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Biological and Conformational Studies on Analogues of a Synthetic Peptide Enhancing HIV-1 Infection

MONICA DETTIN[a,*], CLAUDIA SCARINCI[a], CARLO ZANOTTO[b], ROSSELLA RONCON[a], ANITA DE ROSSI[b] and CARLO DI BELLO[a]

[a] Department of Chemical Process Engineering, University of Padua, Padua, Italy
[b] Department of Oncological and Surgical Sciences, University of Padua, Padua, Italy

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Abstract: We have previously demonstrated that a 23-amino acid peptide derived from the V3 loop of the surface glycoprotein of the HIV-1 strain MN is able to bind CD4 and to enhance HIV-1 infection. Further studies have suggested that the peptide/CD4 interaction induces an increase in both CD4 expression and CD4/gp120 binding affinity. This paper describes the biological and physico-chemical characterization of three analogues of reduced sequence that have been designed in order to identify the minimum active sequence of this peptide corresponding to the MN-HIV-1 principal neutralizing domain. Biological studies indicate that the entire sequence is required for biological activity and that the sequence 1–18 presents an inhibitory activity. CD and FT-IR absorption data are discussed here in order to identify possible structure-function correlations. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: CD; FT-IR; gp120-CD4 interaction; HIV-1; structure-function studies; solid-phase peptide synthesis

INTRODUCTION

H1V-1 infection is triggered by the specific binding of its envelope glycoprotein gp120 to cellular CD4 antigen [1]. The amino acid sequences involved in this binding were localized in the V1 domain of CD4 [2] and in the V4 domain of gp120 [3]. In addition, it was demonstrated that the PND of HIV-1, which is involved in both the fusion and the syncytium formation processes [4,5], plays an important role in the definition of infectivity [6,7] and cell tropism [8,9]. An additional possible target for the PND seems to be the recently described cellular membrane receptor fusin, identified as an entry cofactor for T cell line-tropic HIV-1 isolates [10]. The PND (sequence 307–330) is contained within the disulphide loop in the third hypervariable region V3 of gp120 spanning from residue 303 to 338. While the central portion of the PND is highly kept in different HIV-1 isolates, the amino acids flanking this sequence are variable and antibodies elicited by peptides designed from the PND of different HIV-1 strains are mostly viral-variant specific [11]. We have previously demonstrated that a synthetic peptide corresponding to the PND of the HIV-1 MN strain binds to the CD4 molecule at the V1/V2 domain site identified by monoclonal antibodies MT151 and B66 [12] and enhances HIV-1 induced syncytium formation and infection in CD4+ target cells [13]. The peptide action is dose-dependent and not strain-restricted. We also observed that minor

Abbreviations: AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; DCM, dichloromethane; DIEA, diisopropylethylamine; DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide; Fab, fast atom bombardment; FT-IR, Fourier transformed infrared; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; NMP, *N*-methylpyrrolidone; PAM, 4-(hydroxymethyl)phenyl-acetic acid; PITC, phenyl isothiocyanate; PND, principal neutralizing domain; SDS, sodium dodecylsulfate; TEA, triethylamine; TFA; trifluoroacetic acid; TFE, 2,2,2trifluoroethanol.

* Correspondence to: Dipartimento di Processi Chimici dell'Ingegneria, Universitá di Padova, Via Marzolo, 9-35131 Padova, Italy. Tel.: +39 49 827 5553; fax: +39 49 827 5550; E-mail: peptide@uxl.unipd.it

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structural modifications could result in dramatic changes in the biological activity of the peptide: indeed, substitution of the C-terminal free carboxyl-function with an amide function led to a very significant reduction in biological activity and to the complete loss of CD4 binding capacity [14]. This paper describes the synthesis of the three reduced-sequence analogues that have been designed in order to identify the minimum active sequence of the peptide corresponding to the MN-HIV-1 PND. Since the tip of the V3 loop (Gly-Pro-Gly-Arg-Ala-Phe) is a highly conserved sequence in all viral isolates, we decided to synthesize three reduced-sequence analogues in which this motif is preserved. Biological studies, reported in this paper, indicated that the entire sequence is required to produce the enhancing effect on HIV-1 infection. Interestingly, repeated experiments with the peptide analogue DB3-R (sequence 1–18 of DB3; Table 1) showed a dose-independent inhibitory activity. The fact that deletion of the C-terminal pentapeptide and other major deletions produce analogues with different biological properties could be due to removal of the residues involved in the interaction between the peptide DB3 and the receptor and/or to alteration of the biological active conformation of the peptide. These two hypotheses have been evaluated by synthesizing several point-mutated analogues of DB3 bearing only a single change per sequence [14] and by comparing the conformational differences among the analogues under different experimental conditions, including CD studies both in aqueous and in micellar solutions. The results of CD and FT-IR absorption spectroscopic investigations are discussed in the light of the biological effects, reported in this paper, in order to verify the possible role played by conformational motifs in the secondary structure-function correlation.

MATERIALS AND METHODS

Materials

Protected amino acids and resins were obtained from Novabiochem (Langelfingen, Switzerland). The chemicals used for Boc chemistry peptide synthesis were obtained as follows: acetic anhydride, DIEA, 1 M *N*-hydroxybenzotriazole/NMP, 1 M dicyclohexylcarbodiimide/NMP from Applied Biosystems (Forster City, CA, USA); NMP from Fluka (Buchs, Switzerland); anisole, DMS, DMSO and DCM from Janssen (Geel, Belgium); HF from Union Carbide (Westerio, Belgium). After hydrolysis of peptide samples for 24 h at 110°C in sealed, evacuated vials in constant boiling HCl, amino acids analyses were carried out using a Waters Pico Tag™ work station (Milford, MA, USA) and a HPLC Waters associated system equipped with a programmer Model 660, two pumps Model 6000 and a detector Model 450. Reagents for amino acid derivatization were obtained as follows: PITC and amino acid standard solutions from Pierce (Rockford, IL, USA); TEA from Aldrich (Deisenhofen, Germany); methanol from Fluka (Buchs, Switzerland). The phenylthiocarbammoyl amino acids were separated using a Waters Pico Tag column (3.9 × 150 mm) by means of a binary eluant system. The two eluants used here were purchased from Waters. Peptide HPLC purification was carried out using a Waters 600E HPLC instrument equipped with a Waters 490E programmable multiwavelength detector, whereas peptide HPLC analytical characterizations were obtained using the HPLC system described above. The capillary electropherograms were carried out on an Applied Biosystems instrument Model 270 employing 20 mM sodium citrate buffer pH 2.5 from Applied Biosystems. RPMI medium, 10% fetal calf serum and L-Gln used in biological assays were purchased from Flow Laboratories (Irvine, UK). The 96-microwell plates were obtained from Falcon Microtest III, Becton Dickinson Labware (Lincoln Park, NJ) whereas the kit for p24 HIV-1 capsid antigen quantitative assay was obtained from DuPont de Nemours (Wilmington, DE).

Solid-Phase Peptide Synthesis

The synthesis of peptides was carried out with an automated Applied Biosystems Model 431A apparatus. N_{α}Boc-AA-PAM resin (0.5 mMol/g) was used as a starting solid support. The side-chain protections were: 2-bromo-benzyloxycarbonyl for Tyr; 4-chloro-benzyloxycarbonyl for Lys; benzyl for Thr; tosyl for Arg; and Boc for His. Boc protected amino acids were activated by using *N*,*N*′-dicyclohexylcarbodiimide and introduced as 1-oxy-1,2,3-benzotriazole esters. The coupling media were NMP for the initial 30 min and NMP/DMSO/DIEA for the last 6 min. Incorporation of each residue was followed by 'capping' in acetic anhydride. Double coupling was used throughout the synthesis of DB3-R and DB3-M peptides. Deblocking of side-chain protecting groups and cleavage from the solid support were achieved by means of a treatment with a 10:1:1 mixture HF:DMS: anisole for 60 min at −5°–0°C.

Table 1 Sequences of Synthetic Peptides

Code	Sequence																							
DB3	H-	Tyr	Asn	Lys	Arg	Lys	Arg	Ile	His	Ile	Gly	Pro	Gly	Arg	Ala	Phe	Tyr	Thr	Thr	Lys	Asn	Ile	Ile	Gly	-OH
DB3-R	H-	Tyr	Asn	Lys	Arg	Lys	Arg	Ile	His	Ile	Gly	Pro	Gly	Arg	Ala	Phe	Tyr	Thr	Thr	-OH					
DB3-M							H-	Ile	His	Ile	Gly	Pro	Gly	Arg	Ala	Phe	Tyr	Thr	Thr	-OH					
DB3-S										H-	Gly	Pro	Gly	Arg	Ala	Phe	-OH								

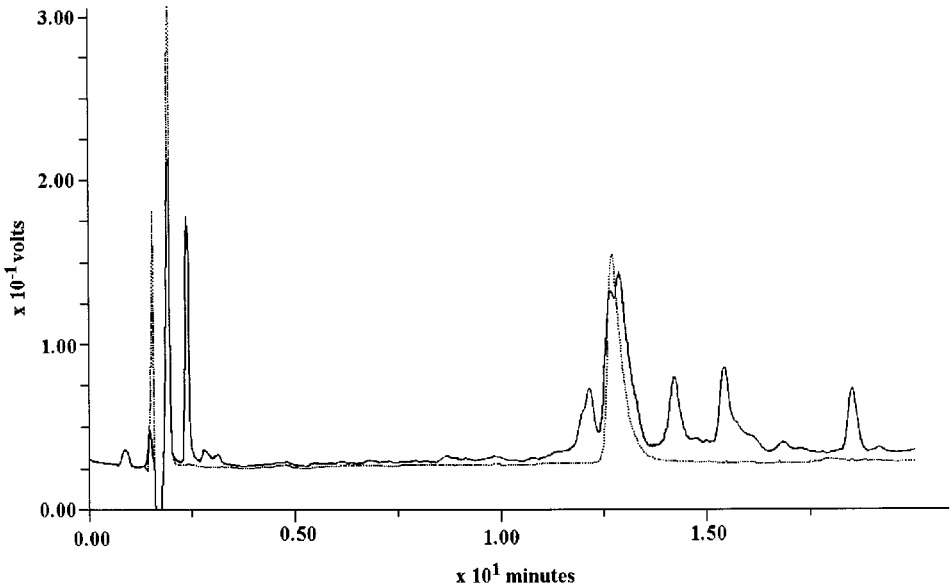

Figure 1 HPLC profiles of crude DB3-R (full line) and of purified DB3-R (dotted line).

HF was evaporated with N_2 and the solid residue was triturated with cold diethyl ether and then with a 30% acetic acid solution. Finally, the solution of the crude peptide was lyophilized.

Purification

The crude DB3-R peptide presented twin peaks with a very similar retention time (Figure 1, full line). An excellent separation of the two peaks was achieved by ion-exchange chromatography using a Water Protein Pak column (SP-5PW, 8 × 75 mm). The binary eluant system was: eluant A: 20 mM NaOAc, pH 5; eluant B: 20 mM NaOAc, 1 M NaCl, pH 5. The gradient was from 0% to 100% of eluant B over 60 min at a flow rate of 4 ml/min. Fractions were examined at 214 nm and those corresponding to the target peptide were collected and desalted. The purified peptide was obtained with a final yield of 4%. The yield is particularly low because of the high quantity of the secondary product that co-elutes with peptide DB3-R (Figure 1, dotted line). Sequencing and mass spectrometric analyses identified the second product with the deleted sequence H-Tyr-Asn-Lys-Arg-Lys-Arg-Ile-His-Ile-Gly-Pro-Gly-Ala-Phe-Tyr-Thr-OH. Crude DB3-M was loaded on a Protein Pak SP-5PW column using the conditions described above for DB3-R ion-exchange chromatography, but the gradient was from 0% to 40% of eluant B over 30 min. After HPLC analysis the best fractions were desalted and further purified on a Delta Pak C_{18} column (15 μm, 100 Å, 7.8 × 300 mm) using the following conditions: eluant A, 0.05% TFA in H_2O; eluant B, 0.05% TFA in CH_3CN; gradient, 18–28% of B over 20 min; flow rate, 4 ml/min; detector, 214 nm and 254 nm. The final yield in the purified peptide was 25%. Crude DB3-S was purified on a Delta Pak C_{18} column using the gradient from 5% to 25% of B over 40 min. The best fractions were collected and lyophilized, and gave a final yield of 39%.

Characterization

The purified DB3-R peptide was eluted on a Delta Pak C_{18} column (5 μm, 100 Å, 3.9 × 150 mm) and gave a single peak at 100% purity grade (Figure 1, dotted line). The chromatographic conditions were: eluant A, 0.05% TFA in H_2O; eluant B, 0.05% TFA in CH_3CN; gradient, 15–25% B over 20 min; flow rate, 1 ml/min; detector, 214 nm. The capillary electrophoretic analysis showed a single component under the following conditions: buffer, 20 mM sodium citrate pH 2.5; temperature, 30°C; voltage, 15 kV; capillary, 72 cm; detector, 214 nm. The amino acid analysis of the DB3-R peptide is the following (theoretical values in parentheses): Asp 0.9 (1); Gly 2.0 (2); His 0.9 (1); Arg 3.0 (3); Thr 2.1 (2); Ala 1.0 (1); Pro 1.0 (1); Tyr 2.1 (2); Ile 2.0 (2); Phe 0.9 (1); Lys 1.9 (2). The exact sequence of the peptide was determined by Edman degradation and its correct mass value was confirmed by Fab mass

spectrometry (experimental value, 2179; theoretical value, 2178).

The analytical HPLC of purified DB3-M gave a single peak at 98.9% purity grade under the following conditions: column, Vydac C_{18} (5 μm, 4.6×250 mm); gradient, 19–27% of B over 16 min. The electropherogram (voltage, 30 kV) gave a 98.4% purity grade. After HCl hydrolysis we obtained the following amino acid analysis: Gly 1.9 (2); His 0.8 (1); Arg 1.0 (1); Thr 2.2 (2); Ala 1.0 (1); Pro 1.1 (1); Tyr 1.0 (1); Ile 1.8 (2); Phe 0.9 (1). Edman degradation and electrospray spectrometry confirmed the identity of the molecule and its high purity grade (experimental value, 1331.98; theoretical value, 1332).

The 100% DB3-S purity grade was determined by HPLC on a Delta Pak C_{18} column using the following gradient, 7–17%B over 20 min. The capillary electrophoresis analysis (voltage, 25 kV) gave a single peak at 100% purity grade. The amino acid analysis gave the following results: Gly 1.9 (2); Arg 1.0 (1); Ala 0.9 (1); Pro 1.2 (1); Phe 1.0 (1). The Fab mass spectrometry gave the expected mass value (experimental value, 605; theoretical value, 603).

Biological Characterization

HIV-1 infection experiments were performed as previously described [13], using CD4+ cells from the MOLT-3 T lymphoid cell line and titred preparations of HIV-1 strain IIIB. Cells were maintained in RPMI medium supplemented with 10% fetal calf serum, 2% L-Gln and 50 μg/ml gentamycin, and cultured under standard conditions. Briefly, 1×10^5 cells were resuspended in 100 μl of medium containing serial two fold dilutions of peptides, and after 1 h infected with HIV-1 (0.01 cpm RT/cell); 24 h later, the plates were centrifuged and washed to remove residual p24 antigen and peptide, and a fresh medium was added. Four days after infection, supernatants were collected and quantitatively assayed for p24 HIV-1 capsid antigen using a commercially available kit. The peptide cytotoxicity was evaluated in parallel cultures, which underwent the same treatment, except for viral infection, by the Trypan blue exclusion assay.

Circular Dichroism

CD spectra were determined at room temperature using a Jasco Model J710 automatic recording circular dichrograph. Cylindrical fused quartz cells of 0.05 cm and 0.1 cm pathlength were used. Spectra are reported in units of mean residue ellipticity (peptide molecular weight/number of amide bonds), $[\Theta]_R$ ($\deg \times cm^2 \times dMol^{-1}$). Each weighed quantity of peptide was dissolved in the minimum amount of water to which 10 mM phosphate buffer pH 6.7, or a micellar solution (14 mM SDS in 10 mM phosphate buffer, pH 6.7), or 2 mM SDS solution, or TFE or CH_3CN was added to a final content of 99% (v/v). Peptide concentration, determined by amino acid analysis, ranged from 4.6×10^{-5} M to 2.36×10^{-4} M.

Fourier-Transform Infrared Absorption

The FT-IR absorption spectrum was measured at room temperature using a nitrogen flushed Jasco Model 300E infrared spectrometer at 2 cm^{-1} nominal resolution. A cell with a CaF_2 window and 0.1 mm pathlength was used. The solution of DB3-S was prepared by dissolving 1 mg of the peptide in 100 μl of D_2O. A spectrum of the solvent was recorded under identical conditions and subtracted from the spectrum of the solution. The curve fitting of amide I spectrum of DB3-S in D_2O (Figure 4) was achieved using a curve fitting program that was purchased from Jasco. The initial peak positions were determined by the second derivative of the experimental spectrum. Position, band width and band shape (Lorentzian or Gaussian) were varied until good agreements was achieved between experimental and simulated spectra. The component bands (Figure 4, solid lines) have been added together to produce a resultant spectrum (dashed line) that matches the observed spectrum closely.

RESULTS AND DISCUSSION

Synthetic DB3, whose sequence reproduces the PND of HIV-l-MN, is able to enhance viral infectivity in a dose-dependent manner and not in a strain-specific one. The search for the minimum sequence still possessing full biological potency has been undertaken in order to identify the segments of the peptide that are essential for the biological activity. Besides supplying structure-function information, this approach should allow the synthesis of shorter peptides with consequent time and cost reduction. The effect of peptides on HIV-l infection has been evaluated by the p24 HIV-1 capsid antigen quantitative determination. The data are plotted as the peptide-treated sample/peptide-untreated control p24 values ratio and represent the mean values of the four separated experiments (Figure 2). The random distribution of p24 ratios around the unit for

DB3-M and DB3-S experiments indicates the inactivity of these analogues. Interestingly, at all of the tested concentrations, the presence of peptide DB3-R produces a 50% lower p24 production compared with untreated infected control. The inhibitory activity of peptide DB3-R appears to be dose-independent. Consequently, our results demonstrate that the deletion of the C-terminal pentapeptide and other major deletions (see Table 1) produce analogues that are not characterized by any biological activity with respect to the peptide DB3 activity or that show inhibitory activity. These results cannot be reconciled with the observations of Nehete *et al.* [15], who claimed that synthetic peptides of different length from the V3 loop regions of HIV-1 IIIB, MN and RF inhibited HIV infection. On the other hand, Yahi *et al.* [16] demonstrated that a synthetic multi-branched molecule, containing eight peptides corresponding to the DB3-S sequence, inhibits HIV-1 entry into CD4+ cells. It is interesting to stress that the inhibitory effect is present only when the multi-branched molecule is added 1 h after the initial exposure of the cells to HIV-1, whereas no inhibitory effect is present if the molecule is added before the incubation with HIV-1, in excellent agreement with our observations.

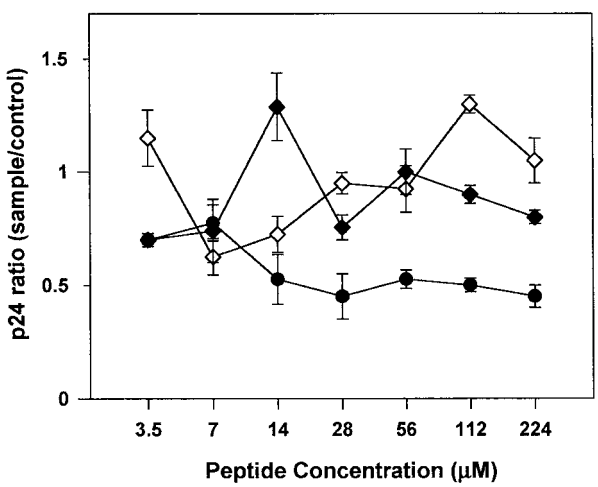

Figure 2 Effect of peptides on HIV-l infection. MOLT-3 cells were incubated with serial two-fold dilutions of peptides (● DB3-R, ◇ DB3-M, ◆ DB3-S) for 30 min at 37°C and then infected with HIV-l. Culture supernatants were removed after 24 h and replaced with a fresh medium without peptide. Viral infection was evaluated by determining the p24 antigen levels in culture supernatants 4 days postinfection. Data are plotted as the ratio of peptide-treated sample/peptide-untreated control p24 values and represent the mean values of four separated experiments.

The fact that deletion of the C-terminal pentapeptide and other major deletions produce inactive analogues could be due to two different reasons, namely the removal of the residues involved in the interaction with the receptor and/or the alteration of the biologically active conformation. While the first hypothesis has been evaluated by synthesizing several point-mutated analogues of DB3 bearing only a single change per sequence [14], the second possibility has been tested in this work by comparing the conformational differences among the analogues resulting from CD studies carried out under different experimental conditions, including both aqueous and micellar solutions. In phosphate buffer all peptides, except DB3-S, showed a negative band between 194 and 197 nm which is typical of randomly coiled conformations (Figure 3). Under the same experimental conditions, even if the bands do not present typical intensities, the spectrum of DB3-S is similar to a type B spectrum (a weak negative band between 220 and 230 nm, a positive band between 200 and 210 nm, and a strong negative band between 180 and 190 nm) which, according to the definition proposed by Woody [17], is indicative of type II β-turn structures in agreement with a recent report by Marbrouk *et al.* [18]. On the other hand, the presence of a type II β-turn structure, involving the Gly-Pro-Gly-Arg sequence, was initially predicted by La Rosa *et al.* [19]. A β-turn structure was also confirmed by NMR studies [20,21] even though the NMR data presented were not sufficient in order to determine the β-turn type. In addition, Ghiara *et al.* [22] showed that the Gly-Pro-Gly-Arg sequence adopts a type-II β-turn structure in the complex between an antibody fragment and a V3 loop peptide.

Interestingly, the presence of a component band at 1639 cm^{-1} in the DB3-S FT-IR absorption spectrum in D_2O (Figure 4), confirms the presence of β-turns. In fact, on the basis of detailed FT-IR studies on a series of bridged proline-containing cyclic peptides with well-established steric structure, Mantsch *et al.* [23] have associated an amide I component band near 1640 cm^{-1} with the acceptor amide C=O of $1 \leftarrow 4$ (C_{10}) H-bonded β-turns. In addition, the bands at 1650 cm^{-1} and at 1664 cm^{-1} are assigned to the remaining solvent-exposed or weakly H-bonded carbonyl groups whereas the bands at 1586 and 1607 cm^{-1} pertain to symmetrical and asymmetrical vibrations of CN bonds of the guanidinium group [24].

The DB3-S CD spectrum is significantly independent from the environment; in fact, very similar

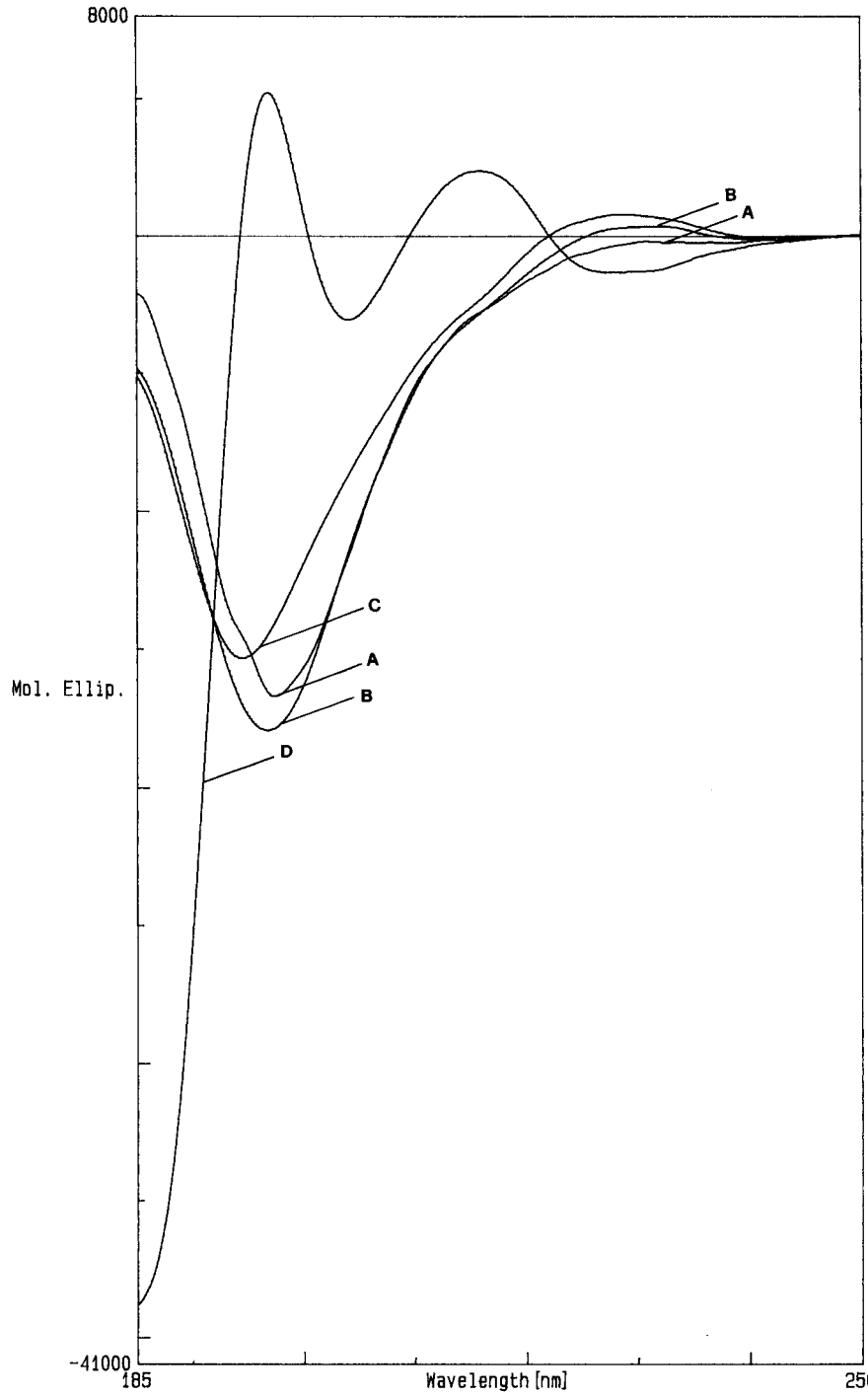

Figure 3 CD spectra of peptides DB3 (A), DB3-R (B), DB3-M (C) and DB3-S (D) in 10 mM phosphate buffer, pH 6.7.

patterns are present in 10 mM sodium phosphate buffer, in 14 mM SDS, in 2 mM SDS, in CH_3CN and in TFE solutions (Figure 5). Titration of the TFE solution with H_2O shows an isodicroic point in the 0–60% range (Figure 6). As it is generally accepted that the spectra identifying a single isodicroic point are representative of mixtures whose compositions differ in the content of only two conformers, it seems reasonable to assume a conformational equilibrium between a type II β-turn and unordered

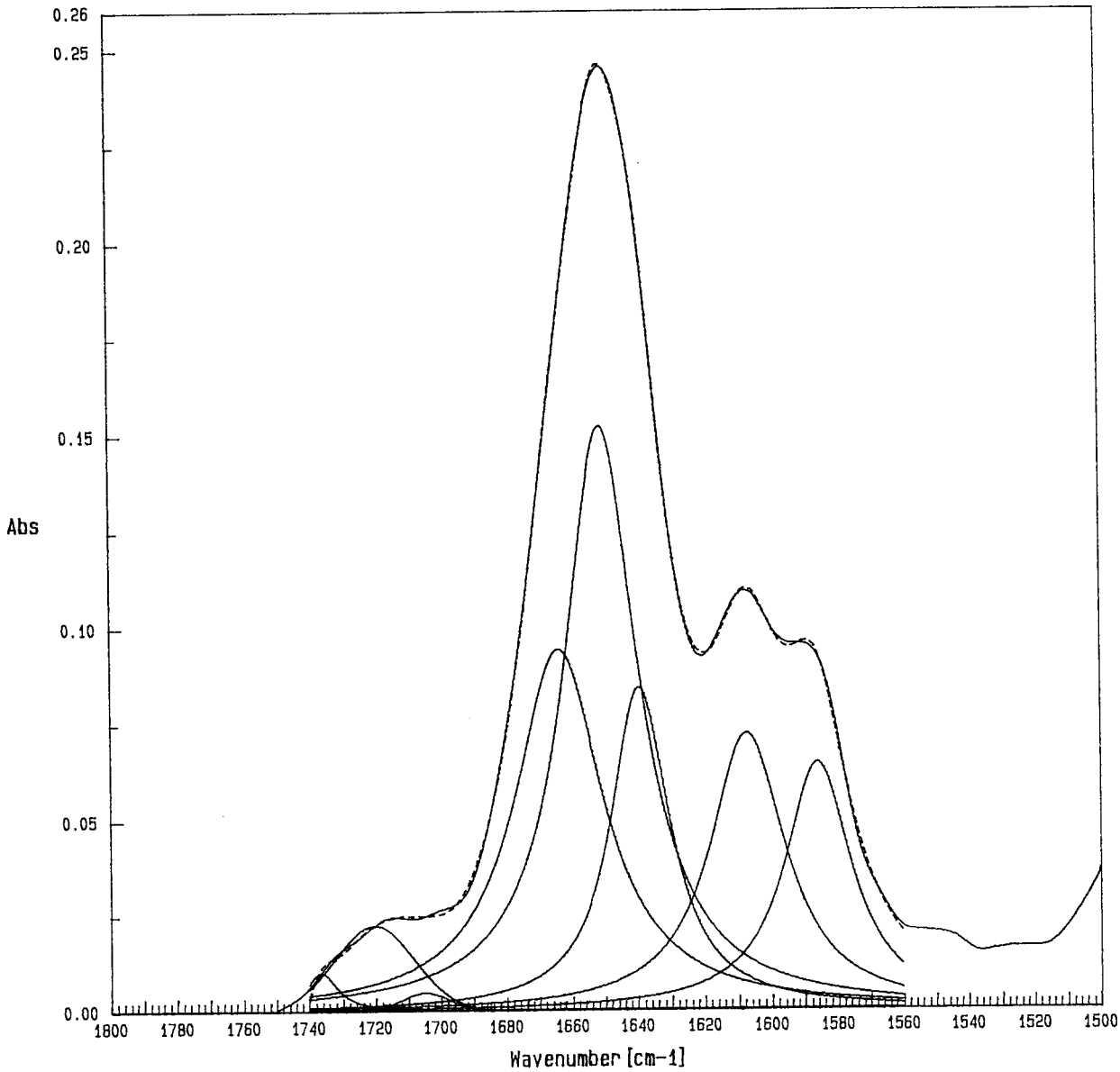

Figure 4 Amide I region in the FT-IR absorption spectrum of the DB3-S peptide in D_2O. The lower lines represent the component bands of the spectrum obtained in a curve-fitting procedure whereas the dashed line shows the sum of the components.

populations, where the type II β-turn conformation is predominant [25].

As shown in Figure 7, the CD spectra of the analogues are significantly different in micellar solution. The spectrum of the biologically active DB3 shows a positive maximum at 185 nm, and two negative maxima the first at 203 nm, and the second (broad) between 215 and 225 nm. A comparison between the spectra in aqueous and micellar solution confirms the transition to more ordered conformations in going towards a more hydrophobic environment. The spectrum of the inactive peptide DB3-S, on the contrary, presents a negative dichroic absorption below 195 nm, a maximum next to 200 nm and negative values above 200 nm, similar to those registered in aqueous solution. The spectrum of the biological inactive peptide DB3-M presents positive values above 195 nm and a negative broad band at 207 nm, indicative of a β-sheet contribution. Finally, the DB3-R peptide, which

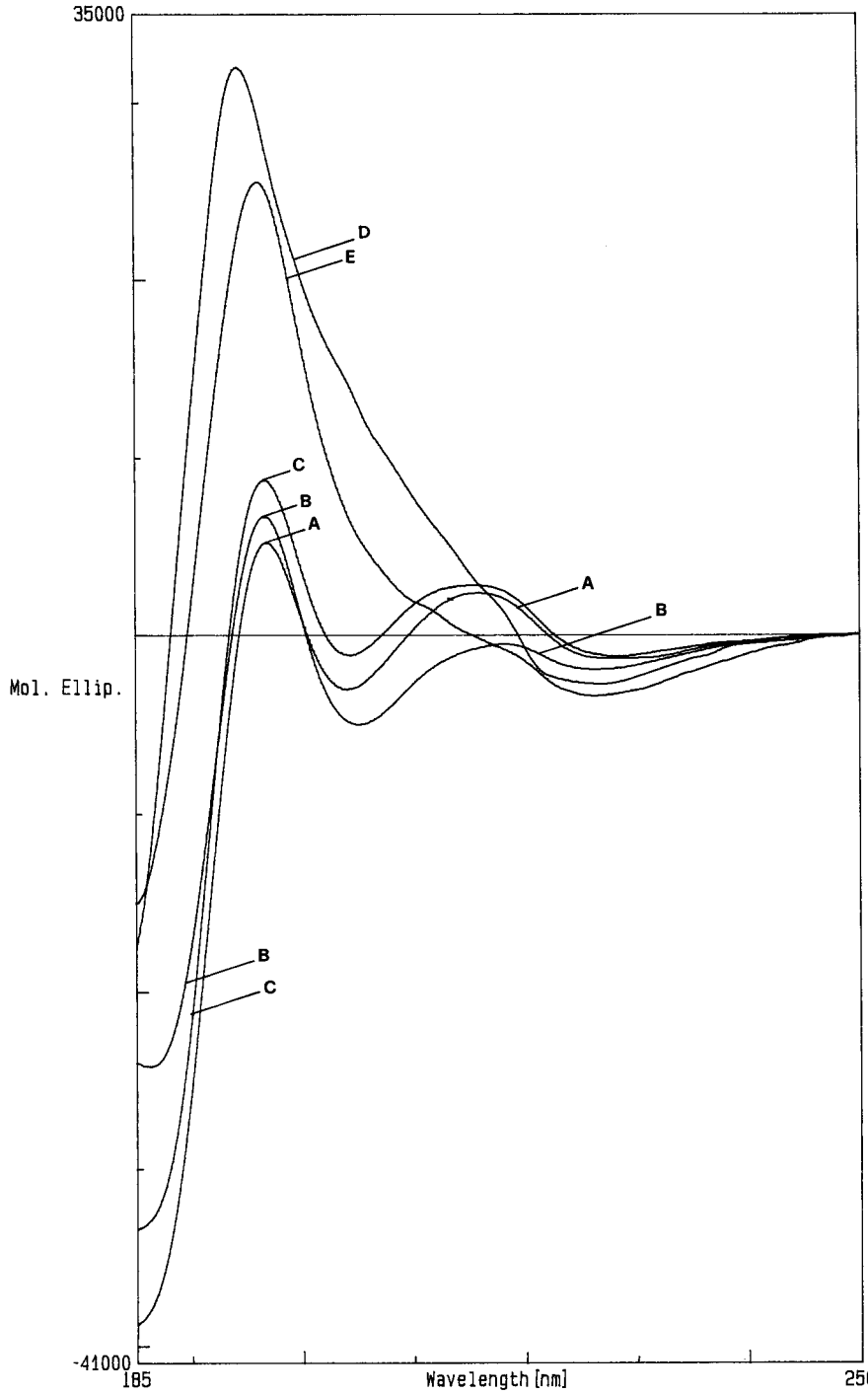

Figure 5 CD spectra of peptide DB3-S in 10 mM phosphate buffer, pH 6.7 (A), in 14 mM SDS (B), in 2 mM SDS (C), in TFE (D) and in CH_3CN (E).

possesses an inhibitory biological effect, gives a spectrum that differs from the spectra of both the biologically active DB3 and the biologically inactive DB3-S, presenting a negative band at 198 nm which is probably due to an unordered structure.

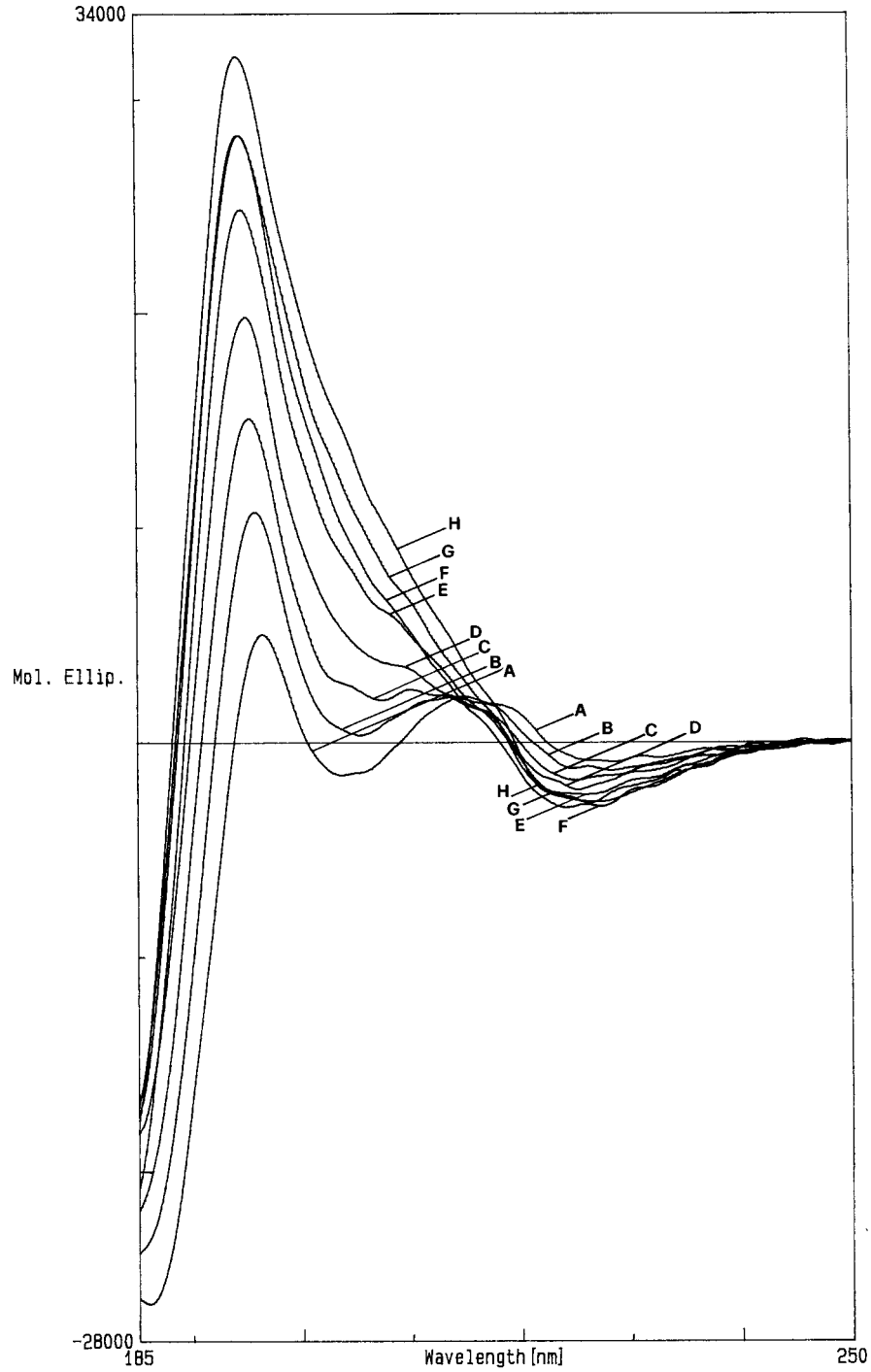

Figure 6 CD spectra of peptide DB3-S in 0% TFE/H_2O (A), in 20% TFE/H_2O (B), in 40% TFE/H_2O (C), in 60% TFE/H_2O (D), in 80% TFE/H_2O (E), in 90% TFE/H_2O (F), in 95% TFE/H_2O (G) and in 100% TFE (H).

CONCLUSIONS

It was demonstrated that a 23-amino acid peptide derived from the V3 loop of gp120 of the HIV-1 strain MN (DB3) is able to bind CD4 and to enhance HIV-1 infection. Three analogues of reduced sequence have been designed to identify the minimum active sequence of the V3 loop peptide. Interestingly the sequence 1–18 presents inhibitory activity, whereas the peptides DB3-M (sequence 7–18 of

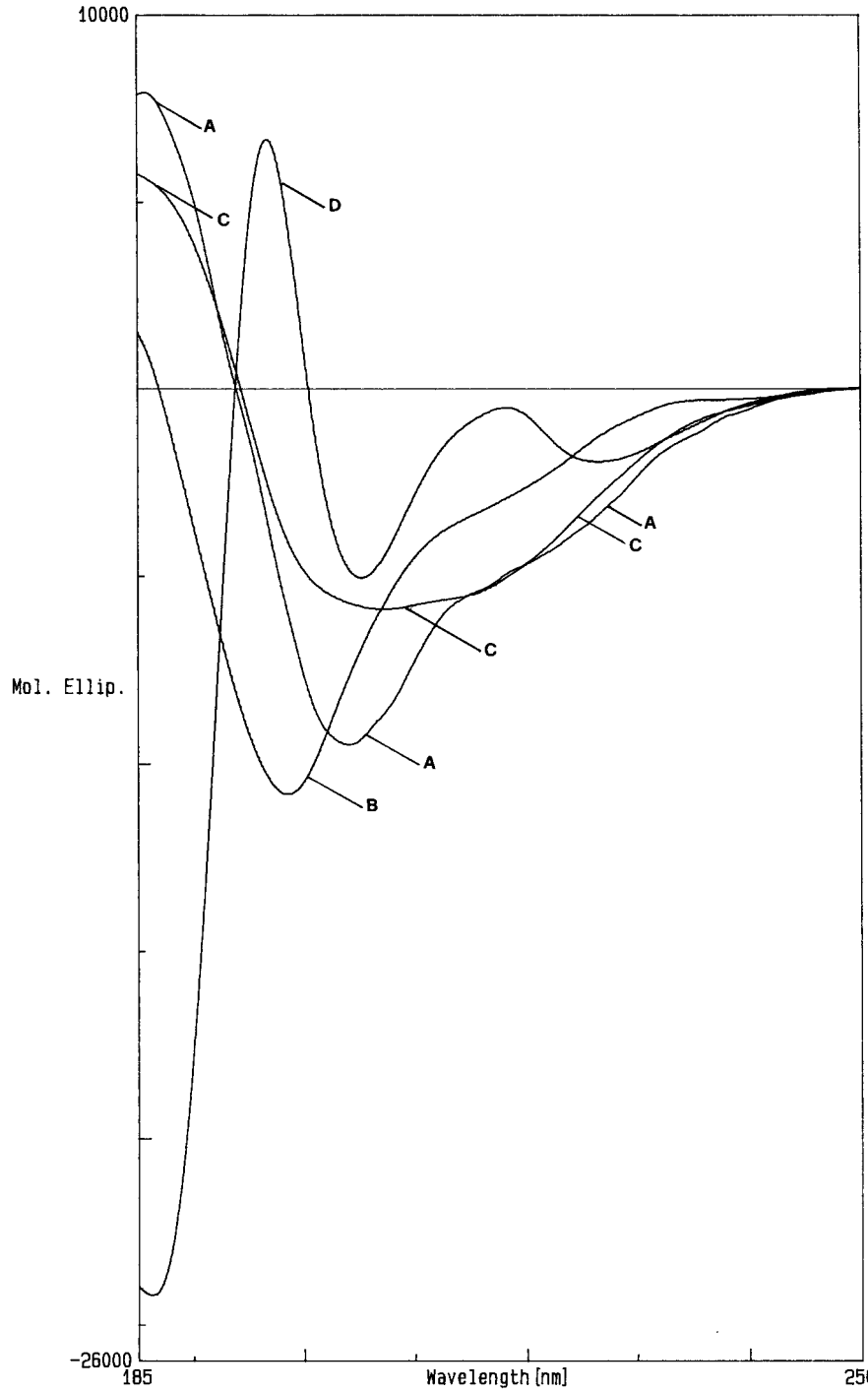

Figure 7 CD spectra of peptides DB3 (A), DB3-R (B), DB3-M (C) and DB3-S (D) in 14 mM SDS, 10 mM phosphate buffer, pH 6.7.

DB3) and DB3-S (sequence 10–15 of DB3) do not show any effect.

The present preliminary conformational studies under different experimental conditions such as 14 mM SDS, TFE and CH_3CN (data not shown) demonstrate the presence of conformational differences among the synthetic peptides substantiating the hypothesis that different conformations may support the different biological activities observed.

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