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Tat Cell-penetrating Peptide Has the Characteristics of a Poly(proline) II Helix in Aqueous Solution and in SDS Micelles

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Abstract: Tat cell-penetrating peptide (GRKKRRQRRRPPQG) is able to translocate and carry molecules across cell membranes. Using CD spectroscopy the conformation of this synthetic peptide was studied in aqueous and membrane-mimicking, micellar SDS solutions at different temperatures. The CD spectrum of the Tat cell-penetrating peptide in SDS micellar solution was virtually unchanged from that in aqueous solution, and at low temperature it was close to that of a poly(proline) II helix. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Tat cell-penetrating peptide; circular dichroism; peptide conformation; poly(proline) II helix

INTRODUCTION

During the past few years it has been discovered that short peptides derived from several proteins, such as HIV-1 Tat, *Drosophila* Antennapedia and kFGF, can be internalized in most cell types and, more importantly, allow the cellular delivery of conjugated biomolecules [1]. Using this approach, a wide range of biomolecules has been delivered. Conjugation of poorly cell permeable drugs to these peptides has thus become a strategy of choice for improving their membrane permeability and consequently their pharmacological properties [2].

This report describes the secondary structure of the cell-penetrating peptide corresponding to the sequence 48–61 (GRKKRRQRRRPPQG) of the HIV-1 Tat protein [3] in aqueous and sodium dodecylsulphate (SDS) micellar solutions. The most interesting features in this sequence are the two prolines at positions 11 and 12, and the two cationic clusters at positions 2–6 and 8–10.

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Unfortunately, the mechanism adopted by this peptide to penetrate the cell membrane and its bioactive conformation are still unclear. Ho *et al.* [4], using LINUS protein structure and GRASP molecular surface predictive programs, suggested that this peptide might have similarities with the amphipathic α-helix structure present in many plasma membrane fusing peptides, such as toxins. However, other groups reported a lack of α-helicity for Tat as detected by circular dichroism (CD) measurements [5,6].

This chirospectroscopic technique is used extensively to study the conformation of proteins and peptides. Among the conformations that a polypeptide chain can assume, the α-helix and β-sheet have well characterized and understood CD spectra [7]. CD spectroscopy is also an excellent technique for detecting left-handed 3_1-helices [8]. Indeed, remarkable and significant differences are observed between the spectra of 3_1-helical and unordered peptides. A characteristic CD feature of a 3_1-helical structure is a small positive band at about 220 nm (with $\Delta\varepsilon = +1.5$ M^{-1} cm^{-1}) and a large negative band at 196 nm (with $\Delta\varepsilon = -11$ M^{-1} cm^{-1}) [9]. This spectrum closely resembles that of the left-handed

poly(proline) II (PP_{II}) secondary structure, allowing for a red shift of ca 10 nm, which can be attributed to the red-shifted absorption spectrum of Pro-Pro tertiary amides compared with that of secondary amides [10]. By contrast, truly unordered peptides with a dynamic structure that samples all the available conformational space, exhibit a similar but somewhat distinct shape. Their hallmark is a negative band at about 225 nm ($\Delta\varepsilon = -1$ M^{-1} cm^{-1}) accompanied by a stronger negative band at 200 nm ($\Delta\varepsilon = -5$ M^{-1} cm^{-1}) [9]. Thus, the shape and magnitude of the CD spectra differentiate truly unordered polypeptides from those with a preference for a 3_1-helical structure.

MATERIALS AND METHODS

Peptide Synthesis

The peptide was synthesized using an Advanced Chemtech model 348Ω peptide synthesizer and the Fmoc/HBTU-HOBt procedure. The peptide was cleaved from the resin using 95% trifluoroacetic acid (TFA), 2% triisopropylsilane, 2% thioanisole and 1% water for 2 h at room temperature and then it was precipitated using diethyl ether after resin separation. This long reaction time was necessary to completely remove Pbf protecting groups from the arginine clusters present in the sequence. Then 41 mg of crude products was purified on a Shimadzu HPLC model LC-8A instrument, using a preparative C_{18} reverse-phase column from Vydac (218TP1022), yielding 29 mg (72%) of pure peptide. Analytical HPLC, performed in a C_{18} column (Jupiter 10 μm, 250 × 4.6 mm; flow rate: 1 ml/min), eluted with a gradient of 5–54% CH_3CN in 0.05% aqueous TFA for 30 min, exhibited a single peak with no detectable impurities in the peptide preparation (t_r: 13.8 min; detection wavelength: 216 nm). Amino acid analysis on a Carlo Erba 3A30 amino acid analyser, after acid hydrolysis, verified the correct peptide composition [Glu 2.01 (2); Pro 1.94 (2); Gly 1.98 (2); Lys 1.97 (2); Arg 6.11 (6)]. The molecular weight of synthetic peptide was determined by ESI-MS on a Mariner (PerSeptive Biosystem) mass spectrometry instrument. The theoretical exact mass for Tat-cell penetrating peptide is 1775.1 ($C_{72}H_{134}N_{36}O_{17}$). The molecular ions $[M + 5H]^{5+} = 356.19$ and $[M + 4H]^{4+} = 444.99$, clearly seen in the ESI-MS spectra (not shown), after calculation give a mass of 1775.9.

Circular Dichroism

A peptide stock solution (10^{-3} M) was prepared in water and then diluted with the appropriate solvent to obtain the desired final solution: 5 mM Tris buffer, pH 6.8; 30 mM SDS or 90% TFE. CD spectra were measured in a 1 mm cell using a Jasco J-715 dichrograph. All data are in $\Delta\varepsilon$ (M^{-1} cm^{-1}) on a *per amide* basis. The CD spectra were repeatable with virtually no variation. The peptide concentration was determined by quantitative amino acid analysis on the stock solution.

RESULTS AND DISCUSSION

Far-UV CD spectra of the Tat cell-penetrating peptide in aqueous buffer solution over the temperature range 10°C–50°C are shown in Figure 1. At 10°C the CD spectrum is characterized by a strong and negative band at 196 nm ($\Delta\varepsilon = -8.00$ M^{-1} cm^{-1}) and a weak and positive band at 223 nm ($\Delta\varepsilon = +0.24$ M^{-1} cm^{-1}). This pattern closely resembles that of a left-handed 3_1-helix conformation. The possibility that polypeptides other than $poly(Pro)_n$ adopt a conformation near the PP_{II} helix has been reported, i.e. PP_{II} helices have been found to occur in globular proteins [11]. These secondary structure elements have been found at the binding sites of many peptide/protein and protein/protein complexes when located on the surface of proteins [11]. Furthermore, CD studies on ionized $poly(Glu)_n$ or $poly(Lys)_n$ peptides by Holzwarth and Doty [12] showed that the spectra of these compounds differ significantly from those characteristic of unordered polypeptides, while resembling that of the PP_{II} helix. In addition,

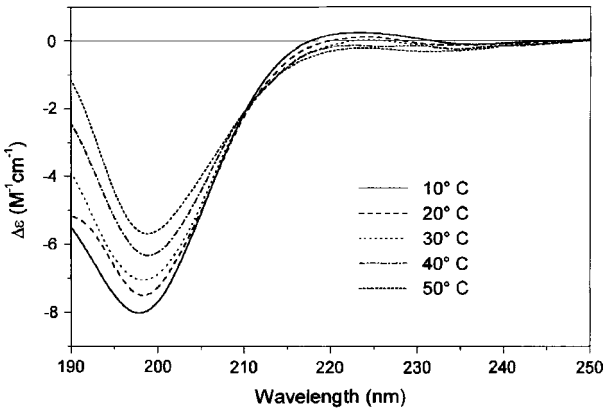

Figure 1 Far-UV CD spectra of Tat cell-penetrating peptide (13.5 μM) in Tris·HCl buffer (5 mM, pH 6.8) as a function of increasing temperature.

Krimm and Mark [13] reported that electrostatic interactions would favour a helical rather than an unordered structure in ionized polypeptides. In these cases, when the steric energy is taken into consideration, the favoured conformation is a left-handed helix having 2.5–3.0 residues per turn.

On raising the temperature, the 223 nm positive band disappears, at 40 °C the CD spectrum becoming negative over the entire wavelength range 250–190 nm. Meanwhile, the absolute value of the negative band at 196 nm decreases from $\Delta\varepsilon = -8.00$ to $\Delta\varepsilon = -5.60$ M^{-1} cm^{-1} upon increasing the temperature from 10 °C to 50 °C. In addition, an isodichroic point at 210 nm is observed. The existence of an isodichroic point implies that the system can be described by two different states, one of which predominates at low temperatures and the other prevails at high temperatures. These data, in combination with recent results from NMR experiments on the shorter segment 47–58 of the HIV-1 Tat protein [14], suggest that the low-temperature state is close to that of a 3_1-helix.

Considering a $\Delta\varepsilon_{196}$ of about -11 M^{-1} cm^{-1} for a fully ordered 3_1-helix [9] and a $\Delta\varepsilon_{196}$ of -2.3 M^{-1} cm^{-1} for a fully random coil conformation [9], the population of the 3_1-helix was estimated to be ~60% at 10 °C and ~30% at 50 °C for the Tat peptide in aqueous solution.

It is well known that the micellar concentration of SDS, as well as the TFE/water mixture, mimic the membrane environment and often induce peptides to become α-helical [15,16]. In previous studies on Antennapedia and kFGF cell-penetrating peptides, 90% TFE induced a large amount of α-helical structure [17]. On the contrary, the CD spectrum of the Tat cell-penetrating peptide in 90% TFE showed only about 20% of α-helix, as judged by the intensity of the negative 222 nm band [18] (data not shown).

The CD spectrum in a micellar concentration of SDS (30 mM) is only partially changed with respect to that of the aqueous solution. The CD spectra of the Tat cell-penetrating peptide in SDS solution over the temperature range 10 °C to 50 °C are shown in Figure 2. At 10 °C, the CD spectrum is characterized by a strong negative band at 199 nm ($\Delta\varepsilon = -7.54$ M^{-1} cm^{-1}), whereas the long-wavelength positive band at 220–230 nm range almost disappears ($\Delta\varepsilon = 0.07$ M^{-1} cm^{-1} at 225 nm), thereby suggesting that at low temperature the Tat cell-penetrating peptide adopts a 3_1-helix-like conformation also in the presence of SDS micelles. However, the near-zero value for $\Delta\varepsilon$ at 225 nm indicates the persistence of a substantial, but not

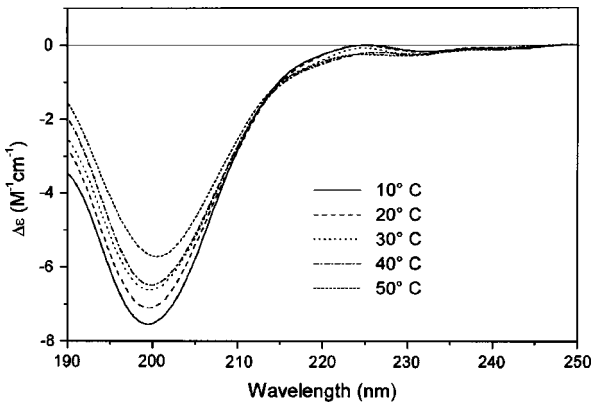

Figure 2 Far-UV CD spectra of Tat cell-penetrating peptide (13.5 μM) in 30 mM SDS solution as a function of increasing temperature.

of a dominant, amount of this conformation (see below). Upon heating, the absolute intensity of the negative CD band decreases, thus indicating that the 3_1-helical structure becomes less populated in favour of the unordered conformation. In addition, at room temperature the CD spectrum is negative over the entire wavelength range. Also in SDS micellar solutions, over the temperature range 10 °C to 50 °C, an isodichroic point at 214 nm is observed. The presence of this spectroscopic feature again permits the description of the system as a combination of two different states, where the low-temperature form is best described by a PP_{II} helix conformation. Under these conditions it is estimated that the population of the 3_1-helix is almost 50% at 10 °C and 26% at 50 °C [9].

In summary, the present CD study demonstrates that the Tat cell-penetrating peptide adopts a left-handed 3_1-helix in aqueous buffer as well as in SDS micellar solutions. In particular, the inability of membrane mimicking environments to induce either an α-helix or a β-sheet structure in the Tat cell-penetrating peptide supports the view that this peptide is folded in a stable 3_1-helix conformation even in aqueous solution. As this structure is characterized by three residues per turn, residues at position i and $i+3$ lie on the same edge of the helix. Thus, the uncharged residues of the Tat cell-penetrating peptide are located on the same edge of the helix, whereas the Arg and Lys residues are mostly located on the other two sides of the PP_{II} helix. It is concluded that this spatial disposition is suitable for the interaction of the peptide with the cellular membrane (the first step of the internalization process).

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REFERENCES

1. Langel Ü (ed.). *Cell-penetrating Peptides. Processes and Applications*. CRC Press: Boca Raton, FL, 2002.
2. Hällbrink M, Florén A, Elmquist A, Pooga M, Bartfai T, Langel Ü. Cargo delivery kinetics of cell-penetrating peptides. *Biochim. Biophys. Acta* 2001; **1515**: 101–109.
3. Prochiantz A. Messenger proteins: homeoproteins, TAT and others. *Curr. Opin. Cell Biol.* 2000; **12**: 400–406.
4. Ho A, Schwarze SR, Mermelstein SJ, Waksman G, Dowdy SF. Synthetic protein transduction domains: enhanced transduction potential *in vitro* and *in vivo*. *Cancer Res.* 2001; **61**: 474–477.
5. Vives E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* 1997; **272**: 16 010–16 017.
6. Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, Sugiura Y. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* 2001; **276**: 5836–5840.
7. Woody RW. In *Circular Dichroism and the Conformational Analysis of Biomolecules*, Fasman GD (ed.). Plenum Press: New York, 1996; 25–67.
8. Toumadje A, Johnson WC. Systemin has the characteristics of a poly(L-proline) II type helix. *J. Am. Chem. Soc.* 1995; **117**: 7023–7024.
9. Woody RW. Circular dichroism and conformation of unordered polypeptides. *Adv. Biophys. Chem.* 1992; **2**: 37–79.
10. Nielsen EB, Schellman JA. The absorption spectra of simple amides and peptides. *J. Phys. Chem.* 1967; **71**: 2297–2304.
11. Adzhubei AA, Sternberg MJE. Left-handed polyproline II helices commonly occur in globular proteins. *J. Mol. Biol.* 1993; **229**: 472–493.
12. Holzwarth G, Doty P. The ultraviolet circular dichroism of polypeptides. *J. Am. Chem. Soc.* 1965; **87**: 218–228.
13. Krimm S, Mark JE. Conformations of polypeptides with ionized side chains of equal length. *Proc. Natl Acad. Sci. USA* 1968; **60**: 1122–1129.
14. Lam SL, Hsu VL. NMR identification of left-handed polyproline type II helices. *Biopolymers* 2003; **69**: 270–281.
15. Gierasch LM. Signal sequences. *Biochemistry* 1989; **28**: 923–930.
16. Waterhouse DV, Johnson WCJ. Importance of environment in determining secondary structure in proteins. *Biochemistry* 1994; **33**: 2121–2128.
17. Ruzza P, Donella Deana A, Calderan A, Brunati AM, Massimino ML, Elardo S, Mattiazzo A, Pinna LA, Borin G. Antennapedia/HS1 chimeric phosphotyrosyl peptide: conformational properties, binding capability to c-Fgr SH2 domain and cell permeability. *Biopolymers* 2001; **60**: 290–306.
18. Chen YH, Yang, JT, Chau KH. Determination of the helix and β-form of proteins in aqueous solution by circular dichroism. *Biochemistry* 1974; **13**: 3350–3359.

