CD STUDIES ON ENKEPHALIN AND ITS Pro^5-ANALOGS

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1. Introduction

Recently some Pro^5-analogs of Met/Leu^5-enkephalin [1] containing a D-amino acid residue in position 2 have been synthesized [2,3]. Of these analogs, (D-Met^2, Pro^5)-enkephalinamide (V, table 1) proved to be one of the most active analgesic of peptide nature [3]. According to more recent in vitro data, its effect is also influenced by Mn^{2+} [4].

The increased activity of analog V can be discussed both in terms of its stability to tissue peptidases and by assuming a conformation different from that proposed for Met^5-enkephalin [5], with favourable transport and/or binding properties [6]. Finally the influence of metal ions on biological activity can be traced back to the formation of metal–peptide or peptide–metal–receptor complexes of proper stability under physiological conditions.

Thus, in order to find a correlation between conformation and biological activity, we performed CD investigations on Met-E and its Pro^5-analogs (table 1). Compounds III–V are Pro^5-enkephalinamides containing D-amino acid residues in position 2. Compound II with Gly in the same position serves as an intermediate between enkephalin and the Pro^5-analogs containing D-amino acids.

2. Experimental

The syntheses of compounds I–V have been reported in [2,3]. CD measurements were performed at room temperature on a Jobin-Yvon-Roussell-Jouan model III dichrograph using spectral grade acetonitrile (AN), double-distilled water and analytical reagent grade salts. Concentration of the samples ranged between 0.25–0.35 mg/ml. Pathlength of the cell used was 0.01 cm. CD is expressed in mean residue ellipticity units (m_Θ, deg. cm^2/dmol). Due to the high noise level of the spectra, only the sign and shape of the CD curves have significance below 210 nm (cf. [7]). The pH of the aqueous solutions was adjusted by HCl or NaOH solutions. The free base form of compounds II–V was prepared using Amberlyst A-26 anion exchange resin. CD titrations were performed in the cell by adding 1–10 μl 1–5 M acetonitrile solution of the salts ($NaClO_4$, $KClO_4$, $Ca(ClO_4)_2$, $Mn(ClO_4)_2$) to the solution (0.7–1.0 ml) of the peptide in AN.

Table 1
Structure and relative antinociceptive potency [3] of Met^5-enkephalin and Pro^5-enkephalinamide analogs

No.	Compound	Potency ratio on a molar basis (morphine = 1)	
		Intravenously	Centrally
I	H–Tyr–Gly–Gly–Phe–Met–OH (Met-E)	0	< 0.02
II	H–Tyr–Gly–Gly–Phe–Pro–NH_2 · HCl	<0.02	< 0.02
III	H–Tyr–D–Ala–Gly–Phe–Pro–NH_2 · HCl	0.22	3.9
IV	H–Tyr–D–Nle–Gly–Phe–Pro–NH_2 · TFA	0.7	18.1
V	H–Tyr–D–Met–Gly–Phe–Pro–NH_2 · AcOH	5.9	78.5

3. Results

CD extrema in the 270–200 nm region of Met-E and Pro^5-enkephalinamides II–V under different conditions are summarized in table 2.

Analogs II–V in water show the same behaviour at pH <7 as Met-E itself (table 2, fig.1; cf. [7]). At pH 11.0, the shape of the spectra of compounds I–V is similar in the 270–210 nm region but differs below 210 nm, where the ellipticity of the Gly^2-analog (II) is negative, contrary to the positive m_Θ values of Met-E and analogs III–V.

CD spectra in AN strongly depend on whether or not the molecule is protonated. In the cationic form, analogs II–V show spectra which are very similar to that found in water at pH <7 (table 2). By contrast, the spectra of the free bases can be characterized by a negative extremum in the 232–239 nm region, a shoulder or a weak positive band at ~220 nm and by positive ellipticity values below 210 nm (see, e.g., fig.1). On addition of 1 equiv. TFA to the solution of the free bases in AN, the spectra of the cationic forms is obtained again.

Met-E in acetonitrile does not exhibit the same

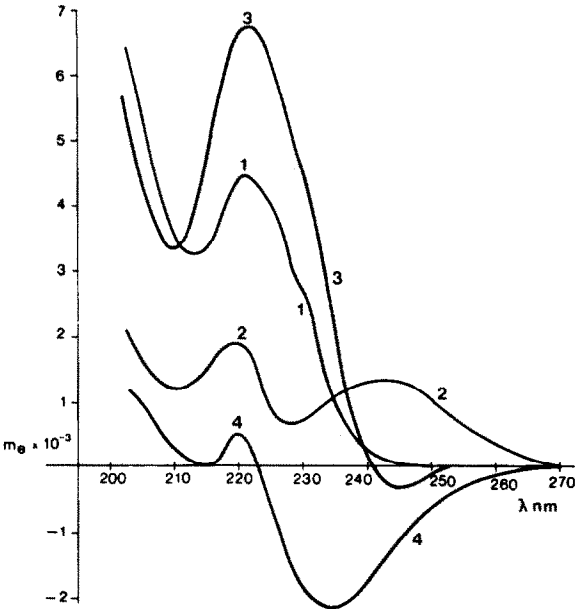

Fig.1. CD spectra of (D-Met^2, Pro^5)-enkephalinamide (V) at 4.6–5.2 × 10^{-4} M in water: pH 3 (1), pH 11 (2) and in acetonitrile:TFA salt (3), free base (4).

Table 2
CD extrema of Met^5-enkephalin and its analogs II–V in water and acetonitrile solution

Compound	λ($m_\Theta \times 10^{-3}$)					
	In water		In acetonitrile			
	pH 3	pH 11	Protonated form	Free base	Free base + 2 equiv. Ca^{2+}	Free base + 2 equiv. Mn^{2+}
I	230[c]	240 (0.5)				
	221 (4.6)	217 (2.3)	228 (6.1)[a]	228 (3.0)[b]	228 (3.1)[b]	231 (1.85)[b]
	<205 (>4.7)	<205 (+)	<205 (>4.6)	207 (2.8)	<205 (<−2.0)	<205 (<−4.1)
II		246 (0.2)	245 (−0.15)[a]	232 (−2.2)[a]	230[c,a]	248[a,c]
	222 (3.6)	228 (−0.5)	230[c]	218[c]	222 (3.5)	227 (1.9)
	<205 (>3.4)	218 (0.5)	223 (4.65)	<205 (>1.6)		213 (−0.3)
		<215 (−)	<205 (>5.1)			<205 (>1.4)
III	227[c]	244 (1.2)		239 (−0.85)[a]		
	221 (5.6)	219 (1.9)	227 (5.15)[a]	221 (2.3)	225 (4.4)[a]	227 (2.3)[a]
	<205 (>6.5)	<205 (+)	<205 (>3.9)	<205 (>4.6)	<210 (−)	209 (−1.95)
IV	227[c]	245 (0.9)	244 (−0.35)	233 (−2.3)	230[c]	234[c]
	222 (5.4)	220 (1.4)	222 (5.4)	219[c]	225 (3.8)	228 (2.4)
	<205 (>6.6)	<205 (>2.0)	<205 (>3.8)	<205 (>3.7)	<210 (<−1.3)	<210 (<−2.4)
V		243 (1.3)	244 (−0.3)			
	230[c]		230[c]	235 (−2.15)	230[c]	230 (2.6)
	221 (4.5)	220 (1.9)	222 (6.75)	220 (0.5)	222 (5.3)	212 (−2.1)
	<205 (>5.4)	<205 (>1.6)	<205 (>4.6)	<205 (>1.0)	208[c]	

[a]With 10% (v/v) HFIP added; [b]Measured as sodium salt, with solvent as at [a]; [c]Shoulder

characteristic spectral change on deprotonation with 0.1 M aqueous NaOH as analogs II–V (fig.2). [The effect on CD spectra of the small quantities of water (4–6 μl/ml AN) can be neglected.]

We also tried to deprotonate the cationic form of analogs II–V (see table 1, compound V was measured as trifluoroacetate salt) by adding 0.1 M aqueous NaOH to their AN solution in the cell. As indicated by the appearance of CD spectra characteristic to the free base form, complete deprotonation of the Gly^2 and $D\text{-}Ala^2$ analogs required 1–1.5 equiv. NaOH, while in the case of the $D\text{-}Met^2$ analog addition of ~5 equiv. NaOH was needed. The most interesting result of our CD measurements is, however, that compound IV, the closest analog of the extremely potent ($D\text{-}Met^2$, Pro^5)-enkephalinamide, showed the same spectrum in AN both with and without addition of 15 equiv. NaOH.

Finally, we measured CD spectra of Met-E and analogs II–V in water and AN solution in the presence of K^+, Na^+, Ca^{2+} and Mn^{2+}. Contrary to the results in [8], the small spectral changes obtained in water on addition of a large excess of salts, were found to be comparable to the high noise level of the spectra. So we continued our measurements in AN. Met-E was measured in the form of its sodium salt, analogs II–V as free bases.

The CD spectrum of compounds I–V in AN is influenced by K^+ only to a negligible extent. Na^+ cause a definite spectral change, but only at higher salt concentrations (see, e.g., fig.3). By contrast, addition of Ca^{2+} or Mn^{2+} perchlorates to the solution of analogs II–V in AN alters the spectrum very strongly (table 2, fig.3).

The spectrum of the deprotonated form of Met-E in AN is influenced by Ca^{2+} and Mn^{2+} especially below 210 nm, where the second positive extremum disappears and the ellipticity values become negative in both cases (fig.2).

4. Discussion

CD spectra of Met-E in water and in the presence of Na^+ and K^+ were first reported in [8]. Its absorption and CD spectra were also measured in aqueous solu-

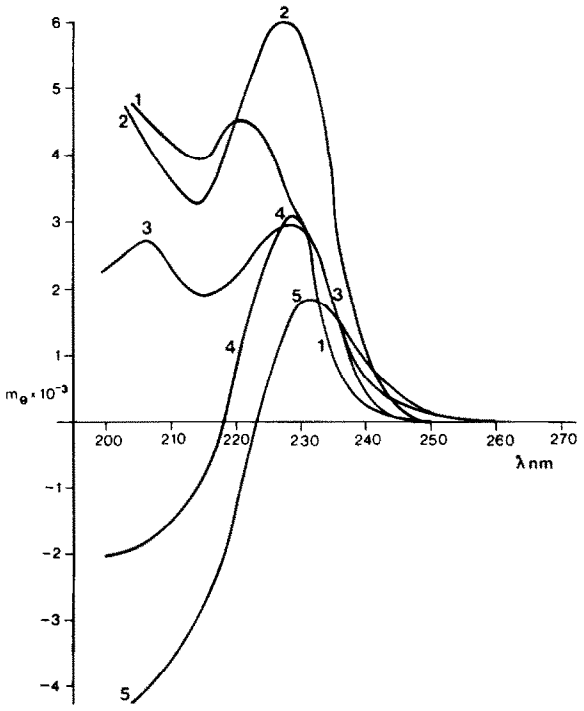

Fig.2. CD spectra of Met^5-enkephalin (I) at 5.5–6.7 × 10^{-4} M in water: pH 3 (1) and in acetonitrile containing 10% (v/v) hexafluoroisopropanol: TFA salt (2), sodium salt (3), sodium salt + 2 equiv. $Ca(ClO_4)_2$ (4), sodium salt + 2 equiv. $Mn(ClO_4)_2$ (5).

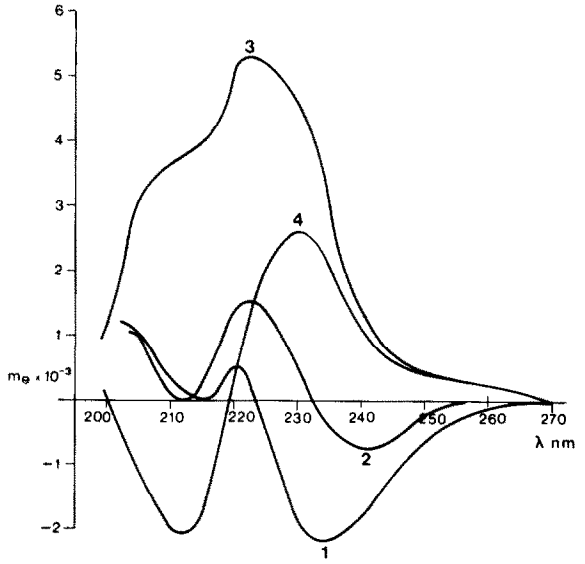

Fig.3. CD spectra of ($D\text{-}Met^2$, Pro^5)-enkephalinamide (free base) at 4.6 × 10^{-4} M in acetonitrile (1) and in the presence of $NaClO_4$ (10 equiv.) (2), $Ca(ClO_4)_2$ (2 equiv.) (3) and $Mn(ClO_4)_2$ (2 equiv.) (4).

tion as a function of temperature and pH, and in trifluoroethanol [7].

Our CD data concerning Met-E in water at pH <7 (table 2) are in good agreement with those of the latter group. As in [7], we could not reproduce the fine structure of the spectrum of Met-E reported in [8]. More recent CD data on Leu^5-enkephalin and its analogs also are qualitatively in agreement with our results [9].

Analogs II–V exhibit similar CD spectra in water at pH 3–5 and practically the same pH dependence, as Met-E itself. Consequently, neither the presence of the side chain of the D-amino acid residues of analogs III–V nor the replacement of methionine with negatively charged carboxylate group by amidated proline can play a dominant role in determining the chiroptical properties and the conformation of compounds II–V in water solution.

Much more important is the degree of protonation of the terminal amino group of tyrosin, especially in the nonpolar solvent AN. Surprisingly, in spite of the considerable difference in the polarity of these solvents, the spectra of the cationic form of analogs II–V in water and AN solution show a great similarity (see, e.g., fig.1). It is very likely that the similarity of CD spectra is connected with the presence of a similar conformation in the two solvents. It may be well assumed that this conformation is fixed by relatively strong interactions between the positively charged alkylammonium group of tyrosine and the carbonyl oxygens of the peptide groups. The complexed cationic group is shielded from the solvent (or base) by the hydrophobic side chains of Tyr^1, Phe^4 and that of the D-amino acids in position 2. The extent of this shielding effect can be well detected by CD titration in AN of the cationic form of analogs II–V with aqueous NaOH. This way the shielding of the alkylammonium group has been found to decrease for analogs: IV >> V > III, II. [In agreement with the above assumption, protonation of the free base form of analog V in AN causes an expressed change in the whole ^{13}C NMR spectrum (M. H., L. Radics (1979) preliminary data).]

As for the biological data, the favourable transport properties going together with this 'inner complexing' can well explain the extremely good intravenous potency of (D-Met^2, Pro^5)-enkephalinamide. Analog IV with D-norleucine in position 2 seems to form the most stable complex. Its decreased in vivo activity may be due to the 'excess' stability of the complex leading to the lack of flexibility, as well.

The formation of inner complexes can also be supported by the general complexing ability of these compounds. According to CD data (table 2, fig.3), Met-E and in a more definite extent analogs II–V form complexes in AN with Ca^{2+} and Mn^{2+}. The shape of CD curves of the Ca^{2+} complexes shows much similarity with the spectra of the parent compounds in protonated form. The Mn^{2+} complex exhibits somewhat different CD spectra.

CD titration performed on (D-Met^2, Pro^5)-enkephalinamide shows that the binding of Ca^{2+} and Mn^{2+} is of a specific nature and the complexes of both ions have a 1:1 stoichiometry, at least at higher salt:peptide molar ratios. By contrast, Na^+ form complexes of much less stability with analog V (fig.4).

The differences in the CD spectra of the Ca^{2+} and Mn^{2+} complexes of Met-E and its analogs II–V are

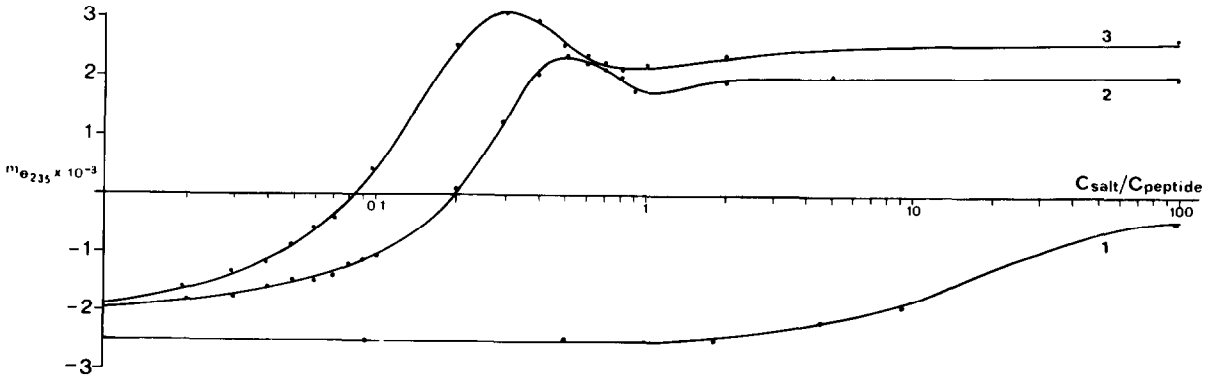

Fig.4. CD titration of (D-Met^2, Pro^5)-enkephalinamide in acetonitrile with Na^+ (1), Mn^{2+} (2) and Ca^{2+} (3).

going parallel with the receptor binding experiments in [10], according to which the inhibition by Met/Leu^5-enkephaline of [^{3}H]naloxone binding is enhanced by Mn^{2+} but not Ca^{2+}. On the basis of our CD experiments, bivalent ions seem to form complexes with the opiate peptides under hydrophobic circumstances, while Na^+ predominantly influence the receptor site conformation. At the same time, the formation of 'inner' and 'outer' complexes of different stability by enkephalin and its analogs may also effect in vitro or in vivo biological activity through the modification of transport properties of the parent molecules.

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