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Influence of side-chain-terminating moieties on the conformation of branched polypeptides and their conjugates with 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone

Ferenc Hudecz [a], Judit Kajtár [a] and Mária Szekerke [b]

[a] *Department of Organic Chemistry, L. Eötvös University Muzeum krt. 4/B, H-1088 Budapest and* [b] *Research Group for Peptide Chemistry, Hungarian Academy of Science, Budapest, Hungary*

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Branched polypeptide; 4-Ethoxymethylene-2-phenyl-5(4H)-oxazolone; Polypeptide-hapten conjugate; CD; Conformation

Poly(Lys-(X_f-DL-Ala_m)) polypeptides carrying hydrophilic (X = His, Glu, Lys) or hydrophobic (X = Nle, Ile, Phe) amino acid residues and their conjugates with 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone were synthesized. The conformational properties of carrier polypeptides and conjugates were studied by circular dichroism (CD) spectroscopy in the wavelength regions 190–250 and 310–380 nm, with the emphasis on analysis under near physiological conditions. Based on CD studies, it could be demonstrated that the helix-forming capacity appears to be related to the hydrophobic nature of the branch-terminating amino acid of the branched polypeptides. With respect to carrier function, the presence of a coupled derivative of oxazolone at the side chain termini generally promotes the formation of helical secondary structure. The absolute configuration of the side-chain-terminating amino acids was found to be important for the local orientation of the hapten molecule in the conjugates.

1. Introduction

Macromolecular carriers have been used to achieve target-specific drug delivery of antitumor drugs, enzymes and toxins [1,2]. In immunological investigations, proteins such as KLH and BSA are frequently used as carriers of artificial synthetic haptens in order to induce high levels of hapten-specific immune response. In order to construct synthetic vaccines, several antigenic determinants of viral or bacterial origin have recently been covalently coupled to proteins or polypeptides [5–7]. For a better understanding of the function of macromolecular carriers, we have developed a versatile model system [8–10]. This group of synthetic, branched polypeptides is based on poly(L-lysine) substituted at the ϵ-amino groups by side chains composed of about three DL-alanine and one additional (X) amino acid residue (fig. 1a). The chemical structure (amino acid composition, side chain distribution and conformation) [10–16] and immunological properties (immune response, immunomodulatory potential) of these polypeptides have been characterized in detail [17–20].

These branched polypeptides were coupled with 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone (oxazolone, Ox) [21], in order to analyse systematically the structural features of macromolec-

Correspondence address: F. Hudecz, Department of Organic Chemistry, L. Eötvös University, Muzeum krt. 4/B, H-1088 Budapest, Hungary.

Abbreviations: in this paper, abbreviations follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature [3] in accord with the recommended nomenclature for graft polymers [4]. KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; Ox, oxazolone, 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone; phOx coupled derivative of Ox; CD, circular dichroism; AK, poly(Lys-(DL-Ala_m)); XAK, poly(Lys-(X_f-DL-Ala_m)); AK-phOx$_j$, poly(Lys-(phOx$_j$-DL-Ala_m)); XAK-phOx$_j$, poly(Lys-(phOx$_j$-X_f-DL-Ala_m)).

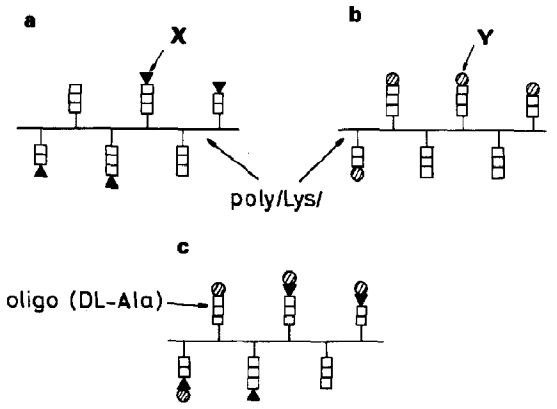

Fig. 1. Schematic representation of branched polypeptides (a) with a single amino acid (X), (b) with 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone derivative (Y), (c) with a single amino acid (X) and 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone derivative (Y) at the side-chain termini.

ular carrier-hapten conjugates. The characteristics of the immune response induced by oxazolone are well-documented in the literature. Therefore, this compound is widely used in basic immunology as a synthetic, antigenic determinant [22–25].

In the present report, we describe the synthesis of oxazolone conjugates with poly(Lys-(X_i-DL-Ala_m)), (XAK), where X = Leu, D-Leu, Phe or D-Phe. This was carried out similarly to the process described previously [26] for oxazolone conjugates with poly(Lys-(DL-Ala_m)) (AK) representing the common inside core of branched polypeptides (fig. 1b and c). Conformational studies were based on the analysis of CD spectra of branched polypeptides containing hydrophilic or hydrophobic side-chain-terminating amino acid residues of the general formula poly(Lys-(X_i-DL-Ala_m)). These spectra were compared with those of the oxazolone conjugates {poly(Lys-($phOx_j$-DL-Ala_m)), (AK-$phOx_j$) and poly(Lys-($phOx_j$-X_i-DL-Ala_m)), (XAK-$phOx_j$)} under near physiological conditions.

2. Experimental

2.1. Materials

Branched polypeptides and their conjugates with 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone were synthesized in our laboratory. Amino acids and 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone were purchased from Reanal (Hungary) and Sigma (U.S.A.), respectively.

2.2. Synthesis of branched polypeptides

Poly(Lys-(DL-Ala_m)) (AK) was prepared by grafting short oligomeric DL-Ala chains onto the ε-amino groups of poly(L-lysine). Poly(Lys-(X_i-DL-Ala_m)) (XAK) was synthesized by coupling a suitably protected amino acid pentachlorophenyl ester or azide to the α-amino groups of AK. Protecting groups were removed completely from the branched polypeptides with HBr in glacial acetic acid. The deprotection was confirmed by ultraviolet spectroscopy at 254 nm. Details of the procedures for synthesis have been reported in previous papers [8,9]. The polypeptides were characterized by amino acid analysis, average relative molar mass determination using sedimentation analysis and gel-permeation chromatography [15]. Characteristic values of the branched polypeptides are summarized in table 1.

2.3. Synthesis of branched polypeptide-$phOx_j$ conjugates

Coupling of 4-ethoxymethylene-2-phenyl-5-(4H)-oxazolone to the branched polypeptides was carried out using the method previously described [22,27] for the modification of proteins by oxazolone, with some alterations. Briefly, the method entailed adding a 1% solution of oxazolone in ethanol in aliquots to a stirred 0.1% solution of the polypeptide in 0.01 mol/l carbonate buffer at pH 9 (in the case of AK), or 0.01 mol/l phosphate buffer at pH 7.5 (in the case of XAK), at 0°C. The input molar ratio of polypeptide to oxazolone is 1 : 10–15 and of buffer to ethanol, 100 : 1 (v/v). Stirring was continued for 2 h and the reaction was allowed to proceed overnight at 4°C. The resulting solution was dialyzed extensively against water or 0.2 mol/l NaCl (pH 7.3) for 4 days.

The phOx content of the conjugates was determined spectrophotometrically as described by Mäkelä et al. [29]. It was assumed that the oxazolone coupled to the α-amino group of alanine (in

the case of AK) or other amino acids such as Phe or Leu (in the case of XAK) has the same molar extinction coefficient at $\lambda = 350–351$ nm as oxazolone coupled to ϵ-aminocaproic acid ($\lambda_{max} = 352$ nm, $\epsilon = 32000$). Analytical data on the conjugates are presented in table 2.

2.4. Spectroscopic measurements

Absorption spectra were recorded on a Specord ultraviolet-visible (Carl Zeiss) spectrophotometer in cells of optical paths 1.0, 0.2 or 0.1 cm at room temperature. The compounds were dissolved in an ethanol/water mixture (1 : 9, v/v) or in 0.2 mol/l NaCl solution at pH 7.3. CD spectra were recorded using a Roussel-Jouan model III dichrograph (Jobin-Yvon) in cells of optical paths 1.0, 0.2 and 0.1 cm at room temperature. The dichrograph was calibrated with epiandrosterone at 304 nm and D-(−)-pantoyllactone at 220 nm [28]. The samples were dissolved in 0.2 mol/l NaCl and the pH was adjusted by the addition of 0.1 mol/l NaOH or 0.1 mol/l HCl. The concentration of the solutions was approx. 0.5 mg/ml. The data from the CD measurements were expressed in terms of $\Delta\epsilon$, calculated for one lysine residue of the main chain carrying a whole side chain.

3. Results and discussion

3.1. Conformation of branched polypeptides

The branched polypeptides studied in this paper are built up of a sequence of three DL-alanine residues with another L-amino acid (His, Glu, Lys, Leu, Nle, Ile or Phe) at their amino-termini. The interpretation of the CD spectra presented in fig. 2 was based on the well-characterized CD spectra of poly(Lys) from which all the branched polypeptides have been derived. In the uncharged state (i.e., alkaline solution), poly(Lys) assumes an α-helical conformation. The CD spectrum is characterized by two negative maxima of approximately equal intensity at 221 and 208 nm. In neutral and acidic solutions, the ϵ-amino groups of poly(Lys) are in the charged state and as a consequence of electrostatic repulsion, the backbone of the polymer adopts an essentially unordered structure. The respective CD spectrum is characterized by a weak negative and weak positive maximum at 218 and 214 nm, respectively, and a strong negative maximum at 199 nm. This spectrum is regarded as belonging to unordered structure [30–32].

In the case of branched polypeptides, the term 'helical conformation' is used mainly when the CD spectrum reflects an ordered spatial arrangement of the backbone. It has been demonstrated [10,13] that in the case of the α-helical conformation, the DL-alanine residues do not contribute to the overall optical activity. It might be assumed that the contribution of the side-chain-terminating L-amino acid residues is much smaller than that of the lysine residues of the ordered main chain. However, the contribution of the branch-terminating L-amino acids to the total CD spectrum proved to be somewhat greater, as significant differences were observed in the CD spectra of polypeptides of different constitution.

The CD spectra of the branched polypeptides studied in 0.2 mol/l NaCl at pH 7.3 are given in fig. 2. The polypeptides containing branches with one Lys, Glu, His, Phe, Nle or Ile residue as chain termini have free basic groups which are charged at neutral pH. The CD spectra of poly(Lys-(His$_i$-DL-Ala$_m$)), poly(Lys-(Glu$_i$-DL-Ala$_m$)) and poly-(Lys-(Lys$_i$-DL-Ala$_m$)) are similar to those of poly-(Lys) under similar conditions and thus are regarded as assuming an unordered conformation [12–14]. In cases where Ile, Nle and Phe are side-chain termini, however, the CD spectra of the polypeptides in 0.2 mol/l NaCl (pH 7.3) display features that are characteristic of the spectrum of an α-helix. A similar effect was observed with Leu [13] and Tyr (unpublished results) coupled to poly(Lys-(DL-Ala$_m$)).

Consequently, the CD measurements carried out at medium ionic strength and at neutral pH (i.e., under near physiological conditions) indicate a marked dependence of the conformation of the branched polypeptides on the identity of the side chain terminating amino acid. These observations are consistent with our earlier findings with branched polypeptides in low ionic strength solutions at neutral pH [10,12–14]. It appears that the

Table 1

Characteristics of branched polypeptides

Compound	Code [a]	Molar ratio of amino acids			$\overline{M}_w$ [c]
		Lys	Ala_m	X_l [b]	
Poly(Lys-(Phe$_l$-DL-Ala$_m$))	FAK	1	3.0	0.9	54700
Poly(Lys-(D-Phe$_l$-DL-Ala$_m$))	D-FAK	1	3.0	0.92	55200
Poly(Lys-(Leu$_l$-DL-Ala$_m$))	LAK	1	2.9	0.76	73500
Poly(Lys-(D-Leu$_l$-DL-Ala$_m$))	D-LAK	1	3.0	0.95	52700
Poly(Lys-(Ile$_l$-DL-Ala$_m$))	IAK	1	3.0	0.95	52700
Poly(Lys-(Nle$_l$-DL-Ala$_m$))	NleAK	1	3.0	0.95	52700
Poly(Lys-(His$_l$-DL-Ala$_m$))	HAK	1	3.0	0.8	56100
Poly(Lys-(Glu$_l$-DL-Ala$_m$))	EAK	1	2.95	0.81	76300
Poly(Lys-(Lys$_l$-DL-Ala$_m$))	KAK	1	2.95	0.60	84500

[a] Based on the single-letter code for amino acids (except Nle).
[b] X = Phe, D-Phe, Leu, D-Leu, Ile, Nle, His, Glu or Lys.
[c] Calculated from the average degree of polymerization of poly(Lys) and the side chain composition.

Table 2

Characteristics of branched polypeptide-phOx$_j$ conjugates

Compound	Code [a]	Molar ratio		$\overline{M}_w$ [b]
		Peptide	phOx$_j$	
Poly(Lys-(phOx$_j$-DL-Ala$_m$))	AK-phOx$_{9.6}$	1	9.6	37800
Poly(Lys-(phOx$_j$-Leu$_l$-DL-Ala$_m$))	LAK-phOx$_{8.0}$	1	8.0	43500
Poly(Lys-(phOx$_j$-D-Leu$_l$-DL-Ala$_m$))	D-LAK-phOx$_{13.0}$	1	13.0	54000
Poly(Lys-(phOx$_j$-Phe$_l$-DL-Ala$_m$))	FAK-phOx$_{7.0}$	1	7.0	56300
Poly(Lys-(phOx$_j$-D-Phe$_l$-DL-Ala$_m$))	D-FAK-phOx$_{6.9}$	1	6.9	56800

[a] Based on the single-letter code for amino acids and phOx, 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone derivative.
[b] Calculated from the average degree of polymerization of poly(Lys) and the side chain composition.

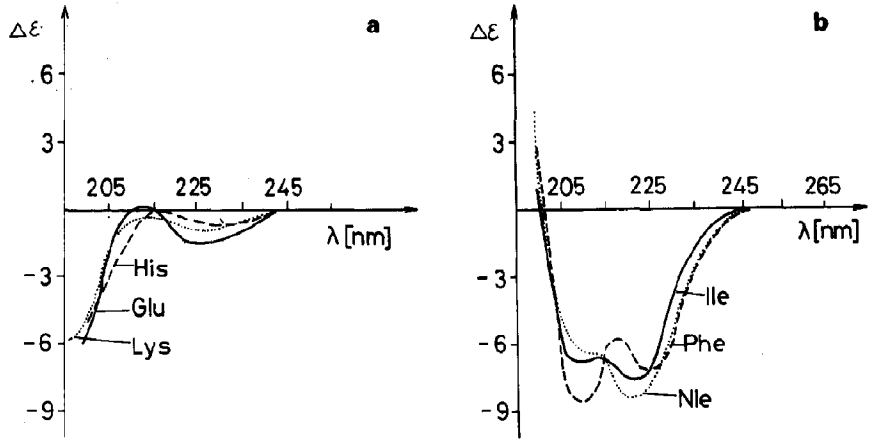

Fig. 2. CD spectra of branched polypeptides in 0.2 mol/l NaCl at pH 7.3. (a) HAK (- - - - -), EAK (———), KAK (· · · · · ·); (b) IAK (———), FAK (- - - - -), NleAK (· · · · · ·).

helix-forming ability of the backbone is related to the hydrophobic nature of the branch-terminating amino acids. The presence of Nle, Ile, Leu or Phe results in a significantly increased tendency toward the formation of ordered structure. In contrast, Lys, His or Glu as side-chain terminal residues favour the formation of an unordered conformation.

3.2. *Conformational characteristics of the branched polypeptide-phOx$_j$ conjugates*

A number of branched polypeptide-phOx$_j$ conjugates were synthesized by spontaneous coupling of 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone to the α-amino groups of polypeptides at slightly alkaline pH. Table 2 lists the abbreviations and characteristic data for the conjugates. Protein and poly(Lys) conjugates of oxazolone were prepared using a similar procedure [22,26,27,29].

The spectroscopic properties (absorption and CD) of polypeptide-phOx$_j$ conjugates were extensively studied in solution. The absorption spectrum of the LAK-phOx$_{8.0}$ conjugate can be regarded as a prototype (fig. 3), as all of the other conjugates reported in table 2 display very similar spectra. As reference compounds, we have recorded the absorption spectra of oxazolone in an ethanol/water (1 : 9, v/v) mixture and of LAK in 0.2 mol/l NaCl at pH 7.3 (fig. 3). The most important observation derived from these spectra was the red-shift in the spectrum of the conjugates compared to that of oxazolone. This change is probably due to covalent bond formation between the ethoxymethylene group of oxazolone and the amino group of the polypeptides [33]. Based on the absorption spectra of the conjugates, the phOx content was determined using $\lambda_{max} = 349–352$ nm and $\epsilon = 32000$ [29]. The average degree of substitution is in the range of 6.9–13.0 oxazolone molecules per molecule, which is similar to data published for protein-phOx$_j$ conjugates [22,26,27, 29,34].

The conformation and pH-induced conformational changes of the conjugates were investigated by CD spectral measurements in the wavelength

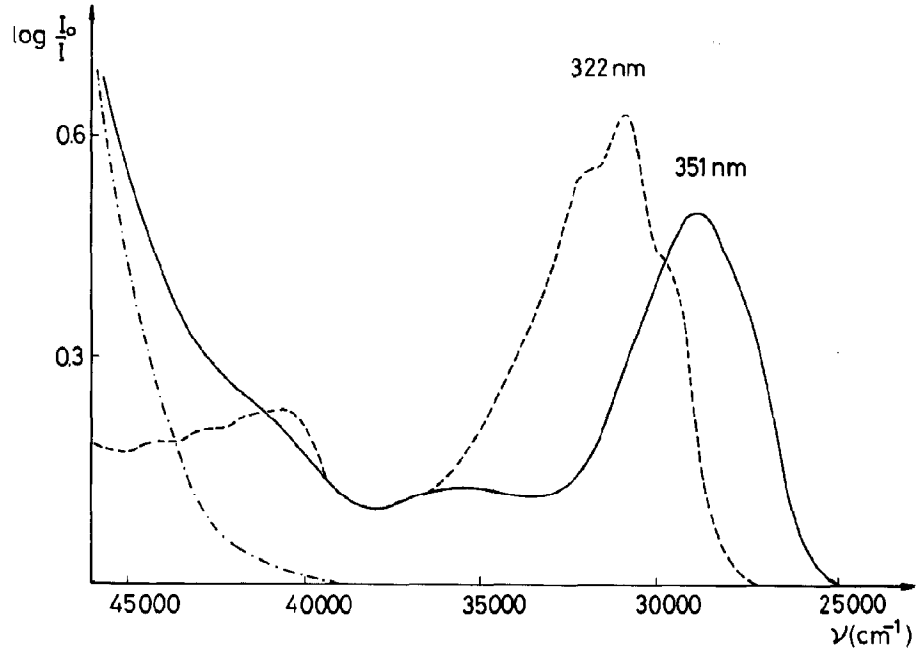

Fig. 3. Absorption spectra of 4-ethoxymethylene-2-phenyl-5(4H)-oxazolone in ethanol/water (1:9, v/v) ($c = 2\times10^{-5}$ mol/l) (- - - - -), LAK in 0.2 mol/l NaCl at pH 7.3 ($c = 2.5\times10^{-5}$ mol/l) (·—·—·) and LAK-phOx$_{8.0}$ in 0.2 mol/l NaCl at pH 7.3 ·($c = 2.7\times10^{-5}$ mol/l) (———).

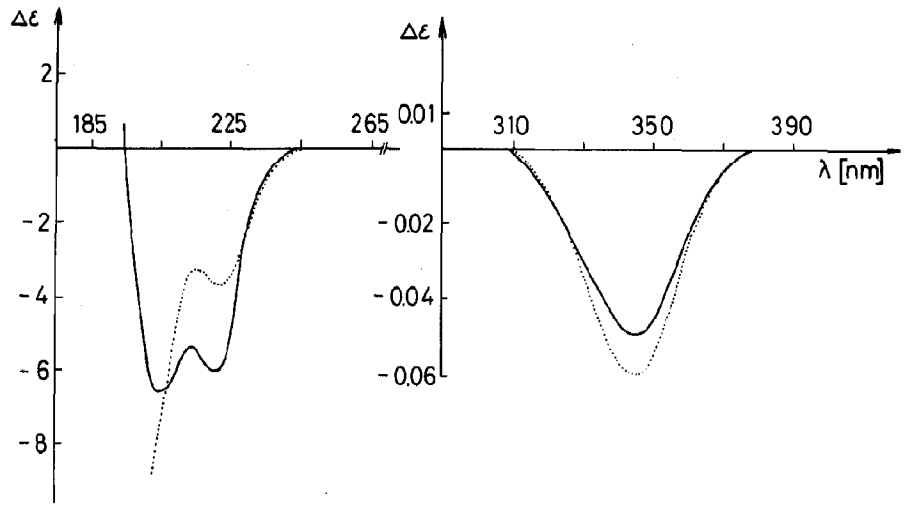

Fig. 4. CD spectra of the AK-phOx$_{9.6}$ conjugate at pH 1.5 (· · · · · ·) and pH 7.3 (———) in 0.2 mol/l NaCl.

region 190–250 nm. The absorbance of the polypeptide-phOx$_j$ conjugates lies between 280 and 400 nm (fig. 3). In the 310–380 nm absorption range, only a weak optical activity was observed, presumably originating from the interaction of the covalently bound oxazolone molecules with their chiral environment in the polymer. This effect can be used for monitoring local structural changes in the conjugates.

The CD curves of the AK-phOx$_{9.6}$ conjugate in 0.2 mol/l NaCl at pH 1.5 and pH 7.3 are shown in fig. 4. In the wavelength region 190–250 nm, the CD spectrum indicates a partially ordered conformation at pH 1.5 as suggested by the appearance of a negative maximum at 222 nm. An increase in pH to 7.3 leads to the formation of a helical structure as indicated by the presence of two negative maxima of approximately equal intensity at 222 and 205 nm. It should be noted that the CD spectrum of the free polypeptide AK corresponds to an unordered conformation under identical conditions [13]. The weak CD band in

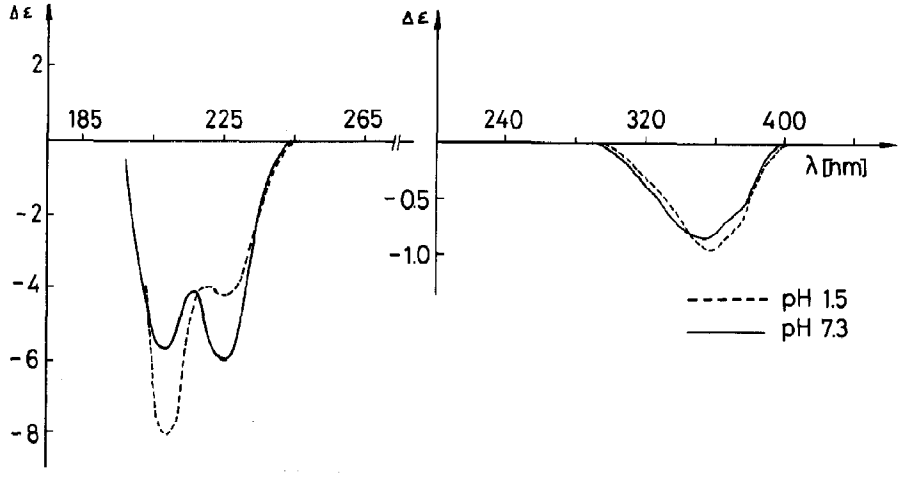

Fig. 5. CD spectra of the FAK-phOx$_{7.0}$ conjugate in 0.2 mol/l NaCl, at pH 1.5 (- - - - -) and pH 7.3 (———).

the 310–380 nm range demonstrates the presence of phOx in the conjugate. The intensity of this negative CD band at 346 nm was slightly higher at pH 1.5 than at pH 7.3.

The CD spectra of conjugates derived from the poly(Lys-(X_i-DL-Ala_m)) carrier polypeptides with the oxazolone derivative located at the ends of their side chains will be discussed using as examples poly(Lys-(phOx$_j$-Phe$_i$-DL-Ala$_m$)) (FAK-phOx$_j$) and poly(Lys-(phOx$_j$-D-Phe$_i$-DL-Ala$_m$)), (D-FAK-phOx$_j$) (figs. 5 and 6). Similar shapes of the dichroic curves were noted in the case of Leu- or D-Leu-containing conjugates (data not shown).

The CD spectrum of the FAK-phOx$_{7.0}$ conjugate between 190 and 250 nm displays a partially ordered conformation at pH 1.5 (intense negative maximum at 225 nm). The shape of the CD spectrum in 0.2 mol/l NaCl at pH 7.3 corresponds to that of a highly ordered structure (fig. 5). The spectra in the range 310–380 nm, at both acidic and neutral pH, are almost identical. The CD band in this region is characterized by a negative maximum at about 355 nm. In acidic solution, the intensity of this band is somewhat higher. It should be mentioned that the conformation of the FAK carrier polypeptide was found to be helical in 0.2 mol/l NaCl at pH 7.3 (fig. 2). However, the CD spectrum obtained at pH 1.5 indicated an unordered structure of the backbone [12].

The effect of pH on the CD spectrum of the FAK-phOx$_{7.0}$ conjugate is analogous to that observed with the AK-phOx$_{9.6}$ conjugate. An increase in pH resulted in the formation of an ordered structure of the conjugate from a partially ordered conformation (figs. 4 and 5). Compared to our previous data with carrier polypeptides [10,12,13], the results presented here lead to the conclusion that the presence of phOx at the end of the side chains in these conjugates promotes the formation of helical secondary structure. As a consequence, poly(Lys-(DL-Ala$_m$)) and poly(Lys-(X_i-DL-Ala$_m$)) (X = Leu or Phe) derivatives carrying the hydrophobic groups of phOx have a more pronounced tendency to assume an ordered conformation, even under 'unfavourable' conditions (i.e., at acidic pH).

A comparison of the CD spectra of conjugates containing Phe or D-Phe in their side chains (FAK-phOx$_{7.0}$ and D-FAK-phOx$_{6.9}$) is demonstrated in fig. 6. The shapes of the CD spectra measured under identical conditions (in 0.2 mol/l NaCl, pH 7.3) in the range 190–250 nm correspond to helical structures. Some quantitative differences can, however, be observed. The band intensities at 225 and 207 nm seem somewhat lower in the spectrum of the D-FAK-phOx$_{6.9}$ conjugate compared with that of its Phe analogue. In the longer wavelength region of the CD spectrum, FAK-phOx$_{7.0}$ shows a negative Cotton effect at

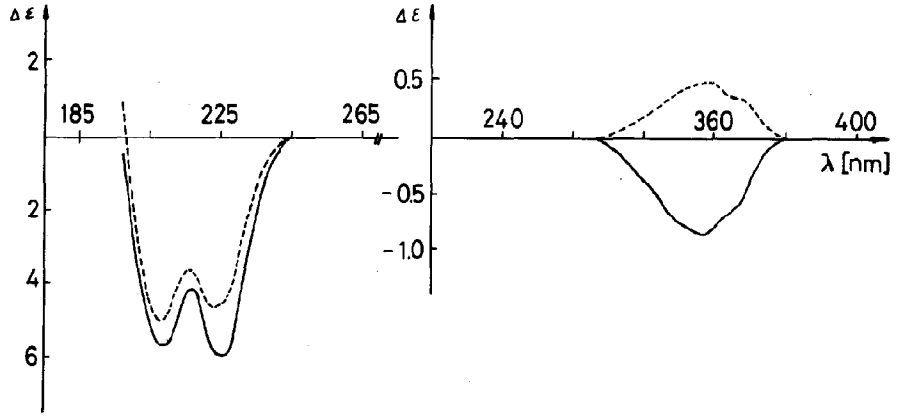

Fig. 6. CD spectra of the FAK-phOx$_{7.0}$ (———) and D-FAK-phOx$_{6.9}$ (- - - - -) conjugates in 0.2 mol/l NaCl at pH 7.3.

355 nm, while its diastereomeric analogue D-FAK-phOx$_{6,9}$ exhibits a positive CD band of similar intensity at the same wavelength. The CD spectra of the two conjugates containing L-phenylalanine or D-phenylalanine residues are mirror images of one another in this wavelength range. Conjugates containing L- or D-Leu residues in the side chains reveal analogous behaviour (data not shown). From these results, it can be concluded that the CD pattern in the range 310–380 nm is strongly dependent on the absolute configuration of the side-chain-terminating amino acid bound to the oxazolone moiety.

4. Conclusion

Analysis of structure-activity correlations points towards the importance of the conformation of branched polypeptides, particularly with respect to their antigenicity and immunomodulatory properties [17–20]. The results of the present experiments, in accordance with our previous observations, demonstrate that the helix-forming ability of the backbone of branched polypeptides seems to be related to the hydrophobic nature of the branch-terminating amino acids.

On the basis of data obtained with model compounds derived by coupling oxazolone to the branched polypeptides, we conclude that the nature of the side-chain-terminating amino acid *and* the hydropathic character of the hapten determine the *overall* conformation of the carrier-hapten conjugates. The CD signal in the wavelength range 310–380 nm suggests that the *local* orientation of the hapten is markedly dependent on the absolute configuration of the branch-terminating amino acids. Therefore, in the designing of such compounds, it should be possible to alter the spatial arrangement of the hapten by proper selection of the absolute configuration of the side-chain-terminating amino acid.

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