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# Three-dimensional structure and antigenicity of transmembrane-protein peptides of the human immunodeficiency virus type 1

## Effects of a neutralization-escape substitution

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A point mutation (Ala-589 to Thr) in the transmembrane protein of the human immunodeficiency virus type 1 (HIV-1) has been shown to decrease the sensitivity of the virus to the neutralizing effect of human HIV-1 specific antibodies [(1990) J. Virol. 64, 3240–3248]. Here 17-residue peptides with the parental and mutant sequences were compared. the parental peptide bound antibodies of sera from HIV-1 infected persons more frequently and with higher affinity than the mutant peptide. However, according to circular dichroism (CD), NMR spectroscopy and molecular modelling the peptides have indistinguishable backbone conformations under a variety of experimental conditions. These techniques showed for both peptides that no ordered helix was present in water solution. However, for both peptides in alcohol–water solutions approximately 60% α-helix could be induced. The three-dimensional structures of these peptides provide a basis for understanding how this mutation in the transmembrane protein may affect the interaction with both the outer envelope glycoprotein and with antibodies.

Antigenic peptide; 3D structure; Escape mutant; Human immunodeficiency virus type 1

## 1. INTRODUCTION

A decrease in sensitivity to the neutralizing effect of antibodies to the human immunodeficiency virus type 1 (HIV-1) in some human sera was previously found to be due to a mutation from alanine to threonine at position 589 in the viral transmembrane protein [1]. Garnier's algorithm [2,3] predicts this region to be α-helical, while the substitution to threonine lowers the probability of formation of an α-helix and increases the probability of formation of a β-structure. We investigated the antigenic and secondary-structural effects of the substitution on the synthetic peptide HIV-env 583–599 using nuclear magnetic resonance spectroscopy (NMR) and circular dichroism spectropolarimetry (CD). Interest in this peptide is further motivated by its immunosuppressive effects in vitro [4,5] and its recently reported binding to a cell-surface molecule of T cells [6].

Both NMR and CD provide conformational information on peptides and small proteins. The NMR data provide specific internuclear distance restraints in solution (i.e. 2D and 3D NOESY experiments), although the technique is limited to interproton distances that are less than 5 Å [7]. Structures of antigenic peptides in solution obtained using NMR have previously been reported [8,9]. Recently, a β-turn was demonstrated by NMR in a peptide with a sequence C-terminally contiguous to the one studied here [10].

In this paper we describe the antigenicity (reactivity with antibodies) of the HIV-env 583–599 peptide and its mutant (Ala-589 → Thr) counterpart. We applied NMR and CD, coupled with molecular modelling, to determine the structures of the two peptides under a variety of experimental conditions.

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*Abbreviations:* A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; G, glycine; I, isoleucine, K, lysine; L, leucine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine, W, tryptophan; Y, tyrosine; NOESY, nuclear Overhauser effect and exchange spectroscopy; NMR, nuclear magnetic resonance, CD, circular dichroism spectropolarimetry.

## 2. EXPERIMENTAL

### 2.1. *Synthetic peptides*

The peptides LQARILAVERYLKDQQL (HIV-env 583–599, the parental peptide) [11], LQARILTVERYLKDQQL (the mutant peptide), and as a control in the competition experiments DRPEGIEEEGGERDRDRS (HIV-env 735–752) [12] were prepared as previously described [11].


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2.2. *Sera*

Sera from 42 persons confirmed by Western blotting to have antibodies to HIV-1 were used. Sixteen HIV-1 negative sera from blood donors served as controls.

2.3. *Enzyme immuno-assays*

Microtitre plates (Immunoplate Maxisorp, Nunc. Roskilde, Denmark) were coated with peptide (10 μg/ml phosphate-buffered saline with sodium merthiolate, PBS-M) or mock-coated with only PBS-M, blocked and washed as previously described [11]. Human serum diluted 1/20, or titrated from 1/20 to 1/2,500, was incubated on the plates; bound antibodies were detected by peroxidase labelled rabbit antibodies to human IgG γ-chains (Dakopatts, Copenhagen, Denmark), and absorbance measurement after color development, as described [11]. The cut-off for positive reactivity in the titrations was an absorbance difference of 0.1.

In the competition assay [13] sera were diluted beyond regions of absorbance plateaus (from 1/20 to 1/2,500) and preincubated with 6–60 μmol/dm$^3$ peptide overnight at 4°C. The enzyme immunoassay was then carried out as described above.

To determine the coating efficiency, both peptides were radiolabelled with $^{125}$I by the lactoperoxidase method to activities of 0.5–$1.0 \times 10^7$ cpm/μg. The iodinated peptide was incubated in immunoplates overnight at 4°C. After five washes the wells were cut apart and their radioactivity measured in a gamma-counter. At the concentration used for coating in the enzyme immunoassay, i.e. 10 μg/ml, 3% of 583–599 and 6% of the mutant peptide had bound. Coincubation with the same amount of the respective unlabelled peptide reduced the binding of both labelled peptides by 60%. We conclude that the unlabelled mutant peptide coated at least as efficiently as unlabelled 583–599.

2.4. *Circular dichroism*

All CD spectra were recorded at room temperature with a J-600 CD spectropolarimeter (Jasco, Tokyo, Japan) with cylindrical cells and pathlengths of 1 and 0.02 cm. Peptide solutions were prepared in $H_2O$ over a range of pH values and in 40% trifluoroethanol (TFE) and 60% $H_2O$ near pH 3 at concentrations of 50–100 μmol · dm$^{-3}$. Experiments were repeated at least three times with varying concentrations. The CD spectra were fitted with the CONTIN fitting algorithm [14] to estimate the amount of secondary structure present. Both peptides were found to have indistinguishable secondary structures and to be largely α-helical in the TFE/$H_2O$ mixture over the pH range of 2.5–9.0. In 100% $H_2O$ they showed no evidence of secondary structure (i.e. they exist as either extended chains or random coils).

2.5. *Nuclear magnetic resonance*

Resonance assignments were achieved using HOHAHA/TOCSY [15] and ROESY (rotating frame NOESY) spectra. The mixing times for the HOHAHA/TOCSY spectra were 60 ms and 120 ms for the ROESY experiment. Distance restraints were obtained from NOESY spectra run at 53°C with a mixing time of 150 ms. The peptide samples were made up in the same solvents as for the CD experiments (but using $d_2$-TFE) at a concentration of 5 mmol · dm$^{-3}$. The presence of sequential NH-NH NOE cross peaks [17] from Ala-585 to Gln-597 in both peptides in TFE/$H_2O$ provided additional data to suggest that the peptides have significant and indistinguishable α-helical contents in the TFE/$H_2O$ mixture.

2.6. *Energy minimization*

Computations were carried out using the QUANTA/CHARMM program package (Polygen Corp., Waltham, MA). Structures were calculated for the peptides both in vacuum and immersed in a box of 500 simulated water molecules [18]. The final low energy structures were obtained by energy minimization calculations which included all of the available distance restraints from the NOESY data.

## 3. RESULTS

3.1. *Immunochemical*

The antibody reactivity of the parental HIV-env 583–599 and mutant '589T' peptides is shown in Fig. 1. Eleven out of 42 HIV-1-positive sera reacted strongly with the parental and, in all but one case, significantly less with the mutant peptide. The other 31 HIV-1-positive sera, which reacted weakly with the parental peptide, had even weaker reactions with the mutant peptide. The 16 HIV-negative sera gave no significant reactions. The 11 HIV-1-positive sera that were strongly reactive with the parental peptide also gave higher reactivity titers with that peptide (average log(dilution factor ± S.D.) = 2.3 ± 0.5) than with the mutant peptide (1.0 ± 0.3).

The results of the competition EIA with immobilized parental peptide and preincubation of eight sera with either parental or mutant peptide are given in Fig. 2. Preincubation with the parental peptide decreased the reactivity with the immobilized peptide more than preincubation with the mutant peptide. Three sera that showed some reactivity with the mutant peptide were similarly preincubated and subsequently tested against immobilized mutant peptide. Also in these cases, preincubation with the parental peptide decreased the reactivity more than preincubation with the mutant peptide. Preincubation with the control peptide, HIV-env 735–752, did not decrease the reactivity of the sera with either immobilized parental or mutant peptide (data not shown).

3.2. *Structural*

The circular dichroism spectra show that the parental

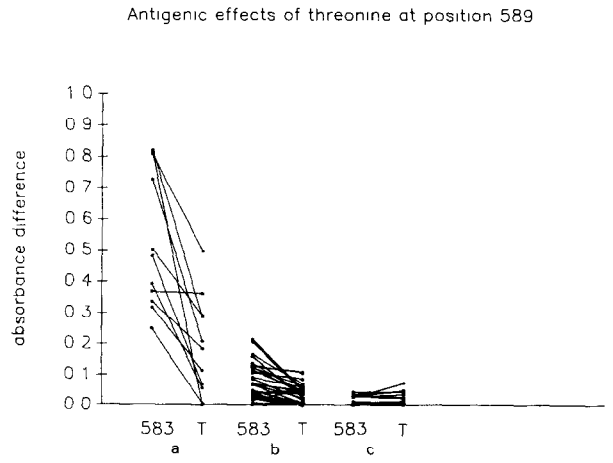


Fig. 1. The reactivity of human sera with the parental peptide HIV-env 583–599 and the 589 threonine mutant peptide (abbreviated 583 and T, respectively, on the abscissa). The normalized absorbance difference between peptide-coated wells and those without peptide are expressed on the ordinate: (a) 11 HIV-1-positive sera strongly reacted with HIV-env 583–599, (b) 31 HIV-1-positive sera with weak or no reactivity to HIV-env 583–599, and (c) 16 HIV-1-negative blood donor sera. The points for each serum have been connected.

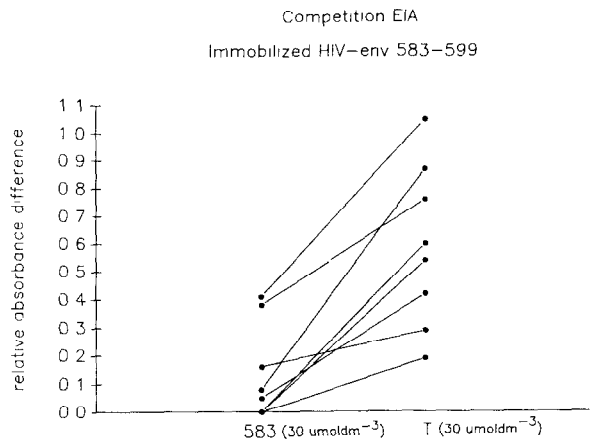


Fig. 2. Competition enzyme immuno-assay with immobilized HIV-env 583–599. The absorbance difference after preincubation of eight sera with peptides divided by their absorbance difference after preincubation without peptide are given on the ordinate. The preincubation peptides and their concentrations are given on the abscissa, where 583 = parental HIV-env 583–599 and T = threonine mutant peptide. The points for each serum have been connected

('589A') and mutant ('589T') peptides have indistinguishable backbone conformations in a TFE/$H_2O$ mixture (Fig. 3). Additional CD spectra were obtained under a variety of solvent conditions. Under any given set of experimental conditions the two peptides always showed indistinguishable conformational behavior.

For both peptides in TFE/$H_2O$ the 2D NOESY spectra (see Fig. 4) had intense sequential $NH_i$–$NH_{i+1}$ cross peaks for all residues except those at the ends of the peptides. Also, the sequential $C_\alpha H_i$–$NH_{i+1}$ cross peaks were quite weak. This cross-peak behavior is characteristic of peptides in α-helical conformations. Energy minimization computations were carried out using the standard value of 2.55 Å as sequential $NH_i$–$NH_{i+1}$ distance restraints. Fig. 5 shows a typical low-energy result obtained for the parental '589A' peptide. A similar result was obtained for the mutant '589T' peptide. The corresponding energies of these structures were similar. The methyl group of the '589A' sidechain is seen here to lie in the space between the side chains of '586R' and '593Y'. This space becomes more crowded when threonine is substituted at position 589.

Interestingly, we observed that the positively charged side chains of '595K' and '592R' are predicted to form salt bridges with the negatively charged side chains of '591E' and '596D', respectively, when the simulations are carried out under vacuum conditions. However, in the presence of the simulated water molecules the distances between those sidechain charges are over 7 Å, which would obviate salt-bridge formation between the ith and (i + 4)th residues [19].

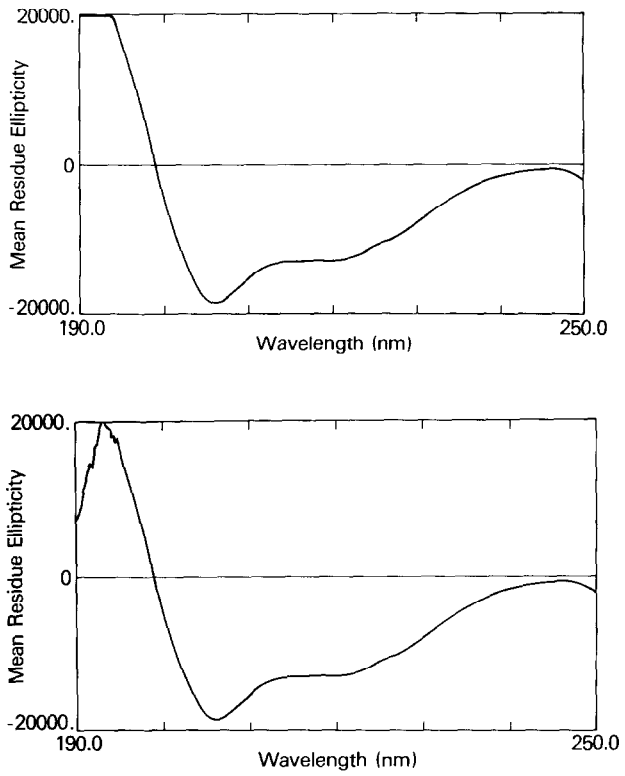


Fig 3 Circular dichroism spectra of (a) '583', LQARILAVERYLKDQQL at pH 2.9; and (b) 'T', LQARILTVERYLKDQQL at pH 2 8. The spectra were obtained at 21°C in a 40% TFE/60% $H_2O$ mixture.

## 4. DISCUSSION

By both CD spectropolarimetry and NMR spectroscopy we found that the parental and mutant HIV-env

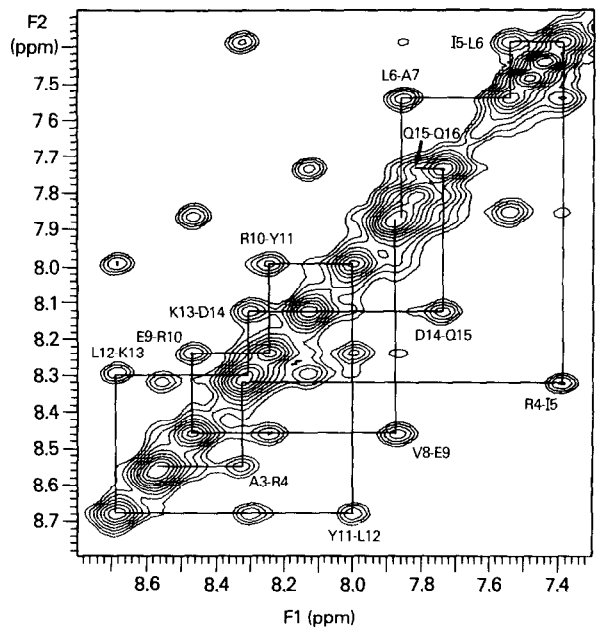


Fig. 4. Two-dimensional NOESY spectrum of amide protons of LQARILAVERYLKDQQL at pH 2.9 in a 40% TFE/50% $H_2O$/10% $^2H_2O$. The temperature was 5°C and the mixing time was 150 ms.

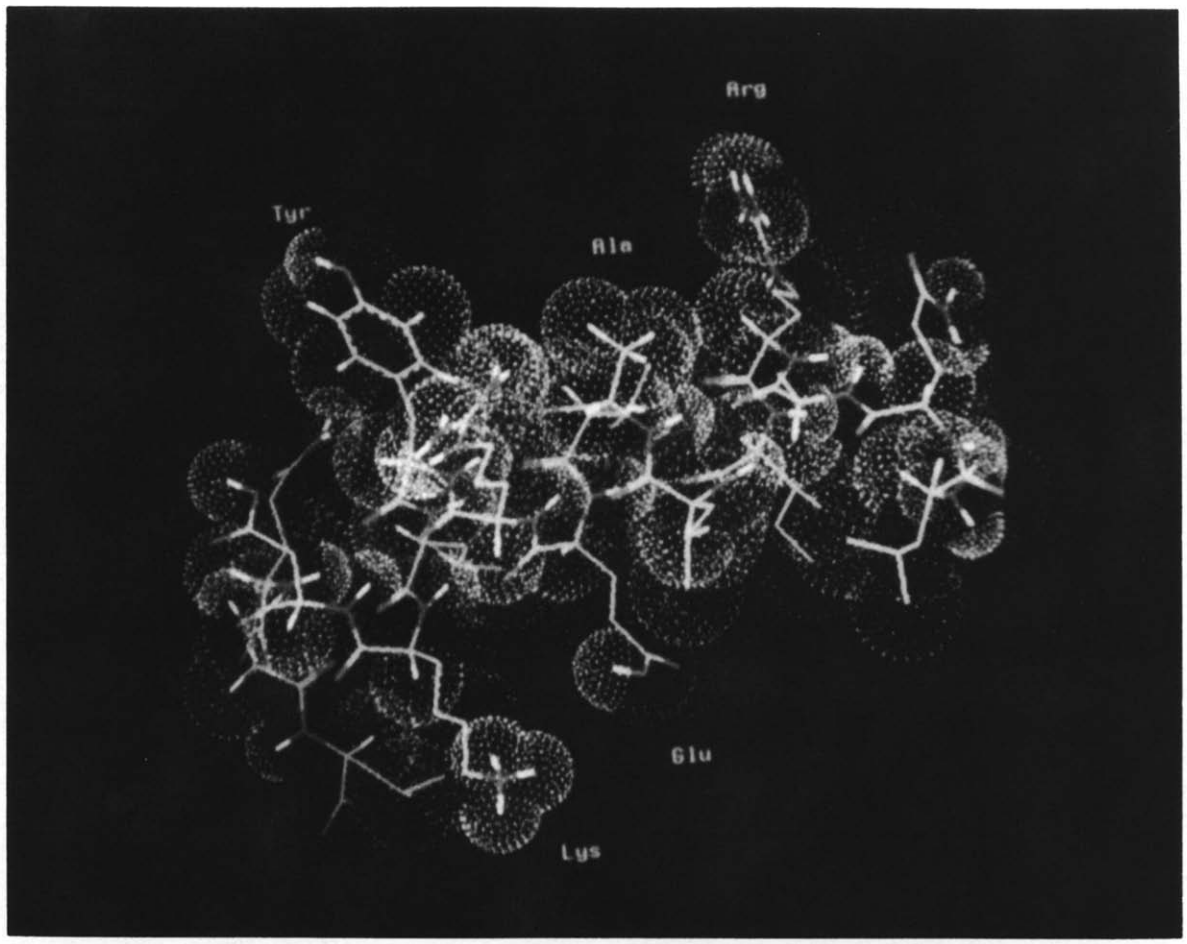


Fig. 5. Space filling model of LQARILAVERYLKDQQL obtained by energy minimization (see text). Several critical amino acid residues are labelled using the three letter abbreviation.

583–599 peptides have indistinguishable backbone conformations and that they form α-helices in a 40% TFE/60% $H_2O$ solvent mixture over a pH range from 2.5–9.0. The A to T mutation, in spite of markedly reducing the binding of human antibodies to the peptide, had no observable effect on the secondary structure of the peptide. Although the structure of the corresponding region in the entire native protein is, as yet, unknown, it can be argued that since the parental and mutant residues occur in identical contexts, similar secondary-structural tendencies of the peptides would correspond to secondary-structural similarities between the mutant and parental proteins in this region of gp41. Thus, the predicted change [2,3] to a β-structure in the mutant gp41 may not occur.

In contrast to HIV-env 583–599 the longer and antigenically stronger HIV-env 586–606 was largely disordered in alcohol/water solution as revealed by the CD spectra (Han et al., unpublished results), although it potentially could form the same salt bridges as are possible for HIV-env 583–599. It cannot yet be settled whether it is the lack of residues 583–585 or the presence of residues 600–606 that disturbs the order. We note, however, that this is a counter example to the suggested rule that strongly antigenic peptides are more ordered than less antigenic ones [8].

Antibodies that react with peptides of this region do not neutralize the virus [1], and thus the antigenic effect on the peptide of the alanine to threonine mutation may be coincidental to the decrease in neutralization. Nevertheless, the substitution may similarly abrogate the binding of hypothetically neutralizing antibodies to an overlapping composite epitope.

Whether the escape mutation in gp41 abrogates the binding of neutralizing antibodies to the virus or interferes with their action in some other manner cannot be answered yet. Even if the region forms α-helices in both parental and mutant forms of the proteins, as do the corresponding peptides, the mutation may still affect the conformation of the envelope glycoprotein complex: the larger side chain in the mutant may use space otherwise occupied by another region of the env-hetero-oligomer [20], e.g. in gp120. The affected neutralization epitope may be distant both in the primary structure and in three-dimensional space (see [21]).

It is noteworthy in this context that glycine and serine

(both less bulky than threonine) can be substituted for alanine at position 589, without rendering the virus resistant to neutralization by human sera [1]. The three-dimensional structure shows that a bulkier residue might increase the free energy of the bound state by filling out a groove on the protein surface. An alternative explanation would be that the free energy of the uncomplexed form is lower for the mutant than for the parental peptide. Either explanation could account for decreased binding of antibodies to the mutant peptide, to a hypothetical composite neutralization epitope, and of gp41 to the outer envelope glycoprotein gp120. This region of gp41 may contact gp120, as mutations both N- and C-terminal to it abrogate gp41–gp120 association [22]. A decreased association of the envelope glycoproteins would also provide a testable explanation of the neutralization-insensitive phenotype; free gp120 can decrease the neutralization titre of human sera [23].

Whether this escape mutation is a general phenomenon and elicits antibodies in vivo, was addressed here by looking for antibodies in 42 sera that would preferentially recognize the mutant peptide. Hypothetically, the mutant but not the wild-type would bind antibodies that could block the binding or action of neutralizing antibodies. We conclude that no such evidence was found; all detectable antibodies were shown to have a higher affinity for the parental than for the mutant peptide.

## REFERENCES


[1] Wilson, C., Reitz Jr., M.S., Aldrich, K., Klasse, P.J., Blomberg, J., Gallo, R.C. and Robert-Guroff, M. (1990) J. Virol. 64, 3240–3248.

[2] Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) J. Mol. Biol. 120, 97–120.

[3] Reitz Jr., M.S., Wilson, C., Naugle, C., Gallo, R.C. and Robert-Guroff, M. (1988) Cell 54, 57–63.

[4] Cianciolo, G.J., Bogerd, H. and Synderman, R. (1988) Immunol. Lett. 19, 7–14.

[5] Ruegg, C.K., Monell, C.R. and Strand, M. (1989) J. Virol. 63, 3257–3260.

[6] Qureshi, N.M., Coy, D.H., Garry, R.F. and Hendersson, L.A. (1990) AIDS 4, 553–558.

[7] Han, K.-H., Niu, C.-H., Roller, P.P. and Ferretti, J.A. (1988) Biopolymers 27, 923–937.

[8] Dyson, H.J., Cross, K.J., Houghten, R.A., Wilson, I.A., Wright, P.E. and Lerner, R.A. (1985) Nature 318, 480–483.

[9] Dyson, H.J., Rance, M., Houghten, R.A., Wright, P.E. and Lerner, R.A. (1987) in: Biological Organization: Macromolecular Interactions at High Resolution (Burnett, R.M. and Vogel, H.S. Eds.) pp. 227–234, Academic Press, New York.

[10] Oldstone, M.B.A., Tishon, A., Lewicki, H., Dyson, H.J., Feher, V.A., Assa-Munt, N. and Wright, P.E. (1991) J. Virol. 65, 1727–1734.

[11] Klasse, P.J., Pipkorn, R. and Blomberg, J. (1988) Proc. Natl. Acad. Sci. USA 85, 5225–5229.

[12] Kennedy, R.C., Henkel, D., Paoletti, D., Allan, J.S., Lee, T.H. and Essex, M. (1986) Science 231, 1556–1559.

[13] Klasse, P.J., Pipkorn, R. and Blomberg, J. (1991) Mol. Immunol. 28, 613–622.

[14] Provencher, S.W. and Glonecker, J. (1981) Biochemistry 20, 33–37.

[15] Bax, A. and Davis, D.G. (1985) J. Magn. Reson. 65, 355–360.

[16] Bothner-By, A.A., Stephens, R.L., Lee, J., Warren, C.D. and Jeanloz, R.W. (1984) J. Am. Chem. Soc. 106, 811–813.

[17] Billeter, M., Braun, W. and Wüthrich, K. (1982) J. Mol. Biol. 155, 321–346.

[18] Han, K.-H., Syi, J.-L., Brooks, B.R. and Ferretti, J.A. (1990) Proc. Natl. Acad. Sci. USA 87, 2818–2822.

[19] Marqusee, S. and Baldwin, R.L. (1987) Proc. Natl. Acad. Sci. USA 84, 8898–8902.

[20] Pinter, A., Honnen, W.J., Tilley, S.A., Bona, C., Zaghouani, H., Gorny, M.K. and Zolla-Pasner, S. (1989) J. Virol. 63, 2674–2679.

[21] Perry, N., Fox, G., Rowlands, D., Brown, F., Fry, E., Acharya, R., Logan, D. and Stuart, D. (1990) Nature 347, 569–572.

[22] Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Goh, W.C., Terwilliger, E., Dayton, A., Rosen, C., Haseltine, W. and Sodroski, J. (1987) Science 237, 1351–1355.

[23] McKeating, J.A., McKnight, A. and Moore, J.P. (1991) J. Virol. 65, 852–860.