. The observed changes in fluorescence may be due to this antibody residue which may be in contact with the peptide.

Effects of Mutations in the Peptide on Binding—Arginine residues at positions 4, 8, and 11 in the RP135 peptide, which correspond to positions 2, 6, and 9 in the peptides we synthesized and to positions 311, 315, and 318 in gp120, were replaced by alanine. The dissociation constants for the interaction of these peptides with the 0.5β Fv fragment were determined by fluorescence enhancement titration as shown in Fig. 3. Free energies of binding were calculated from the measured dissociation constants using Equation 3 (Table I). In the absence of salt, the dissociation constant for the interaction between wild-type peptide and the Fv fragment is about 2 nM. Skinner et al. (23) reported dissociation constants of 5 nM and 14 nM at 20 °C for the interaction of the full 0.5β antibody with gp120 and the RP135 peptide, respectively. The free energy of binding of the wild-type peptide to the Fv fragment is, in the absence of salt, −11.91 (± 0.16) kcal mol\(^{-1}\). The mutations R4A, R8A, and R11A reduce the binding free energy by 0.22 (± 0.20), 4.32 (± 0.16), and 1.58 (± 0.17) kcal mol\(^{-1}\), respectively.

Effect of Salt on Binding—Free energies of binding of the wild-type and mutant peptides to the 0.5β Fv fragment were determined also in the presence of 1 M NaCl (Table I). The changes, upon addition of 1 M NaCl, in the free energies of binding of wild-type peptide and the R4A, R8A, and R11A mutants are 1.76 (± 0.17), 1.78 (± 0.13), 1.21 (± 0.03), and 0.79 (± 0.07) kcal mol\(^{-1}\), respectively. In the presence of 1 M NaCl, the mutations R4A, R8A, and R11A reduce the binding energy to the 0.5β Fv fragment by 0.24 (± 0.09), 3.77 (± 0.08), and 0.61 (± 0.09) kcal mol\(^{-1}\), respectively.

**DISCUSSION**

Amino acid sequence information had suggested that electrostatic interactions are of special importance in the binding of the RP135 peptide antigen to the 0.5β Fv antibody fragment. The 20-mer peptide contains 6 positively charged residues (4 arginines and 2 lysines) and no negatively charged residues whereas the antibody's complementarity determining regions contain many negatively charged residues and only a few positively charged residues. In addition, model building (5) showed that a shallow concave groove is formed by the 6 complementarity determining regions of the antibody and by two frame-
work residues (Tyr-49(H) and Trp-47(H)), and that this potential antigen-binding site contains many negatively charged side chains. Electrostatic interactions were previously demonstrated to be important in formation of other antibody-antigen complexes (24).

In order to determine the contribution of electrostatic interactions, we established a quantitative and highly accurate binding assay for the interaction of the RP135 peptide with the 0.5β Fv fragment. We then analyzed the effects of mutations of arginine residues in the peptide on its interaction with the 0.5β Fv fragment both in the absence and in the presence of 1 M NaCl (Table I). All arginine residues in the peptide were replaced except Arg-15 which was not, owing to its role in stabilizing the peptide conformation (7, 25). The free energies of binding of the wild-type and all of the mutant RP135 peptides to the 0.5β Fv fragment are found to decrease in the presence of 1 M NaCl demonstrating the importance of multiple electrostatic interactions in this system.

The mutations R8A and R11A, but not R4A, are found to have large effects on binding of the RP135 peptide to the 0.5β Fv fragment. Our precise measurements are in agreement with the more qualitative findings of Okada et al. (26) that mutation of the corresponding arginine residues in gp120 affect its binding to the 0.5β monoclonal antibody. Okada et al. (26) also showed that mutation of these arginine residues affects virus infectivity and syncytium-inducing ability. Our results are also consistent with a previous epitope mapping study by NMR (5) which showed that the antigenic determinant recognized by the Fab fragment of the 0.5β antibody consists of 16 residues (Lys-5 to Ile-20). Interestingly, in most HIV isolates there is a deletion in the V3 loop of two residues corresponding to Arg-8 and Gin-7 in RP135 (27) which may explain why the 0.5β antibody is strain-specific.

The salt-dependent components of the contributions to the binding energy of the arginine residues mutated in this study were isolated by invoking thermodynamic cycles shown in Fig. 4. By analogy to double-mutant cycles (28), the cycles in Fig. 4 consist of two different steps: (i) a mutation and (ii) transfer from 0 M NaCl to 1 M NaCl. The coupling free energies for such cycles are calculated using Equation 4, and they reflect to what extent the effect of the mutation is salt-dependent. If the coupling energy is zero, then the effect of the mutation is salt-independent. If the coupling energy is different from zero, then the effect of the mutation is salt-dependent. Surprisingly, it may be seen from Fig. 4 that there is no correlation between the magnitude of the salt-dependent contribution to the binding energy of a given residue and its apparent overall contribution.

### Table I

| Peptide       | ΔG₁   | ΔG₂   | ΔGἀave⁺ |
|---------------|-------|-------|---------|
| Wild-type (0 M salt) | -11.91 (±0.24) | -11.90 (±0.20) | -11.91 (±0.16) |
| R4A (0 M salt) | -11.76 (±0.15) | -11.62 (±0.19) | -11.69 (±0.12) |
| R8A (0 M salt) | -7.51 (±0.02)  | -7.66 (±0.02)  | -7.59 (±0.01)  |
| R11A (0 M salt) | -10.39 (±0.08) | -10.27 (±0.07) | -10.33 (±0.05) |
| Wild-type (1 M salt) | -10.23 (±0.09) | -10.07 (±0.10) | -10.15 (±0.07) |
| R4A (1 M salt) | -9.95 (±0.09)  | -9.87 (±0.07)  | -9.91 (±0.06)  |
| R8A (1 M salt) | -6.51 (±0.05)  | -6.24 (±0.03)  | -6.38 (±0.03)  |
| R11A (1 M salt) | -9.40 (±0.06)  | -9.67 (±0.08)  | -9.54 (±0.05)  |

*ΔGἀave⁺ = 1/2(ΔG₁ + ΔG₂).*

**Fig. 4. Thermodynamic cycles showing the coupling between effects of high salt and mutations in the peptide antigen on its binding to the 0.5β Fv fragment.** The Fv fragment and the wild-type peptide antigen are designated by Ab and Ag (wt), respectively. Single-letter notation for amino acids is used. Free energy values are given in kcal mol⁻¹ (Table I). The coupling free energies, ΔGint, were calculated using Equation 4.
For example, the apparent contribution ($\Delta G_{\text{app}}$) of Arg-8 to the binding energy is $-4.32 \pm 0.16$ kcal mol$^{-1}$ whereas the salt-dependent component of its contribution is only $-0.55 \pm 0.18$ kcal mol$^{-1}$. In contrast, the apparent contribution of Arg-11 to the binding energy is $-1.58 \pm 0.17$ kcal mol$^{-1}$ and the salt-dependent component of its contribution is $-0.97 \pm 0.19$ kcal mol$^{-1}$. We can define $\phi$ as the ratio between the salt-dependent contribution, $\Delta G_{\text{salt}}$, and the apparent total contribution ($\phi = \Delta G_{\text{salt}}/\Delta G_{\text{app}}$). The $\phi$ values for Arg-4, Arg-8, and Arg-11 are $-0.09 \pm 0.99$, $0.13 \pm 0.04$, and $0.61 \pm 0.14$, respectively. There is uncertainty regarding the $\phi$ value for Arg-4 but it is clear that the salt-dependent contribution is more dominant in the case of Arg-11 compared with Arg-8. In principle, two reasons may account for this difference: (i) incomplete shielding by salt, i.e. Arg-11 is more solvent-exposed than Arg-8 in the Fv fragment-peptide complex or (ii) Arg-8 is involved in nonelectrostatic interactions. Further structural and energetic studies now in progress are required in order to distinguish between these possibilities.

The strength of exposed and buried salt bridges has been determined in other systems. In T4 lysozyme, a buried salt bridge was found to contribute 3-5 kcal mol$^{-1}$ to protein stability (29) whereas solvent-exposed salt bridges in barnase were found to contribute only about 1 kcal mol$^{-1}$ to its stability in the absence of high salt which had a strong masking effect on them (30). Here, both the magnitude of the mutational effect ($\Delta G_{\text{app}} = -4.32 \pm 0.16$ kcal mol$^{-1}$) and the extent of shielding by 1 M NaCl ($\phi = 0.13 \pm 0.04$) suggest that Arg-8 is involved in a buried salt bridge. The magnitude of the mutational effect in the case of Arg-11 ($\Delta G_{\text{app}} = -1.58 \pm 0.17$ kcal mol$^{-1}$) and the extent of masking by salt ($\phi = 0.61 \pm 0.14$) suggest that this residue is probably involved in a more solvent-exposed electrostatic interaction.

Acknowledgments—We thank Dr. S. Matsushita for the 54/C81 hybridoma cell line producing the 0.5$\beta$ monoclonal antibody. We also thank S. Rubinarburt and Prof. M. Fridkin for peptide synthesis, Dr. M. Peretz and Prof. Y. Burstein for help with peptide purification, and Dr. S. Cabilly for advice at the early stages of this project.

REFERENCES
1. Matsushita, S., Robert-Guroff, M., Rusche, J., Kato, A., Hattori, T., Hoshino, H., Javaherian, K., Takatsu, K., and Putney, S. (1988) J. Virol. 62, 2107–2114
2. Emini, E. A., Schleif, W. A., Nunberg, J. H., Conley, A. J., Eda, Y., Tokiyoishi, S., Putney, S. D., Matsushita, S., Cobb, K. E., Jett, C. M., Eichberg, J. W., and Murthy, K. K. (1992) Nature 355, 728–730
3. Goudsmit, J., Debuck, C., Meloen, R. H., Smit, L., Bakker, M., Asher, D. M., Wolff, A. V., Gibbs, C. J., J., and Gajdusek, D. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4478–4482
4. Palter, T. J., Clark, M. E., Langlois, A. J., Matthews, T. J., Weinhold, K. J., Randall, R. R., Bolognesi, D. P., and Haynes, B. F. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1932–1936
5. Zvi, A., Kustanovich, I., Gefel, D., Levy, R., Eisenstein, M., Matsushita, S., Richael-Scerdesc, P., Regenmortel, M. H. V., and Anglister, J. (1995) Eur. J. Biochem. 229, 178–187
6. Rini, J. M., Stanfield, R. L., Stura, E. A., Salinas, P. A., Proty, A. T., and Wilson, I. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6325–6329
7. Ghiara, J. B., Stura, E. A., Stanfield, R. L., Proty, A. T., and Wilson, I. A. (1994) Science 264, 82–85
8. Winter, G., and Milstein, C. (1991) Nature 349, 293–299
9. Skerra, A. (1993) Curr. Opin. Immunol. 5, 256–262
10. Skerra, A., and Plückthun, A. (1988) Science 240, 1038–1041
11. Riechmann, L., Foote, J., and Winter, G. (1988) J. Mol. Biol. 203, 825–828
12. Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margalies, M. N., Ridge, R. J., Brucelletti, R. E., Haber, E., Cis, R., and Oppermann, H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5879–5883
13. Bird, R. E., Hardman, K. D., Jackson, J., W., Johnson, S., Kaufman, B. M., Lee, S.-M., Lee, T., Pope, S. H., Riordan, G. S., and Whittow, M. (1988) Science 242, 423–426
14. Better, M., Chang, C. P., Robinson, R. R., and Horwitz, A. H. (1988) Science 240, 1041–1043
15. Riechmann, L., Cavanagh, J., and M unans, S. (1991) FEBS Letters 287, 185–189
16. Marasco, W. A., Hasefitin, W. A., and Chen, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7889–7893
17. Matsushita, S., Maeda, H., Kimachi, K., Eda, Y., Maeda, Y., Murakami, T., Tokiyoishi, S., and Takatsu, K. (1992) AIDS Res. Hum. Retroviruses 8, 1107–1115
18. Orflandi, R., Güssow, D. H., Jones, P. T., and Winter, G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3833–3837
19. Mead, D. A., Szczesna-Skupura, E., and Kemper, B. (1986) Prot. Eng. 1, 67–74
20. Sayers, J. R., Schmidt, W., and Eckstein, F. (1988) Nucleic Acids Res. 16, 791–802
21. Gill, S. C., and von Himmel, P. H. (1989) Anal. Biochem. 182, 319–326
22. Snyder, S. L., and Sobocinski, P. Z. (1975) Anal. Biochem. 64, 284–288
23. Skinner, M. A., Ting, R., Langlois, A. J., Weinhold, K. J., Lyerly, H. K., Javaherian, K., and Matthews, T. J. (1988) AIDS Res. Hum. Retroviruses 4, 187–197
24. Novotny, J., and Sharp, K. (1992) Proc. Biol. Med. 58, 203–224
25. Zvi, A., Hiller, R., and Anglister, J. (1992) Biochemistry 31, 6972–6979
26. Okada, T., Patterson, B. K., Otto, P. A., and Gurney, M. E. (1994) AIDS Res. Hum. Retroviruses 10, 803–811
27. La Rosa, G. J., Davide, J. P., Weinhold, K., Waterbury, J. A., Proty, A. T., Lewis, J. A., Langlois, A. J., Dreesman, G. R., Boswell, R. N., Shadlock, P., Hidley, L. H., Karplus, M., Bolognesi, D. P., Matthews, T. J., Emini, E. A., and Putney, S. D. (1990) Science 249, 932–935
28. Horwitz, A., and Fersht, A. R. (1990) J. Mol. Biol. 214, 613–617
29. Anderson, D. E., Beddell, W. J., and Dahlquist, F. W. (1990) Biochemistry 29, 2403–2408
30. Horwitz, A., Serrano, L., Avron, B., Bycroft, M., and Fersht, A. R. (1990) J. Mol. Biol. 216, 1031–1044
31. Laemmli, U. K. (1970) Nature 227, 680–685