Self-association and Domains of Interactions of an Amphipathic Helix Peptide Inhibitor of HIV-1 Integrase Assessed by Analytical Ultracentrifugation and NMR Experiments in Trifluoroethanol/H₂O Mixtures*

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EAA26 (VESMNEELKIQVRAQAEHLKTY) is a better inhibitor of human immunodeficiency virus, type 1, inte grase than its parent Lys-159, reproducing the enzyme segment 147–175 with a nonpolar-polar/charged residue periodicity defined by four helical heptads (abcdefg) prone to collapse into a coiled-coil. Circular dichroism, nuclear magnetic resonance, sedimentation equilibrium, and chemical cross-linking were used to analyze EAA26 in various trifluoroethanol/H₂O mixtures. In pure water the helix content is weak but increases regularly up to 50–60% trifluoroethanol. In contrast the multimerization follows a bell-shaped crease regularly up to 50–60% trifluoroethanol. In contrast the multimerization follows a bell-shaped curve with monomers in pure water, tetramers at 10% trifluoroethanol, and dimers at 40% trifluoroethanol. All suggest that interhelical interactions between apolar side chains are required for the coiled-coil formation of EAA26 and subsist at medium trifluoroethanol concentrations. The N₂ temperature coefficients measured by nuclear magnetic resonance show that at low trifluoroethanol concentration the amide groups buried in the hydrophobic interior of four α-helix bundles are weakly accessible to trifluoroethanol and are only weakly subject to its hydrogen bond strengthening effect. The increased accessibility of trifluoroethanol to buried amide groups at higher trifluoroethanol concentration entails the reduction of the hydrophobic interactions and the conversion of helix tetramers into helix dimers, the latter displaying a smaller hydrophobic interface. The better inhibitory activity of EAA26 compared with Lys-159 could arise from its better propensity to form a helix bundle structure with the biologically important helical part of the 147–175 segment in integrase.

The well defined conformational properties exhibited by many peptides in solution have led to their use as models for protein folding and stability but also for the design of peptide inhibitors of enzymes (1–3). The model peptides are either artificial (4–6) or may reproduce protein segments (7–13). When they have defined conformations, the peptides can provide useful information on the relationships between local interactions, secondary structures, and tertiary structures (14–17).

We have previously tested the hypothesis that synthetic peptides reproducing amphipathic helical segments of enzymes may interact with these segments and interfere with the catalytic properties. Examples concern topoisomerase II (10, 11), an enzyme involved in the maintenance of DNA topology, and also retroviral integrase (IN) (12, 13) that catalyzes the integration of viral DNA into host cellular DNA (two recent reviews, Refs. 18 and 19). IN has no cellular counterpart and can be therefore considered as a specific target for development of anti-HIV therapy (20). In an earlier study, we have reported (13) that Lys-159, a peptide corresponding to the 147–175 segment of HIV-1 IN, inhibited the integration catalyzed by IN, most likely through specific binding to its counterpart in the protein. Such a specific binding was plausible since in the crystal structure of the catalytic core IN 50–212, the major part of the target 147–175 segment (from residue 147 to 166) is stabilized under a characteristic amphipathic helix (helix α4) (Fig. 1) with most of its hydrophobic side chains strikingly oriented outward from the molecule (21). The accessibility of the 147–175 segment to ligands has then been confirmed by the use of antibodies raised against the peptide Lys-159 (12). The antibodies not only bind specifically to IN and its catalytic core but also exhibit strong inhibitory activity against the enzyme, confirming both the surface exposure of the 147–175 segment and its biological importance. In fact, this segment has been shown to contain several critical residues for both the binding of IN to the LTR (long terminal repeats) ends of the viral genomic DNA and the DNA end processing (22, 23). These include, for instance, residues Glu-152, Lys-156, Lys-159, and Arg-166 oriented on the polar/charged face of the α4 amphipathic helix (Fig. 1).

The peptide, EAA26, was primarily conceived to provide a
better inhibition of the catalytic activity of IN than its parent peptide Lys-159 (12, 13, 24) that comprises four typical heptad repeats abcedfg (25), with the a and d positions being all apolar residues (except position a of the first and second heptads occupied by Q and N, respectively). EAA26 results from Lys-159 through several modifications (three substitutions are as follows: K156E, G163A, and D167A; and four deletions are as follows: Ser-147, Gln-148, Gly-1149, and Val-150) tending to increase the helical stability and the packing potential of helices (Fig. 1). We used data from previous reports showing that helical content can be augmented by creating interactions between side chain residues (26–28), salt bridge formation (29–31), and introduction of alanine residues (32). We were also guided by the AGADIR predictions for the helix potential (33–35) and by the COILS (36) and PAIRCOILS (37) algorithms for the α-helical bundle propensity of the selected peptides. EAA26 was studied in various 2,2,2-trifluoroethanol (TFE)/H2O mixtures, TFE being the solvent widely used that favors helical structure in polyampholytes (38). The main effect of TFE is supposed to occur through either a direct or an indirect strengthening of the intrahelical hydrogen bonds (39–42), although it has been recently suggested that helix stabilization by TFE would not depend on such a strengthening (43). Actually, TFE may exert more specific effects depending on the chemical structure of the studied individual peptide or the individual peptide region. For instance, TFE can stabilize not only helices but also β-hairpin structures (44–45) and molten globule-like folding intermediates (46–48). Obviously, effects on the balance of intrahelical hydrogen bonds are not the same at low and high TFE concentrations (9, 39, 41, 42). At concentrations above 40% TFE, the medium becomes less like bulk water and more like bulk alcohol. Under the latter conditions, TFE disturbs the helical structures stabilized by hydrophobic interactions, entailing the exposure of apolar side chains (49).

In the present study, the analysis of EAA26 has been carried out by combining circular dichroism (CD), NMR, chemical cross-linking, and sedimentation equilibrium. CD spectroscopy is a useful tool to study the formation of α-helices in aqueous and organic solutions. There is, however, a controversy on its ability to distinguish safely between straight α-helices (free helices or helices packed into bundles) and α-helices wrapped into coiled-coil structures (50). NMR which is very potent to analyze both secondary and tertiary structure also fails to discriminate between intra- and inter-monomer NOE connectivities in symmetric multimers, as only one set of resonances arises from the monomer (51). In contrast, equilibrium sedimentation can be safely used to establish the multimerization of biological molecules (Ref. 52 and references therein).

The present work follows three previous studies by our group on the IN fragment 147–175 and the properties of derived peptides (12, 13, 24). Here, we focus more particularly on the propensity of EAA26 to form in TFE a stable amphipathic helix and the impact of the latter on the α-helical bundle formation through hydrophobic contacts. We show that the way in which TFE affects the helix stability is subtle. The major idea is that in four-helix bundles, predominant at low TFE concentration, the amide groups of the residues involved in the hydrophobic core do not interact directly with TFE and do not show strengthened hydrogen bonds. The helix stabilization is induced through the interaction of TFE with the amide groups of residues belonging to the hydrophilic stripe and propagates through the peptide backbone to the residues situated within the hydrophobic stripe. Indeed, the overall stabilization of the α-helical conformation is reflected by the NOE and CSI values of all residues. In contrast, only those amide groups belonging to the residues of the outer hydrophilic stripe and that have direct access to TFE demonstrate an increase in the N2 temperature coefficients. However, increasing the TFE concentration destabilizes the hydrophobic core of the four-helix bundles and leads to dimeric coiled-coils. Concomitantly, TFE gains better access to the amide groups of even the apolar residues, and consequently, we observe the formation of additional intrahelical hydrogen bonds in these dimeric structures.

MATERIALS AND METHODS

Structure Predictions—α-Helix secondary structure predictions were carried out using the AGADIR computer program kindly provided by the authors (33–35). AGADIR considers short range interactions between residues at different pH values and temperatures and allows us to predict the helicity per residue of peptides lacking tertiary interactions in solution. Structure predictions reliable for peptide segments (in the protein environment) were also performed using the GOR algorithm (53, 54). Propensity of peptides to adopt coiled-coil structure was assessed using the COILS and PAIRCOILS programs (36, 37), based on the relative frequency of amino acid occurrence at each position (a–g) of the coiled-coil heptad repeat.

Synthesis—The sequence of EAA26 compared with the segment of the native peptide Lys-159 (sequence 147–175 of IN) is shown in Fig. 1. EAA26 peptide was assembled according to a standard Fmoc (9-fluorenylethoxycarbonyl) procedure on an Applied Biosystems model 432A peptide automatic solid-phase synthesizer. Peptidomimetics were synthesized by solid-phase methodology using the phosphoramidate procedure on an Applied Biosystems 318B automatic apparatus. All DNA strands were purified through 15% denaturing (7 M urea) polyacrylamide gels and labeled at their 5’ end with γ-32P (12).

Circular Dichroism—CD spectra were acquired using a Jobin-Yvon Mark VI spectrometer linked to a PC microprocessor. Measurements were calibrated with (+)-10-camphorsulfonic acid. Samples prepared in aqueous solution at pH 3.5 and in appropriate H2O/TFE mixtures were placed in thermally jacketed cuvettes with 0.1, 1, and 10 mm path lengths. Spectra recorded with 1 nm steps and corrected for the baseline were averaged over 10 scans. Before spectra recording, samples were incubated 10 min at the chosen temperature (25 °C) to allow the solutions to reach their equilibrium state. Effects of TFE addition (from 0 to 90% in aqueous solution) and of peptide concentration (from 10 to 100 μM) on α-helix contents were determined at 25 °C. The α-helix content was estimated by direct reading of the circular dichroism, 𝛁 (m–1 cm–1) at 222 nm, as described previously (13, 55).

Analytical Ultracentrifugation—Samples of the peptide were dissolved and equilibrated on a Sevaphex G-25 column (Amersham Pharmacia Biotech) in water/TFE (0, 10, or 40% TFE) containing 0.1 μM NaCl at pH 6.2, 6.6, and 6 respectively. 0.15 ml of sample solution was introduced in the centrifugation cell over 50 μl of fluoro-carbon FC43. The corresponding equilibration buffer (0.21 ml) was introduced in the reference sector of each centrifugation cell. Centrifugation was performed in a Beckman Optima-XLA analytical centrifuge, using standard double sector cells with 1.2-mm thick aluminum centes. A temperature of 30 °C was chosen to minimize the formation of micro-particles of TFE as reported previously (56). The cells were scanned at the wavelengths indicated in the text, which was selected to keep the absorbance below 1 in the entire cell at equilibrium. After reaching the selected centrifugation speed, radial scans were recorded at 2-h intervals for at least 18 h to check that equilibrium was reached. When equilibrium was achieved, 10 successive scans were recorded and averaged to improve the signal to noise ratio in the recordings. Scans at 360 nm, a wavelength at which neither the buffers nor the sample absorb light, were also recorded at equilibrium to check the absence of optical artifacts that might affect the base line (because of the low molecular weight of the peptide, meniscus clearance could not be achieved even at 60,000 rpm, thus precluding the determination of the base line).

The protein distribution at equilibrium was analyzed with the Origin-based Optima XL-A Data Analysis Software (Beckman). The different fitting models (Ideal 1, Ideal 2, and Ass4) for single data sets (XLA-Single program) were systematically tested, and the best fitting was retained on the basis of both the χ value and the lack of systematic deviation of the residuals. In all cases, the base line was fixed at 0 absorbance and not allowed to float during the fitting.
procedure. The value of the partial specific volume used for molecular weight calculations was 0.747 ml/g, as determined according to Cohn and Edsall (57) from the amino acid composition of the EAA26 peptide. The densities of the NaCl solutions containing 0, 10, and 40% TFE were determined by use of a volumetric pycnometer (Prolabo, Paris, France) and found to be 1.004, 1.051, and 1.178 g/ml, respectively.

Chemical Cross-linking— Chemical cross-linking was performed with glutaraldehyde for EAA26 under three experimental conditions, namely 0, 10, and 40% TFE solutions containing 0.1M NaCl, at pH 6.2, 6.6, and 6, respectively. The peptide EAA26 was dissolved at a final concentration of 50 mM in a total volume of 100 ml. After 15 min equilibration period, glutaraldehyde was added to 0.001% (mass/vol- ume). 50 ml of the reaction mixture were withdrawn at 180 min. The reaction was stopped with 5 ml of NaBH4, 2 M. Each aliquot was mixed with 10 ml of sample buffer (300 mM Tris-HCl, pH 6, 8 50% glycerol (mass/volume), 0.05% bromphenol blue (mass/volume), and 10% SDS (mass/volume). The cross-linked products were analyzed by Tris/ Tricine/SDS-polyacrylamide gel electrophoresis (58). The separating gel (16% acrylamide) resolution was increased by inserting a 10% spacing gel between stacking and separating gels. The separated products were detected by silver staining (59).

NMR—One- and two-dimensional proton NMR spectra were recorded at 500 MHz in a Bruker AMX 500 spectrometer and processed on a Silicon Graphic Station with the FELIX (MSI) software. Samples of EAA26 (~2 mM) were analyzed under three different conditions as follows: aqueous solution (90% H2O, 10% D2O); TFE 20%, H2O 80%; and TFE 60%, H2O 40%. The pH, not adjusted, measured by a glass electrode was nearly 3.0. All the samples contained 1 mM EDTA and a small amount of TSP, introduced as an internal chemical shift reference. Two-dimensional 1H NOESY and TOCSY experiments were recorded at 10, 20, 30, and 40 °C in order to determine the NMR temperature coefficients. Solvent suppression was achieved either by presaturating the solvent signal or by using a gradient pulse WATER- GATE (60). These experiments were typically acquired with 2048 × 512 complex t1 points, a spectral width of 11 ppm, and a relaxation delay of 1–1.5 s. Mixing times were in the 80–120 ms range for TOCSY experiments and in the 50–300 ms range for NOESY experiments. Spectra were zero-filled (2048/1024 real points in t2/t1 dimensions) and subjected to shifted sine bell weighting functions (P6 generally) in both dimensions before Fourier transformation. Quantification of sequential NOEs was achieved by calculating the relative intensities in contour plot spectra between sequential dαNN or dβNN and intra-residue dααN connectivities to the sequential dααN ones, taking into account (averaging) the parts below and upper the diagonal of spectra.

Molecules were visualized and plotted using the Insight II program (Molecular Simulations Inc., San Diego, CA) on a Silicon Graphic workstation O2 R10000.

RESULTS

In a previous paper (24) we have mentioned that EAA26 was a better inhibitor of IN compared with Lys-159 (segment 147–175 of HIV IN). Its amino acid sequence is shown in Fig. 1, together with that of its parent Lys-159 and the profile of

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**Fig. 1.** Conformation of the 147–175 segment of IN as found in the crystal structure of the catalytic core (21, 89). A ribbon representation showing the periodicity of polar (red) and apolar (blue) residues in the amphipathic α4-helix. Residues of the intrahelical loop are in yellow, and those starting the α5-helix are in green. Figure also shows the sequence of the 147–175 segment of IN with the pattern of polar (red) and nonpolar (blue) amino acids in the α4-helix (Val-151 to Val-165), the interhelical loop amino acids (yellow), and the amino acids starting the α5-helix (green). The sequence of the designed EAA26 peptide is given below with the secondary structure helix predictions (GOR and AGADIR) and the tertiary structure coiled-coil predictions (COILS and PAIRCOILS). Deleted amino acids are indicated by asterisks, and substituted amino acids are given in red. Y has been added at the C terminus and is used to determine the peptide concentration (see "Materials and Methods").
The effect of peptide concentration on CD spectra was then examined in both aqueous solution and H₂O/TFE mixtures. The profile of helix content, obtained from direct reading of Δε at 222 nm, as a function of TFE concentration is shown in Fig. 3. TFE is a helix stabilizing solvent supposed to proceed through the strengthening of intrahelical hydrogen bonds, and TFE/H₂O mixtures are commonly used to stabilize peptide helices since their introduction by Goodman and Litowsky (38). In aqueous solution, the α-helix content is found low (−20%) in agreement with AGADIR. At 10% TFE, EAA26 displays a helix content of −45%, i.e. −35% of helix increase compared with pure H₂O. As is often the case the maximum of helix stabilization (−65%) is reached at higher co-solvent concentrations (−45% TFE) (61).

The effect of peptide concentration on CD spectra was then examined in both aqueous solution and H₂O/TFE over a 100-fold range of concentrations (10 to 1000 μM) (not shown). In H₂O, the helicity slightly increased from −15 to −27% suggesting the existence of a weak structure stabilizing association. In contrast, neither in 20% TFE nor in 40% TFE did the CD spectra show a detectable change. Particularly, the CD bands at 222 and 208 nm do not show measurable variation, whereas their ratio is often used to identify the coiled-coil formation in solution (62).

**Analytical Centrifugation**

The ability of EAA26 to adopt multimeric structures was assessed by both ultracentrifugation at sedimentation equilibrium and chemical cross-linking experiments. Sedimentation equilibrium is one of the most powerful techniques to assess the association properties of macromolecules in solution. Samples of the EAA26 peptide at 0.7 mg/ml in 0, 10, and 40% TFE containing 0.1 M NaCl at pH 6.2, 6.6, and 6, respectively were submitted to sedimentation equilibrium to determine the association state of the peptide at each TFE concentration. Equilibrium was first achieved at 50,000 rpm, and the cells were scanned at 360 nm to check for possible optical artifacts due to the redistribution of TFE in the centrifugation field that might create a Schlieren effect resulting in a distorted base line. While a flat base line centered close to 0, apparent absorbance was observed practically throughout the cells with 0 or 10% TFE, the cell with 40% TFE showed a strongly distorted base line that precluded any reasonable analysis of the peptide distribution. The three cells were scanned at 287 nm. They were analyzed as indicated under “Materials and Methods” and provided the following results. For EAA26 in aqueous solution in the absence of TFE, an excellent fitting was obtained assuming a unique species with ideal behavior ($\chi^2 = 1.8 \times 10^{-5}$; random distribution of the residuals) with an estimated molecular mass of 3,300 Da. Attempts to use a model with an oligomerization equilibrium did not improve the quality of the fitting. Moreover, they provided nearly the same molecular mass for the monomer and extremely small values for the association constants. The peptide thus appeared to behave as a monomer in TFE-free solution. Possible reasons why the molecular mass obtained experimentally was larger by 10% when compared with that calculated from the amino acid composition of EAA26 (2,996 Da) will be discussed later.

At 10% TFE, assuming a unique species with ideal behavior provided a very poor fitting ($\chi^2 > 10^{-4}$; nonrandom distribution of the residuals) and a molecular mass of about 4,800 Da,

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**Fig. 2.** Projection of segments 155–167 of both helix α of HIV-1 IN (A) and peptide EAA26 (B) down the helix axis. Polar amino acids are in red and nonpolar in blue.
inconsistent with either a monomer or a dimer. Very good fittings were, however, obtained assuming either a monomer-tetramer equilibrium or multiple equilibria between monomers, dimers, and tetramers. Practically the same $\chi^2$ value ($2.5 \times 10^{-5}$) was obtained with both models, both showed random distributions of residuals, and both gave similar monomer molecular masses (3,170 and 3,240 Da, respectively). In the multiple equilibria model, the monomer to dimer association constant was found extremely low, suggesting the absence of significant amounts of dimers. Thus, at 10% TFE and in the concentration range between 0.2 and 0.7 mM, the solution behaved essentially as a mixture of monomers and tetramers in equilibrium.

At 40% TFE, no satisfactory fit could be obtained with any model we tried, confirming that the distortion of the base line reported above prevented analysis of the equilibrium.

To overcome this difficulty, the centrifugation speed was reduced to 35,000 rpm, which was expected to minimize the redistribution of the TFE in the cell and therefore to minimize the optical artifacts due to Schlieren effects. Indeed, when equilibrium was reached, the base line observed at 360 nm was flat and close to 0 practically throughout the cell. Only the data points close to the bottom of the cell containing 40% TFE still showed non-negligible distortions. The three cells were then scanned at 287 nm and the diagrams analyzed as above. The equilibrium distributions thus obtained are shown on Fig. 4. Essentially the same results as those reported for 50,000 rpm were obtained for the 0% TFE solution. At 10% TFE, a slightly better fitting, in terms of both lower $\chi^2$ and random distribution of residuals, was obtained with the multiple equilibria model (monomers, dimers and tetramers) than with the single monomer to tetramer equilibrium. The distribution at 40% TFE was analyzed in the region of the cell where no significant distortion of the base line was detected. Assuming a unique species with ideal behavior, the best fitting ($\chi^2 = 3.6 \times 10^{-3}$; nonrandom distribution of residuals with downward curvature characteristic of a nonideal solution) was obtained with molecular mass of 6,050 Da. Attempts to fit the data on the basis of a model with a unique or multiple equilibria failed to improve the quality of the fitting and provided a molecular mass for the “dissociated” species of 6,240 and 6,280 Da, respectively. Moreover, a very small value was obtained for the monomer/oligomer association constant. These results strongly suggested that the peptide exists mainly as dimers at 40% TFE.

In an attempt to minimize further the optical artifact due to the redistribution of TFE, the centrifugation speed was again reduced, this time to 30,000 rpm, and the cells scanned again when equilibrium was reached. Scans at 360 nm showed that the optical artifact had completely disappeared since flat base lines centered very close to 0 were observed in all the cells. The cells were then scanned at 287 nm and analyzed as above. Again, the best fittings were obtained for a unique, ideal, monomeric species at 0% TFE and for mixed equilibria between monomers, dimers, and tetramers at 10% TFE. At 40% TFE, a reasonably good fitting, which provided an estimated molecular mass of 6,270 Da, was obtained with the assumption of a unique, ideal species ($\chi^2 = 5.8 \times 10^{-5}$). But again, the residuals showed a nonrandom distribution characteristic of a nonideal solution. New fittings were therefore attempted using as a model a unique, nonideal solute. Introducing the second virial coefficient to take nonideality into account reduced the $\chi^2$ value to $2.6 \times 10^{-3}$ and significantly improved the distribution of the residuals. Yet, some systematic deviation of the residuals remained clearly visible near the meniscus and bottom of the solution. The molecular mass obtained from the fitting with a nonideal model was 6,120 Da.

The results of the centrifugation studies are summarized in Fig. 4 and Table I. It can be seen that the molecular weight estimates are systematically higher, sometimes by as much as 15%, than the real molecular weight of the EEA26 peptide. A likely explanation is that the partial specific volume used for the calculations may be erroneous, since it was determined from the weight average of the partial specific volumes of the residues. Whereas this is acceptable for large globular proteins (57), the calculated partial specific volume may, in the case of a small peptide, differ significantly from the real one because of preferential hydration or solvation by the peptide.

Despite the relatively low precision on the molecular weight estimates, the results in Table I clearly point to the following conclusions. In the absence of TFE, the peptide is monomeric and shows no tendency to self-associate. In the presence of 10% TFE, monomers, dimers, and tetramers of EEA26 coexist in equilibrium with each other. In the presence of 40% TFE, the peptide is essentially dimeric.

**Chemical Cross-linking**

A confirmation of the analytical centrifugation results came from chemical cross-linking experiments performed with glutaraldehyde at peptide concentration and solvent conditions similar to those used in analytical centrifugation. Results can be seen on the gels reported in Fig. 5. In aqueous solution most of the peptide was found monomeric, although the presence of
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NMR Assignments and Helical Structure in TFE

In a previous study we have reported some of the helix inducing properties of TFE at low concentration on EAA26 (63). Here, studies are extended to the analysis of the higher concentrations of TFE in order to assess better the respective role of the intrahelical hydrogen bonds (strengthened by TFE) and of the hydrophobic contacts (reduced by TFE) on the stability of the coiled-coil structures adopted by EAA26.

NOE Connectivities—Fig. 6 contains the $H_{\alpha}H_{N}$ and $H_{N}H_{N}$ fingerprint regions of NOESY spectra for EAA26 in aqueous solution and 20% TFE. Some of the NOEs cannot be measured because of signal overlap. In aqueous solution, the number of NOE connectivities is at a minimum and signal intensities are globally weak (Fig. 6A), and the $d_{NN}$ NOEs suggest the presence of an only weak helical content. This is corroborated by the sequential $d_{i,N}$ connectivities that are stronger than intraresidue ones. Only two medium range connectivities $d_{i,N} (i, i + 3)$ are detected in the N-terminal part (residues 154–157 and 155–158). In 20% TFE, stronger $d_{NN}$ NOE connectivities form now an almost continuous stretch for residues 154 to 166, and numerous medium range NOE connectivities, namely $d_{NN,i+4}$ and $d_{NN,i+3}$ NOEs, appear in the $H_{\alpha}H_{N}$ region (Fig. 6B).

Fig. 7 summarizes the NOE data obtained for EAA26 in H$_2$O, 20% TFE, and 60% TFE, qualitatively classified as strong, medium, and weak. Typically, the higher intensity of intraresidue $d_{NN}$ connectivities compared with sequential ones, $d_{i,N}$ and $d_{NN}$, constitutes a good criterion for helical stabilization. All indicate that the helical content is weak in H$_2$O. In contrast, the number of sequential and medium range NOEs is a maximum in TFE and clearly parallels the helix contents reflected by CD spectra measured in this organic solvent. Mean relative intensities $d_{NN,seq}/d_{NN,seq}$ are equal to 17% in H$_2$O and 57% both in 20 and 60% TFE; and $d_{i,N}$ $d_{NN}$ $d_{NN,i+4}$ NOEs are equal to 32% in H$_2$O, 72% in 20% TFE, and 69% in 60% TFE (24, 63–65).

However, more medium range NOE connectivities, typical of the helix structure, are present at 60% TFE stipulating that the helix is longer by two residues compared with that observed at 20% TFE (Met-154 to Thr-174 versus Met-154 to Leu-172). Globally, the occurrence in TFE solutions of $d_{NN,seq}/d_{NN,seq}$ $d_{NN,i+4}$ NOEs indicates that a significant population of the peptide is now under an α-helical structure. The maximum number of $d_{NN} (i, i + 4)$ NOEs is measured at 60% TFE, stipulating that a major stabilization occurs in these solvent conditions. Moreover, between 20% TFE and 60% TFE, there is also a redistribution of the $d_{NN} (i, i + 3)$ and $(i, i + 4)$ and $d_{NN} (i, i + 3)$ NOEs, suggesting some change in the helix areas.

$H_{\alpha}$ Chemical Shifts—$H_{\alpha}$ proton chemical shifts undergo a mean upfield shift of about ~0.4 ppm from random coil values during helix formation (66, 67) so that helical residues have negative CSI. Note that the $H_{\alpha}$ resonances present very little intrinsic sensitivity to TFE (68), thus the CSIs are mostly related to changes occurring in the peptide secondary structures. The patterns of CSIs (Fig. 8), like NOE connectivities (Fig. 7), account for a major conformational transition caused by TFE additions. From 0 to 20% TFE, the main α-helix stabilization involves the central region of the peptide spanning a very weak amount of dimer was also detected. However, addition of TFE promoted the formation of oligomeric species. At 10% TFE, tetramers become the most populated species, whereas at 40% TFE only the dimeric species are observed. Thus, globally, the cross-linking data parallel the analytical centrifugation ones, both techniques reflecting the existence of a multimerization process that follows a bell-shaped curve as a function of TFE concentration.
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TABLE I

| Solvent  | 0% TFE | 10% TFE | 40% TFE |
|----------|--------|---------|---------|
| Fitting program | Speed (in 1000 rpm) | Ideal 1 | 35 | 50 | Assoc4 | 35 | 50 | Nonideal | 35 |
| Monomer $M_r$ | 3460 | 3050 | 3340 | 3220 | 3110 | 3240 | 3130 | 3310 | 3020 |
| Oligomeric state | 1 | 1 | 1 | 1.2 | 1.2 | 1.4 | 2 | 2 |
| $T_e$ | 1.2 | 1 | 1.8 | 1.5 | 6 | 2.5 | 2.6 | 3.6 |

FIG. 5. Glutaraldehyde cross-linking of EAA26 analyzed by SDS-polyacrylamide gel electrophoresis. Experiments were carried out in H$_2$O/TFE mixtures at different TFE contents: 0, 10, and 40%, like in ultracentrifugation experiments. $T$ corresponds to uncross-linked peptide. The products monomer ($M$), dimer ($D$), trimer ($Tr$), and tetramer ($T$) are revealed by silver staining. $MW$ refers to the molecular mass standards.

roughly the amino acids Met-154 to Ala-167. From 20% up to 60% TFE, they are mainly the C-terminal residues that are concerned by the helical stabilization. However, whatever is the medium, the residues occupying the peptide central region are those displaying the more negative CSIs, the results paralleling the NOE data presented above. Globally, the CSI, the NOE, and the CD data all together argue for a major helical stabilization from 0 to 20% TFE. More subtle effects are observed beyond 20% TFE, including an extra stabilization of the secondary structure at the peptide C terminus and perhaps a weak destabilization for residues Glu-157 and Leu-158 at the N terminus.

$N_H$ Temperature Coefficients—Fig. 9 summarizes the effects of temperature on the amide hydrogen chemical shifts measured in the three solvent conditions. In aqueous solution, of 26 residues 10 residues are found to display amide $N_H$ temperature coefficient values clearly above the admitted threshold ($-$6 ppm/K) (69), and 6 residues have temperature coefficient values either at or very close to the threshold. Met-154, Asn-155, Leu-158, Ile-161, Ile-162, and Val-165 display low temperature coefficients incompatible with intrahelical hydrogen bonds. Note that all these residues, except Asn-155, possess apolar side chains forming a hydrophobic stripe along the amphipathic $\alpha$-helix in the crystal structure of the IN catalytic core (Fig. 1) (21). At 20% TFE, the Met-154, Asn-155, and Leu-158 residues show even lower $N_H$ temperature coefficients stipulating that their intrahelical hydrogen bonds are weakened compared with H$_2$O, in apparent disagreement with both the NOE and CSI features arguing that these residues are involved in stable helical structures. Under the same conditions, the residues of the hydrophilic stripe on the opposite side of the hydrophobic stripe (Fig. 1) exhibit as expected helical hydrogen bonds strengthened by TFE, in agreement also with the NOE and CSI data. In the contiguous C-terminal region (from Arg-166 to Ala-175), represented by an interhelical loop in the crystal structure (21) and predicted to be only weakly structured by AGADIR (Fig. 1), an usual strengthening of the intrahelical hydrogen bonds is induced at 20% TFE.

At 60% TFE, most of the $N_H$ temperature coefficients exhibit higher values at the N terminus and lower values at the C terminus, whereas in the central region some residues show either higher (Leu-158 and Val-165) or slightly lower (Ile-162) values. Note, that it is in the C-terminal region that the hydrogen bonding pattern is the most disturbed by increasing the TFE concentration, while at the same time, the CSIs, the NOEs, and the CD criteria all together reflect a helix stabilization. Thus, the C-terminal region that is mostly unordered in the crystal display fluctuating helical conformations in solution, with preferred helical dihedral angles rather than helical hydrogen-bonded amides at the high TFE concentrations (48).

There is thus a very good agreement between the NOE and CSI methods, both reflecting a helix stabilization in EAA26 induced by TFE solvent. The CD results further parallel the NMR data. Yet, the peptide appears structurally heterogeneous since the response of the peptide to TFE is not the same along the whole EAA26 sequence. In the central region represented by an amphipathic helical structure in the crystal, most of the helix stabilization occurs between 0 and 20% TFE and changes only weakly beyond. In contrast, in the C-terminal region the helix seems to increase gradually from 0 to 60% TFE, according to the geometrical parameters inferred from CSIs. At the same time, the $N_H$ temperature coefficients reflect puzzling events occurring in the central region of EAA26 and can be explained only by taking into account the property of the peptide to adopt oligomeric structures in which the amide groups of apolar residues are in the hydrophobic interior of packed helices and are thus sheltered from the TFE molecules (Fig. 10). The propensity of EAA26 to adopt oligomeric structures has been shown by ultracentrifugation and chemical cross-linking and has been predicted by COILS.

DISCUSSION

We used a strategy based on complementary spectroscopic techniques to assess the propensity of a natural peptide, designed for its inhibitory activity against the HIV-1 IN, to adopt folded tertiary structures in aqueous as well as in low and in high TFE concentration solutions.

In the crystal structure of the catalytic core of IN most of the 147–175 segment (region spanning amino acids 147–166) has been found to be involved in a typical amphipathic $\alpha$-helical structure ($\alpha$4-helix) (21) with several of its hydrophobic residues strikingly oriented outward at the surface of protein. The C-terminal portion (segment 167–175) adopts a loop structure, with a $\beta$-turn (residues Ala-169, Glu-170, His-171, and Leu-172) in its center, and its three ending residues included in the $\alpha$5-helix (21). Moreover, the importance of the 147–175 segment for the LTR ends recognition by IN has been highlighted by several groups (22, 23, 70–74). We have previously shown that the peptide Lys-159 reproducing the 147–175 segment has a helical content of $-5$% in aqueous solution and of $-25$% in 20% TFE (13). At the same time, Lys-159 presented coiled-coil...
forming properties according to both CD and cross-linking experiments and exhibited weak inhibitory activity against IN in in vitro assays (12, 13). In a subsequent paper (24), we have mentioned that a designed peptide, EAA26, behaved as a twice better inhibitor compared with Lys-159 in in vitro assays and that the inhibition proceeded through the blocking of the IN binding to the LTR 3' end (12). Now, in order to go further in the search of more potent inhibitors, we asked the question whether the extra inhibitory activity demonstrated by EAA26 is related to its greater ability to form an α-helix secondary structure and a coiled-coil/α-helical bundle motif regarding the parent peptide Lys-159, assuming that improving the latter properties in the designed peptide will help to increase its energy of interaction with the target segment in the protein.

Identification of Stable Helical Regions in EAA26—The ability of EAA26 to adopt a more stable α-helix compared with Lys-159 results mainly from the substitution of α-helix breaker residues by the α-helix inducer Ala residue and a better balance of stabilizing interactions versus destabilizing interactions in residue side chains. α-Helix stabilization in peptides implies intrahelical hydrogen bonds between amide groups and also favorable interactions between side chains. These can be either hydrophobic (Van der Waals, π/π, . . . ) or electrostatic (hydrogen bonds, ionic bridges, . . . ). Among these, a critical role can be attributed to i, i + 3 or i, i + 4 intrahelical or hydrophobic interactions (9, 41, 75, 76) and to the helix inducing amino acids Ala and Leu for their particular side chains (77).

Assuming that the EAA26 region spanning amino acids Val-151 to Val-165 adopts an amphipathic helical structure similar to the α4-helix in the crystal (21), then 1) three ionic interactions (two of type i, i + 3, Glu-156 to Lys-159 and Glu-157 to Lys-160; one type i, i + 4, Glu-156 to Lys-160) versus one ionic repulsion (of type i, i + 4, Glu-152 to Glu-156) are possible along the hydrophilic stripe, and 2) six nonpolar interactions (three of type i, i + 3, Val-151 to Met-154, Leu-158 to Ile-161, and Ile-162 to Val-165; and three of type i, i + 4, Met-154 to Leu-158, Leu-158 to Ile-162, and Ile-161 to Val-165) can occur along the hydrophobic stripe. It can be noted that the gain in helicity expressed by EAA26 is nicely predicted by the AGADIR algorithm and is further confirmed by both CD and NMR experiments (13, 24). Experimentally, the peptide appears structurally heterogeneous in the three media considered with the highest helix content within the central region (Met-154 to

Fig. 6. Fingerprint d$_{NN}$ and d$_{NN}$ NOESY regions for EAA26 in aqueous solution (A) and 20% TFE (B). Intraresidue peaks are labeled by corresponding amino acid residues (one-letter code and position in the sequence), whereas sequential and medium range connectivities are indicated by residue positions in the sequence. The peptide numbering from V1(151) through A25(175) is used. Represented spectra were recorded at 20 °C, pH ~ 3.0, 300-ms mixing time, using a WATERGATE solvent suppression scheme.
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Ala-167) and a rather flexible peptide portion at the C terminus, corroborating the crystal structure data and the AGADIR predictions. Yet, TFE at low concentration induces the α-helix in the C-terminal portion highlighting that the helix can be promoted in a peptide region devoid of defined secondary structure.

Autoassociation of EAA26 Molecules in TFE/H2O Mixtures—Typically, the reduced polarity of TFE/H2O mixtures compared with pure H2O increases the strength of hydrogen bonds (39–43), but it is supposed to decrease the contribution of hydrophobic interactions and thereby to hinder the coiled-coil formation (39, 40, 42, 61). Here, both the chemical cross-linking and sedimentation equilibrium experiments show the propensity of the EAA26 molecules to multimerize in TFE/H2O mixtures. This indicates that hydrophobic interactions persist in TFE, even at relatively high concentration of this solvent, in agreement with the recent observations of Padmanabhan et al. (41). However, the stabilizing effects of TFE are not so straightforward and may vary from one peptide to another according to the amino acid sequence and the propensity of the peptide for a given secondary structure (44, 45). Moreover, molten globule-like folding intermediates can be stabilized by TFE (46–48).

Here, by using combined techniques we can demonstrate that there is a correlation between the stabilization of the helical secondary structure and the formation of fixed associated structures, at least at low TFE concentration. The concentration of TFE is an important factor that governs the size of oligomers that appears to be at a maximum at 10% TFE, where tetramers have been found to dominate over both the dimers and the monomers, the latter occurring at higher TFE concentration. According to these results, it is likely that the amphipathic helical structure induced by low TFE concentration in EAA26 helps to drive the polypeptide to fold into a tertiary structure. The stability of the latter requires hydrophobic forces still important at low TFE ratio.

However, present results prove that CD spectra cannot be safely used to detect the association of helical peptides into ternary structures (50, 62). Peptide concentration effects are present in the CD spectra recorded in water, while under these conditions no oligomeric structures are detected by either ultracentrifugation or chemical cross-linking. In contrast, in TFE mixtures the increase in peptide concentration does not alter the CD spectra, whereas the ultracentrifugation and the cross-linking show molecular associations. A possible explanation for such results could be that in TFE the peptide concentration does not or only weakly affects the helix packing and that the packing does not induce significant change on the helix backbone angles, thus preventing any detection by CD.

Packing Properties of EAA26 Molecules—The reduction of the oligomer size at the higher concentration of TFE naturally drew our attention to the possible role of hydrophobic forces in the maintenance and the geometry of the associated structures. Interfaces between amphipathic helices are generally formed by adequately repeated hydrophobic side chain residues, the best examples being the coiled-coils in which two to five α-helices are wrapped around each other with a superhelical twist (78). Typically, such structures originate from 7-residue sequence motif, (abcdefg), repeating every two α-helical turns (25, 36, 79–85), but as shown very recently, they may also arise from 11-residue sequence motif (abcdefgij), repeating every three α-helical turns (86–88).

According to COILS the propensity of EAA26 for coiled-coil/α-helix bundle formation is high in its central region (Met-154 to Ala-167) and significantly weak at its N terminus (incomplete heptad) and its C terminus. The central region displays a well defined amphipathic helical structure (α4-helix) in the crystal (21, 89) with some of its hydrophobic side chains interacting with the α5-helix and the others oriented at the surface of the molecule and free of interactions. Dealing with a peptide fragment, instead of a peptide segment, no extra constraints may arise from the protein context and results will transduce the interactive properties of the sole peptide modulated by the surrounding solvent. Domains of protein interactions are generally assessed by hydrogen-deuterium exchange using both NMR (87, 88) and mass spectroscopy (90). The method provides fruitful information on protein-protein interfaces, as an ob-
groups (39). On another hand, the H$_2$O molecules as they are smaller than the TFE molecules can occupy the hydrophobic core and bind the carbonyls of buried amide groups, conferring thus aqueous-like properties to the hydrophobic core. Such H$_2$O molecules bound to amide groups have been found inside the hydrophobic core of four $\alpha$-helix bundles (92) and in the hydrophobic cavity of proteins (93). In the hydrophobic core formed by the interacting helices at 20% TFE, the intra- and interhelical interactions taking place between apolar side chains spaced ($i$, $i+3$) and ($i$, $i+4$) in the sequence (41) partly counterbalance the lack of hydrogen bond stabilization by TFE molecules. Decrease of solvent polarity (40–60% TFE) causes the reduction of the size of associated species from tetramer to dimer, in response to the reduction of hydrophobic contacts. The concomitant increase of helix content observed by both CD and NMR parameters (NOEs and CSIs) is caused by the strengthening of intra-helical hydrogen bonds related to the gain of access of TFE molecules to the amide groups previously buried in tetramers. Although the accessibility of amide hydrogens to TFE is higher in dimers than in tetramers, an amphipathic helix pattern of hydrophobic and hydrophilic residues persists for the central region spanning amino acids from Leu-158 to Val-165 arguing that dimerization concerns only this region. There is no sign of hydrophobic-hydrophilic residues patterning in the C-terminal region particularly at high TFE concentrations. The weakening of hydrogen bonding shown by $N_{	ext{H}}$ temperature coefficients is inconsistent with the helical features deduced from the arrays of NOEs and CSIs under the same conditions. The overall pattern at 60% TFE suggests that the secondary structure at the C terminus differs substantially from both the fully hydrogen-bonded $\alpha$-helix generated at 20% TFE and the loop structure observed in the crystal (21, 89). Similar results have been reported for the tandemstat protein (48) and the hen egg white lysozyme (94) at high TFE concentrations. In these two examples, helical structures of greater flexibility are generated in segments having loop or random structure in the native state, and which might correspond to fluctuating conformations with preferred helical angles but lacking stable hydrogen bonds (48, 94).
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TFE/H₂O mixtures. At the same time, both sedimentation equilibrium and chemical cross-linking studies show the high propensity of the helices for helix bundle formation through hydrophobic interactions under the dependence of TFE concentration. Certainly, the analysis of the hydrogen bond network stabilizing the EAA26 helix in various TFE/H₂O mixtures deserves a particular study using the new NMR scalable coupling method (95, 96). However, this method requires a peptide uniformly labeled with ¹⁵N and ¹³C. Yet, our results already support a model for the central portion of EAA26 that forms an interhelical turn between the structurally rather well defined central region, the C-terminal amino acids are shown in blue, and polar amino acids are shown in red. Amino acids are numbered according to the corresponding positions in the α4-helix.

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