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Cationic oligopeptides with the repeating sequence L-lysyl-L-alanyl-L-alanine: conformational and thermal stability study using optical spectroscopic methods

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The infrared (IR), vibrational circular dichroism (VCD), and electronic circular dichroism (ECD) spectra of short cationic sequential peptides (L-Lys-L-Ala-L-Ala)$_n$ (n = 1, 2, and 3) were measured over a range of temperatures (20–90 °C) in aqueous solution at near-neutral pH values in order to investigate their solution conformations and thermally induced conformational changes. VCD spectra of all three oligopeptides measured in the amide I′ region indicate the presence of extended helical polyproline II (PPII)-like conformation at room temperature. UV-ECD spectra confirmed this conclusion. Thus, the oligopeptides adopt a PPII-like conformation, independent of the length of the peptide chain. However, the optimized dihedral angles ϕ and ψ are within the range −82 to −107° and 143–154°, respectively, and differ from the canonical PPII values. At elevated temperatures, the observed intensity and bandshape variations in the VCD and ECD spectra show that the PPII-like conformation of the Lys-Ala-Ala sequence is still preferred, being in equilibrium with an unordered conformer at near-neutral pH values within the range of temperatures from 20 to 90 °C. This finding was obtained from analysis of the temperature-dependent spectra using the singular value decomposition method. The study presents KAA-containing oligopeptides as conformationally stable models of biologically important cationic peptides and proteins. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cationic peptides; conformation; thermal stability; vibrational circular dichroism

Introduction

The determination of the solution structure of peptides and proteins is one of the central themes of modern biophysical research, and many experimental and theoretical methods have been developed to deduce this information. Small peptides, with less than 20 amino acids, are known to fulfill a number of biological roles. For instance, sequential cationic oligopeptides containing positively charged amino acids, such as lysine (Lys), arginine (Arg) or ornithine (Orn), which are the subject of this work, have been suitable models of biologically important small-sized proteins – histones – located in the chromosomes of eukaryotic cells. They are able to interact with negatively charged functional groups of many biologically important molecules including DNA [1,2], polyuronic acids [3], and porphyrins [4–8], and thus influence a broad range of biological functions; for instance, transcription or packing of DNA, gene regulation and others. Therefore, the studies of the structure and stability of peptides as models of protein structural elements have long been the subject of numerous spectroscopic studies [9–12].

Most optical spectroscopic secondary structure studies of peptides and proteins involve the use of electronic circular dichroism (ECD) [13] measured in the far UV for the n–π^* and π-π^* transitions of the amide linkage. Coupling of the involved electronic transition dipoles within this chiral molecule leads to the extended optical activity, which is characteristic of the chain conformation, and results in an observed ECD spectrum possessing a sign/frequency profile that is characteristic of different secondary structures [14]. Since the corresponding bands are usually broad and overlapping, ECD is less sensitive to conformation and subtle conformational changes of peptides than another chiroptical technique, vibrational circular dichroism (VCD) spectroscopy [15,16]. In the mid-IR spectral region, VCD combines the advantages of the group selectivity and frequency resolution of IR with the chiroptical specificity of ECD and provides a number of resolved, relatively localized transitions that correspond to specific bond types. Most of the peptide and protein VCD studies have been focused on the amide I region (or amide I′ in deuterated solvents) where the C=O stretching vibration of a peptide bond is localized. VCD is a powerful tool, not only for characterizing the type of qualitative secondary structure in peptides and proteins [17–20], but also for its potential for monitoring the changes of host oligopeptide conformations caused by local interactions with guest molecules [4,8] or by variation of physico-chemical conditions (pH, ionic

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strength, temperature, etc.) in solution [19,21]. For qualitative analyses one can utilize the spectral bandshape and the band position to predict the dominant secondary structural type in a peptide or protein [17,19]. However, the frequencies used as markers might be problematic in short peptides due to solvent effects and the inhomogeneity of the peptide secondary structure. Quantitative predictions of secondary structure for proteins are possible with bandshape analyses of VCD, and more detailed insight into secondary structure aspects can be obtained in combination with ECD and other spectroscopies.

In this work, solution conformations of sequential cationic oligopeptides of three different peptide chain lengths, H-(L-Lys-L-Ala-L-Ala)$_n$-OMe [(KAA)$_n$; n = 1, 2 and 3], were investigated in the temperature range 20–90 °C, using a combination of spectroscopic techniques with emphasis on VCD, ECD, and non-polarized IR absorption. The repeating sequence KAA was chosen for this study because it belongs to the family of simplest positively charged sequences, which are models of biologically important cationic peptides and proteins (antimicrobial peptides and others).

Materials and Methods

Materials

The cationic tripeptide H-(L-Lys-L-Ala-L-Ala)-OH was purchased as an acetate salt 99% in purity from Bachem AG (Germany). The hexapeptide H-(L-Lys-L-Ala-L-Ala)$_2$-OH and nonapeptide H-(L-Lys-L-Ala-L-Ala)$_3$-OH were synthesized using common methods of solid-phase peptide synthesis [22,23]. They were purified by HPLC (high-performance liquid chromatography) and their identity was confirmed by amino acid analysis, HPLC, and electrospray ionization mass spectrometry (ESI-MS). The results of analysis are summarized in Table 1. ESI-MS spectra were measured using Agilent 5075B MSD from Agilent Technologies (USA). HPLC was carried out on a 5 µm RP-18 Vydac 25 × 1 cm column with TSP instrument assembled from an SP 8800 pump, an SP 4290 integrator and TSP Spectra 100 UV detector, flow rate 3 ml min^{-1} at 220 nm, using an isocratic 0.05% TFA/aq (0–10 min) and then gradient 0–50% CH_3CN in 0.05% TFA/aq (10–60 min) mobile phase. Samples for the amino acid analysis were hydrolyzed with 6 M HCl containing 3% phenol at 110 °C for 20 h. The amino acid analyses were performed on a Bachem 20 instrument (Pharmacia, Sweden). The C-terminus of the peptides was then esterified by the procedure described elsewhere [8,24]. The esterification efficiency was checked by paper electrophoresis, which was carried out in a modified moist chamber apparatus [25] on Whatman No. 3MM paper at 20 V/cm, with 6% aqueous acetic acid (pH 2.4) and pyridine-acetate buffer (pH 5.7) as the electrolytes, for 45 min. Electrophoretic mobility (E) was expressed as the ratio of the distance from the start line of the compound concerned and the reference amino acids (Gly or His), as indicated by superscripts for tripeptide: H-Lys-Ala-Ala-OMe ($E^{Gly}_{2.4}$ 2.15; $E^{His}_{2.4}$ 1.21; $E^{His}_{5.7}$ 1.32) compared with H-Lys-Ala-Ala-OH ($E^{Gly}_{2.4}$ 1.92; $E^{His}_{2.4}$ 1.05; $E^{His}_{5.7}$ 0.85). Finally, the ESI-MS spectra confirmed the C-terminus esterification (for H-Lys-Ala-Ala-OMe: $C_{13}H_{26}N_4O_4$ – calculated 302.38; found m/z 303.21).

All three compounds were then repeatedly treated (typically three times) with 0.1 mol l^{-1} DCl in D_2O, and lyophilized to remove the acetate and TFA ions that can interfere with the amide I′ band in IR and VCD spectra. This process, which was continued until the IR absorbance of the acetate and TFA was eliminated, also resulted in a substantial H-D exchange of the amide groups.

Table 1. Analytical data of hexa- and nonapeptide sequential oligomers

Peptide	Formula Calc./found (m/z)	HPLC RT (min)	Amino acid analysis Lys	Ala
H-(Lys-Ala-Ala)$_2$-OH	$C_{24}H_{46}N_8O_7$ 558.68/559.35	8.73	1.00	1.98
H-(Lys-Ala-Ala)$_3$-OH	$C_{36}H_{68}N_{12}O_{10}$ 829.02/829.52	13.87	1.00	2.02

Sodium deuteroxide (NaOD, 99.5% D) and deuterated phosphoric acid (D_3PO_4, 99% D) were purchased from Aldrich and used to prepare 0.2 mol l^{-1} deuterated sodium phosphate/D_2O buffer solution (pH = 6.1 uncorrected); D_2O (99.9% D) was purchased from Isosar GmbH (Germany).

FTIR and VCD Measurements

The samples for IR-absorption and VCD temperature-dependent measurements were prepared by dissolving the oligopeptides at a concentration 66 g l^{-1} in 0.2 mol l^{-1} sodium phosphate/D_2O buffer (pH = 6.1). Prior to each spectral measurement, the pH values were measured using a glass pH-microelectrode (9802BN, Orion) and a digital pH-meter (Cole-Parmer) without correction for the isotope effect. The solutions were placed in a demountable electrically heated cell (P/N20500, Specac, UK) constructed of CaF_2 windows separated by a 25 µm Teflon spacer and equipped with the heated jacket controller (3000 Series, Specac, UK). The temperature range studied was 20–90 °C.

IR and VCD spectra were recorded on an IFS-66/S FT-IR spectrometer equipped with the VCD/IRRAS module PMA 37 (Bruker, Germany), as described elsewhere [26]. The spectral resolution was 8 cm^{-1}. VCD spectra were measured in three blocks of scans; each block of 3680 scans was accumulated for 20 min. Each spectrum was processed with a zero-filling factor of 4. The final VCD spectra were averaged from these three blocks and baseline correction was performed. The quality of our VCD measurements was demonstrated by a typical noise spectrum calculated as the standard deviation of all three blocks of VCD scans.

ECD Measurements

The ECD spectra of peptides at concentrations from 0.50 to 0.69 g l^{-1} were recorded on a Jasco J-810 spectropolarimeter (Japan) equipped with a thermostatted cell holder attached to a Jasco Peltier temperature control system PTC-423S with an accuracy of ±0.2 °C. The temperature range from 10 to 90 °C was used in discrete 10 °C steps. The spectra were measured in a 1 mm quartz cell with 1 nm bandwidth, 2-s response and 50 nm min^{-1} scanning speed. Four blocks of scans were recorded, averaged and baseline subtracted using a spectrum of the solvent obtained under the same experimental conditions.

Molecular Modeling

Molecular modeling and geometry optimization were performed using the semi-empirical PM3 and AM1 methods provided in the commercially available HyperChem 7.52 package (Hypercube, Inc., Waterloo, Ontario, Canada). The geometry was first optimized for

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the KAA sequence and then this geometry was used to build up the KAAKAA and KAAKAAKAA structures. Finally, the hexapeptide and nonapeptide geometries were optimized. All optimizations were performed with the Polak-Ribiere algorithm, using an root mean squared gradient of 0.1 kcal Å^{-1} mol^{-1}.

Data Processing

To help understand the thermal stability of oligopeptides studied, the singular value decomposition (SVD) method of factor analysis (FA), a common technique for the analysis of multivariate data, was applied. This method can identify those subspectral components that represent the major, thermally dependent band shape variances in our datasets. The data were treated using a SVD/FA software package developed in the laboratory of Prof. Jiří Bok (Institute of Physics of Charles University, Czech Republic) and described in literature [27,28].

Results and Discussion

IR and VCD Spectra of KAA and KAAKAA

The IR spectra of the tripeptide KAA and hexapeptide KAAKAA measured in the amide I′ region at different temperatures are presented in Figure 1. At 20 °C, the amide I′ band maximum occurred at 1651 cm^{-1} for KAA and at 1649 cm^{-1} for KAAKAA. These band positions might be indicative of the polyproline II (PPII)-like conformation, a left-handed 3_1 helix ('extended' helix) of *trans* peptides [19,29]. In agreement with the literature [30], at shorter oligomer chain lengths, a shift up in frequency was observed. When gradually heated to 90 °C, a variation of peak intensities and a shift of the absorption maxima to 1660 and 1656 cm^{-1} were observed for KAA and KAAKAA, respectively (Figure 1, insets). Due to the presence of isosbestic points in the spectra of both peptides (located at ~1655 and 1653 cm^{-1} for KAA and KAAKAA, respectively), the possibility of a multi-state unfolding mechanism during the heating was very low. Rather, the IR spectra of both peptides indicate a slight change of the conformation ordering or a loss of the PPII-like structure at high temperatures.

In order to support this hypothesis, both sets of temperature-dependent IR spectra were analyzed by SVD/FA method for the spectral region shown in Figure 1. The first two component spectra (factors) described more than 98% of the total bandshape changes and, therefore, became the focus of our attention. The results are presented in Figure 2. In general, the first component spectra S1 and their coefficients (loadings) C1 represent the average intensity of the IR pattern, which varied little due to the small variation of C1 values. The second components S2, which had a derivative shape, contributed to the major thermal spectral variation and represent the frequency shift of the amide I′ absorption maximum. Their loadings C2 increased monotonically with increasing temperature, corresponding to an insignificant change of the PPII-like conformation for either KAA or KAAKAA. Insignificant loadings of the third component (describing less than 1% of spectral changes, not shown) indicate negligible contribution to the intensity from other well-organized conformations whose populations would grow in during the thermal process. SVD/FA analysis of IR spectra thus indicated that only one component (C2) significantly contributes to the bandshape variation. Generally, if just one component is important and its coefficient would track the steady loss in intensity, then

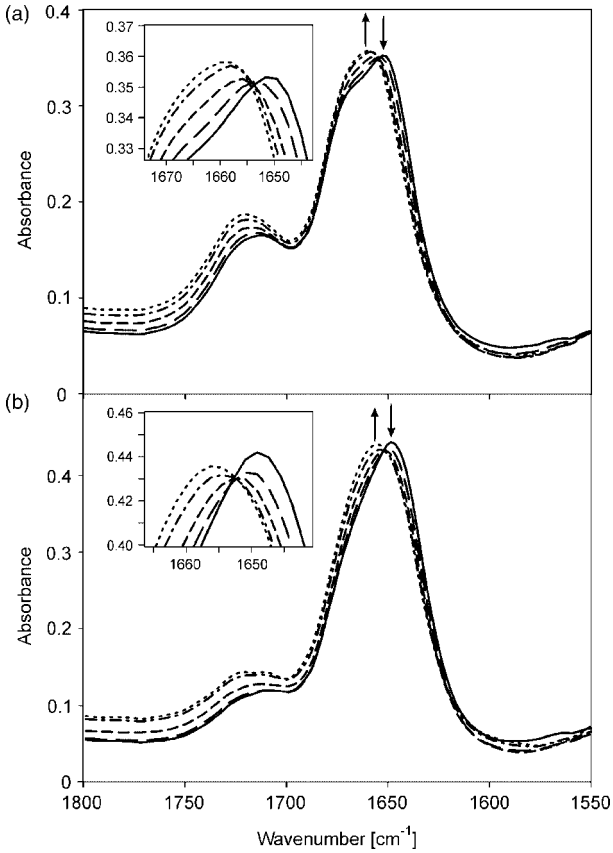

Figure 1. Amide I′ temperature-dependent IR spectra of KAA (a) and KAAKAA (b) in deuterated phosphate buffer pH = 6.1. (Solid line, 20 °C; long dash, 40 °C; short dash, 60 °C; dash-dot, 80 °C; dotted, 90 °C). Arrows indicate the direction of intensity change with increasing temperature. Insets: the region close to isosbestic point.

the data reflect a steady structural loss of a single conformational type. Thus, an unordered conformer might be populated at the expense of the PPII-type conformer at higher temperatures. As a consequence, the extended PPII-like conformation coexists with an unordered structure of KAA and KAAKAA at elevated temperatures.

Since the intrinsic sensitivity of VCD to the local structure of peptides and proteins has been proven in previous studies, e.g. [17,19,20], temperature-dependent VCD spectra of KAA and KAAKAA were measured (Figure 3) and analyzed in the same spectral region as used for IR. At 20 °C, the VCD spectra of both oligopeptides showed a negative couplet consisting of an intense negative band located at 1650–1640 cm^{-1} and a weak positive band at about 1675 cm^{-1}. According to the literature [4,19,29], this pattern is characteristic of the PPII-like conformation, a structure with left-handed local ordering. This finding thus fully supports our IR observation.

It should be noted here that since VCD is sensitive to relatively short-range interactions, it detects residual structures in oligopeptides as short as the tripeptide, even when the long-range order is impossible [12]. Therefore, the conformation of a short tripeptide is best viewed as an ordered left-handed turn or left-handed twist that, at least locally, is similar to that of a left-handed helical PPII-like conformation. This is consistent with the earlier findings of the Keiderling group [19], who studied the conformation of analogous, but more strongly charged, oligopeptides (oligomers and polymers based on lysine) as a

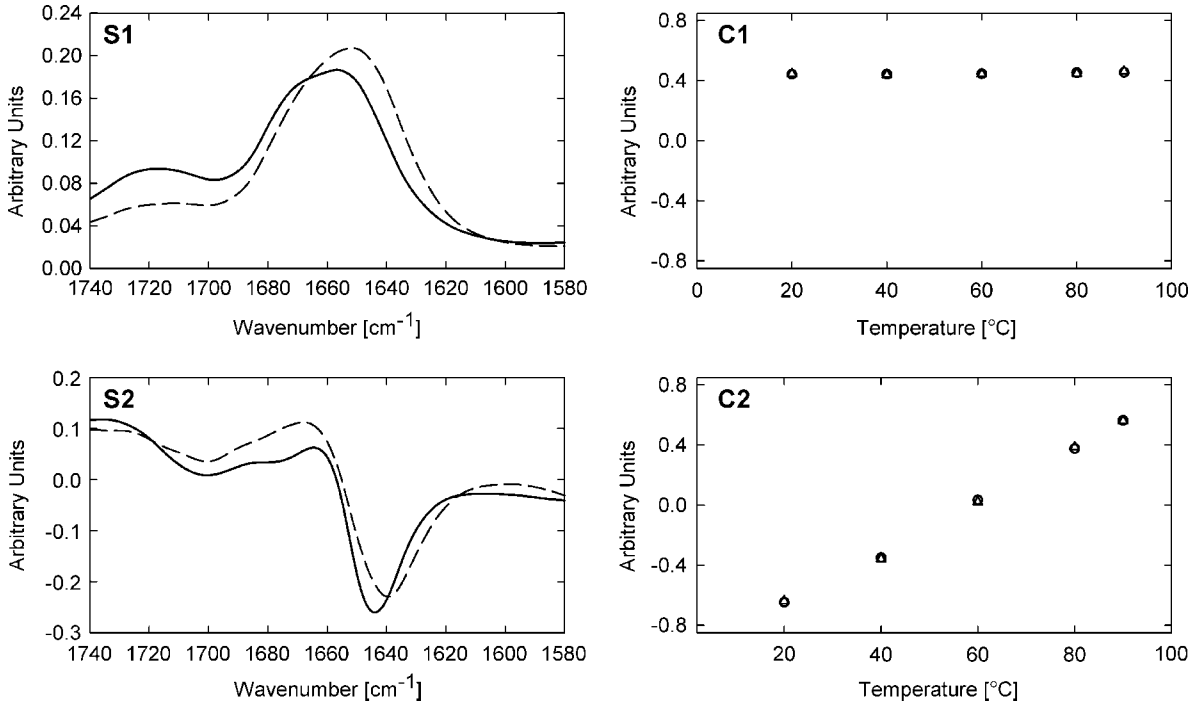

Figure 2. First and second component spectra (S) and their coefficients (C) derived from the amide I′ IR spectra for KAA and KAAKAA using the SVD method (data in Figure 1). Component spectra: KAA, full line; KAAKAA, dashed line. Coefficients: KAA, circles; KAAKAA, triangles.

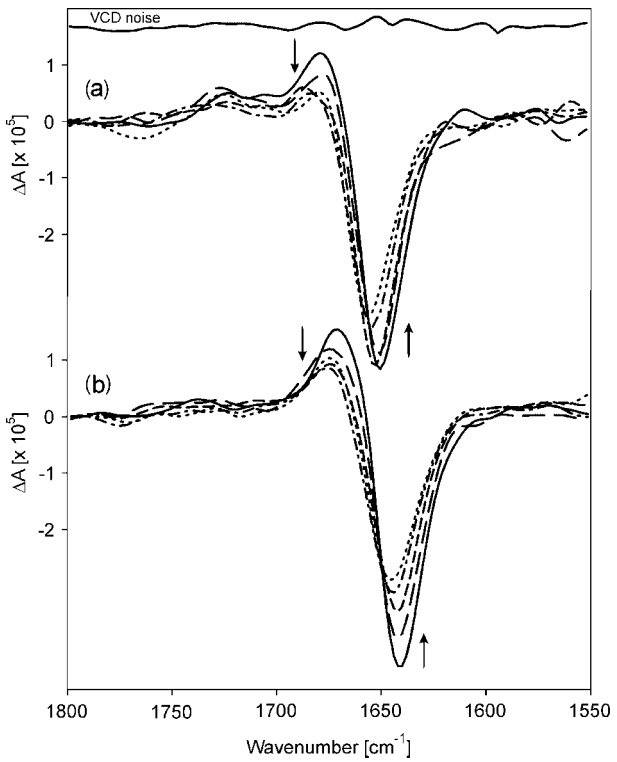

Figure 3. Amide I′ temperature-dependent VCD spectra of KAA (a) and KAAKAA (b) in deuterated phosphate buffer pH = 6.1. (Solid line, 20 °C; long dash, 40 °C; short dash, 60 °C; dash-dot, 80 °C; dotted, 90 °C). Arrows indicate the direction of intensity change with increase in temperature. The typical VCD noise spectrum is also shown.

function of peptide length. They found residual local ordering in these so-called random coils, thereby proving that such conformations maintain some short-range structure even when the long-range order is lost. This means, therefore, that the random coil conformation is not actually random, but rather ordered, depending on the amino acid composition. In addition, Eker *et al*. [10–12] have confirmed that even very small peptide fragments such as tri- and tetrapeptides can adopt a PPII-like conformation if they contain the appropriate amino acid residues. The same authors also found that trilysine and alanine-based tripeptides containing Lys residues exhibit a substantial population of the PPII-like conformation. Our findings that tripeptide KAA can adopt the PPII-like conformation is thus not surprising.

At elevated temperatures, the VCD of both peptides was notably reduced in intensity. While the hexapeptide showed a more pronounced intensity change in the amide I′ region, the shape of the VCD spectra characteristic for PPII-like conformation was maintained for both the peptides even at 90 °C. This indicates considerable conformational uniformity and stability of the KAA sequence independently of chain length and temperature. As discussed above for IR, it is reasonable to assume that the decrease in the VCD signal at higher temperatures is consistent with a partial loss of PPII-like structure, along with a higher population of an unordered conformer in these peptides due to enhanced chain motions.

UV-ECD Spectra of KAA, KAAKAA and KAAKAAKAA

Temperature-dependent ECD spectra of the tripeptide, hexapeptide, and nonapeptide are shown in Figure 4. The spectra of all three peptides at room temperature showed approximately the same band positions: a strong negative band at ~193 nm and a weak positive band at ~217 nm. In accordance with the literature [14,31], this bandshape is characteristic of the PPII-like conforma-

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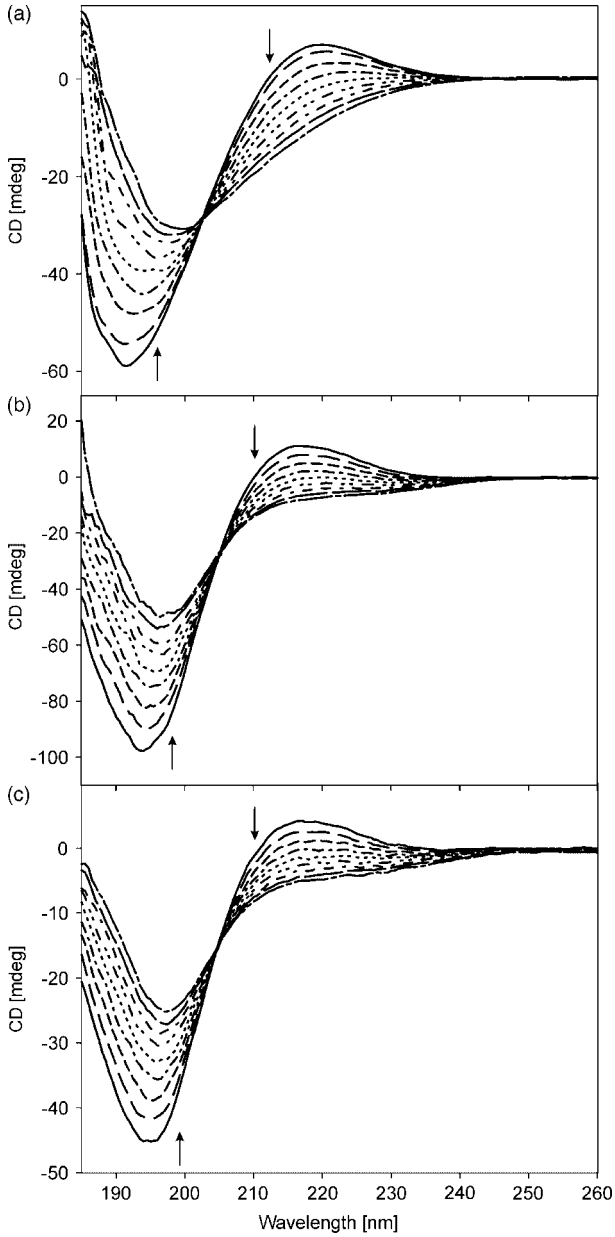

Figure 4. ECD spectra of KAA (a), KAAKAA (b) and KAAKAAKAA (c) in deuterated phosphate buffer pH = 6.1. Samples were heated from 10 to 90 °C in steps of 10 °C. Arrows indicate the direction of intensity change with increase in temperature.

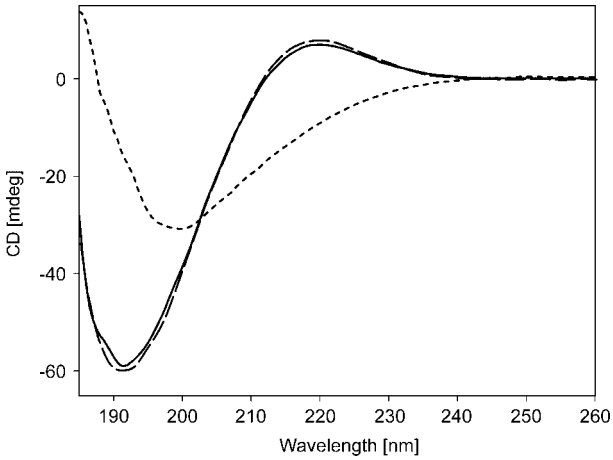

Figure 5. ECD spectra of KAA in deuterated phosphate buffer pH = 6.1 measured at 10 °C (full line) before heating to 90 °C (short dash) and then immediately after cooling back to 10 °C (long dash).

tion. Therefore, this observation fully supports the IR and VCD results discussed above.

As the temperature was increased, a gradual shift of the negative band to ~200–197 nm and a decrease in intensity were observed for all the peptides, independently of peptide chain length. In fact, the positive bands at ~217 nm disappeared and the negative bands decreased in intensity to about 52, 51, and 55% for KAA, KAAKAA, and KAAKAAKAA, respectively, when reaching 90 °C. Then, the samples were immediately cooled back to 10 °C and the spectra showed a full reversibility of the ECD signal, as presented for the tripeptide in Figure 5. The spectra for the hexa- and nonapeptide are not shown because of similar signal reversibility as observed for the tripeptide. Such reversibility suggested a full reversibility of the PPII-like structure upon cooling. This means that all the peptides adopt a well-organized PPII-like conformation, which has the same ordering as before the thermal treatment.

Because the spectra measured at high temperatures deviated considerably (in terms of band positions and intensities) from the canonical curves of the three standard conformations [α-helical; β-sheet; 'random coil' (PPII)], as measured, for instance, on poly-L-lysine by Greenfield and Fasman [14], we compared our results with studies describing temperature-dependent ECD experiments on similar molecules. For instance, Tiffany and Krimm [32], who investigated the temperature-dependent ECD spectra of negatively and positively charged polypeptides, found similar variations, as we have observed in Figure 4, in their ECD spectra of poly-L-glutamic acid and poly-L-lysine. At elevated temperatures, the ECD patterns weakened (positive bands disappeared and the negative bands decreased significantly in intensity), and changed their shape (shift of the negative bands to higher wavelengths). They interpreted such spectral changes as an increasing portion of the chains developing unordered conformations. The ECD spectra would then consist primarily of a superposition of the ECD spectra of unordered and of PPII-like conformations. One might argue that the temperature dependence of the ECD spectra shown in Figure 4 indicates the presence of at least another ordered conformer. However, the coexistence of only two conformations (two-state model) was supported by the presence of isodichroic points in our ECD spectra (Figure 4), as well as by results of their SVD analysis. The first two component spectra, shown in Figure 6, describe more than 95% of the total bandshape changes. The contribution from the third components was lower than 3% for all the peptides and is therefore not discussed here. Loadings C1 and C2 of the three peptides showed a monotonic change with increasing temperature, corresponding to a significant population of a PPII-like conformation within the range of temperatures studied. SVD/FA analysis of ECD thus confirmed our IR and VCD results suggesting that the PPII-like conformation of KAA, KAAKAA, and KAAKAAKAA remains preferred conformation and coexists with an unordered conformation at elevated temperatures.

Finally, in order to visualize presumable conformations of all three oligopeptides, we modeled and optimized their structures using the semi-empirical AM1 and PM3 methods. No angles or other parameters were fixed during the optimization. It was found by both methods that the averaged torsion angles ϕ

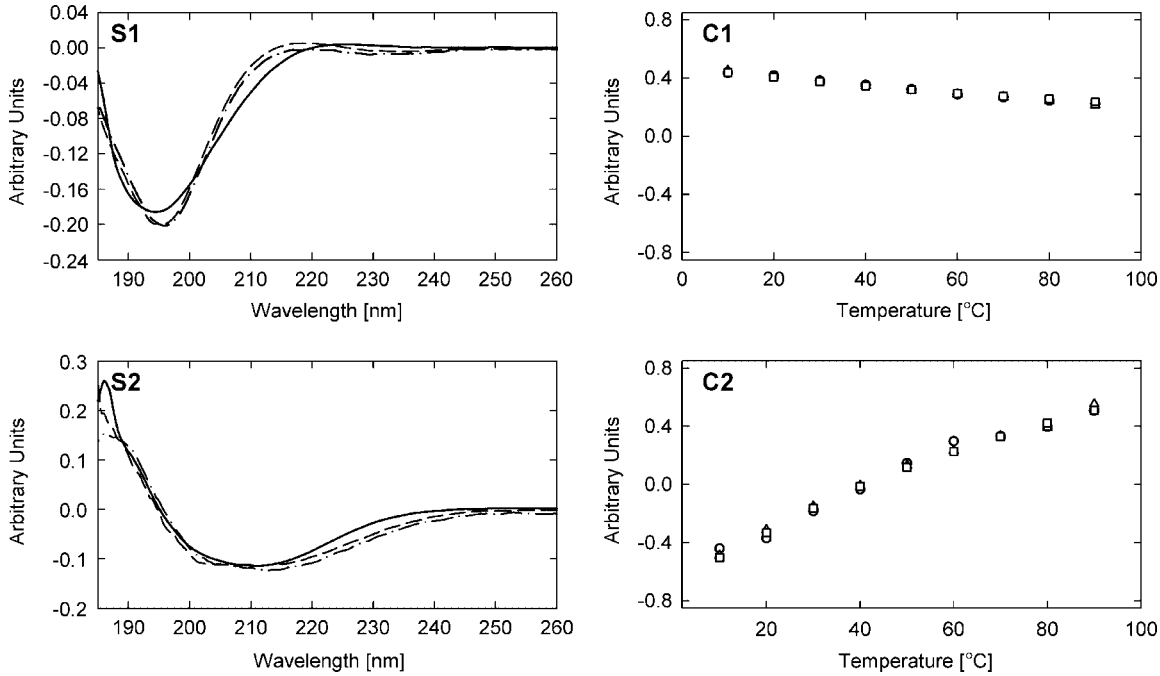

Figure 6. First and second component spectra (S) and their coefficients (C) derived from the ECD spectra for KAA, KAAKAA, and KAAKAAKAA using the SVD method (data in Figure 4). Component spectra: KAA, full line; KAAKAA, dashed line; KAAKAAKAA, dash-dotted line. Coefficients: KAA, circles; KAAKAA, triangles; KAAKAAKAA, squares.

Table 2. Averaged torsion angles (°) of KAA, KAAKAA and KAAKAAKAA: geometry optimized using semi-empirical AM1 and PM3 methods

Peptides	ϕ		ψ	
	AM1	PM3	AM1	PM3
KAA	−105.2	−82.2	150.5	153.9
KAAKAA	−106.8	−101.1	152.9	150.1
KAAKAAKAA	−101.1	−92.8	143.9	143.9

and ψ (Table 2), essential dihedral coordinates determining the backbone conformation of a polypeptide chain [33], lay in the upper left quadrant of the Ramachandran plot [33,34] that consists of the broad allowed region comprising PPII-type, β-turn, and β-strand conformations. Compared with these conformations, the ϕ and ψ values we have found are in a good agreement with the values reported for PPII-like conformation (canonical PPII shows $\phi \sim -75^{\circ}$ and $\psi \sim 145^{\circ}$) [34]. Our ϕ values, however, were more negative and ψ values slightly more positive than found for the canonical PPII, but still located in the general PPII-type region (more displaced from the ideal PPII toward the upper border of the Ramachandran plot), indicating the presence of an extended PPII conformation. These results reflect accurately our IR-absorption, VCD and ECD interpretations discussed above. The preferred structures of the investigated peptides obtained by PM3 method are shown in Figure 7.

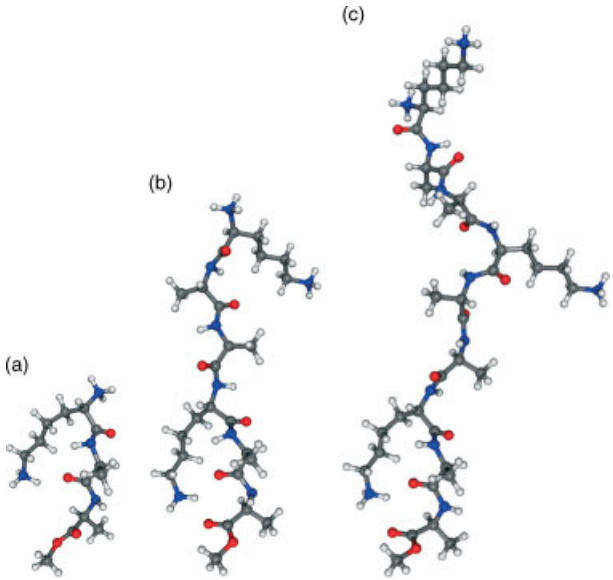

Figure 7. Optimized structures of KAA (a), KAAKAA (b), and KAAKAAKAA (c), using the semi-empirical PM3 method.

Conclusion

In this work, we focused on the conformation and thermal stability of KAA-containing cationic sequential oligopeptides in aqueous solution. We found, based on IR-absorption, VCD and ECD results, that $(KAA)_n$ oligopeptides, where $n = 1$, 2, and 3, adopt a left-handed polyproline II (PPII)-like conformation at room temperature, when dissolved in aqueous solution at near-neutral pH values. In case of the shortest peptide, KAA, such a conformation is best viewed as a left-handed turn or left-handed twist, which, at least locally, is similar to that of a left-handed helical PPII. Our data suggest that the temperature and peptide chain length impact the conformation of these oligopeptides to a low degree. Thus, PPII-like conformation is preferred

conformation, which may be in a thermodynamic equilibrium with an unordered conformation at higher temperatures. It was also confirmed by SVD analysis of temperature-dependent IR and ECD spectra that the population of significantly different, somehow ordered, conformations is negligible. Therefore, the study presents KAA-containing oligopeptides as conformationally stable models of biologically important cationic peptides and proteins. Furthermore, this study demonstrates convincingly that the combination of IR-absorption, VCD and ECD spectroscopies provides a powerful tool for the determination and monitoring of the secondary structure of peptides in solution.

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