Circular Dichroism Study on Fully Bioactive CCK-Peptides of Increasing Chain Length

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Z. Naturforsch. **48b**, 1419–1430 (1993); received July 13, 1993

CCK-Peptides, Structure-Function Relationship, Bioactivities, Circular Dichroism, Lipid Interaction

A CD conformational analysis has been performed on CCK-peptides elongated at the N-terminus in sequence mode beyond the naturally occurring CCK-8 up to the pentadecapeptide sequence. By extending N-terminally the CCK-8 sequence an intramolecular salt bridge between the tyrosine-O-sulfate and the arginine guanido function is allowed to be established. However, this intramolecular electrostatic interaction was not found to affect the bioactivities of the CCK-peptides indicating that induction of such salt bridge at the level of the ligand molecule does not prevent a similar interaction at receptor level by exchange of the counterion partner. As expected for unconstrained short linear peptides the dichroic properties in aqueous solution were indicative of predominantly random coil structure. Conversely, in aqueous TFE the CD spectra were consistent with the presence of γ-type turns similarly to what has been observed under identical conditions for small size peptides related to the homologuous gastrin hormone. In surfactant solutions the CCK-peptides were found to assume β-type structures by inserting at least the C-terminal portion of the bioactive core into more hydrophobic compartments of the surfactant micelles, whereas the hydrophilic charged N-termini of the CCK-peptides of increasing chain length are exposed to the water phase in random coil structures as suggested by the CD spectra. This contrasts previous findings related to the homologuous gastrin peptides, where identical CD spectra were recorded in aqueous TFE and in presence of micelles. This observation strongly suggests that gastrin and CCK related peptides exhibit distinct conformational preferences, despite their high degree of sequence homology, and fully agrees with the ability of CCK to interact specifically with different receptors.

Introduction

Since the initial discovery and isolation of CCK from pig intestine as a 33-membered linear peptide [1] a surprising size-heterogeneity of the circulating forms of this hormone has been detected both in gastrointestinal tissues and brain (for recent reviews see ref. [2–4]). In fact, processing of prepro-CCK leads to CCK-58 as the largest circulating form and further enzymatic trimming is producing CCK-39, CCK-33, CCK-25, CCK-22, CCK-18, CCK-8 as well as CCK-5 and CCK-4, whereby the distribution of these CCK-peptides was found to vary among the tissues. Moreover, sulfation of the tyrosine residue in the C-terminal portion of the CCK-molecule was recognized as essential for the hormonal activities in the peripheral system (*e.g.* gall bladder contraction, pancreatic enzyme secretion, gut motility), but not for its function as neurotransmitter in the central nervous system. Correspondingly, a classification of the CCK-receptors into peripheral type A (sulfate-dependent) and central type B (sulfate-independent) receptors has been proposed [5]. Extensive studies with both synthetic and natural CCK-peptides have clearly shown that the C-terminal octapeptide (CCK-8) possesses the whole range of biological activities. However, it remains still controversial whether

Abbreviations: CCK, cholecystokinin; CCK-peptides are defined by the number of amino acid residues which constitute the biologically active C-terminal sequences of CCK-33, *i.e.* CCK-8 = CCK-(26–33), (Thr, Nle)-CCK-9 = (Thr[28], Nle[31])-CCK-(25–33), CCK-10 = CCK-(24–33), Ser(dGal)-CCK-11 = Ser(dGal)[23]-CCK-(23–33), CCK-12 = CCK-(22–33), CCK-13 = CCK-(21–33), CCK-15 = CCK-(19–33), [125]I-BH-(Thr, Nle)-CCK-9 = 3-(4-hydroxy-3-[[125]I]iodophenyl)propionyl-(Thr[28], Nle[31])-CCK-(25–33); Ser(dGal), 2-deoxy-α-D-galactosyl-serine; HG-17, human little-gastrin; TFE, trifluoroethanol; CD, circular dichroism; NMR, nuclear magnetic resonance; TRIS, tri(hydroxymethyl)aminomethane; SDS, sodium dodecylsulfate; OGP, octyl-β-D-glucopyranoside; CTAH, hexadecyltrimethylammonium hydroxide; cmc, critical micellar concentration.

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Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0932–0776/93/1000–1419/$ 01.00/0

Table 1. Sequences of the C-terminal CCK-peptides used in this study and for comparison the sequence of sulfated and non-sulfated norleucine[15]-human-little-gastrin.

H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	CCK-8
H-Arg-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Nle-Asp-Phe-NH_2	(Thr, Nle)-CCK-9
H-Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	CCK-10
H-Ser(dGal)-Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	Ser(dGal)-CCK-11
H-Ile-Ser-Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	CCK-12
H-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	CCK-13
H-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	CCK-15
Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2	non-sulfated (Nle^{15})-HG-17
Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr(SO_3H)-Gly-Trp-Nle-Asp-Phe-NH_2	sulfated (Nle^{15})-HG-17

and to what extent its N-terminal extension to larger size CCK forms are affecting the potency of the hormone [6–9]. Nevertheless most of the structure-function studies have been focused so far on the CCK-8 molecule in the attempt of uncovering as much as possible of the functional information encoded in the single structural elements of this relatively short bioactive peptide.

The specific interaction of the CCK hormone with several types of receptors could imply that the highly charged and flexible linear peptide is capable of adopting different bioactive conformations in the dynamic process of recognition and binding by the receptor. In this context extensive conformational analyses have been performed on CCK-8 and related shorter fragments using fluorescence transfer energy techniques, ^{1}H-NMR and computational procedures [10–17]. The results underline the difficulties encountered in determining the conformational preferences of this molecule and thus its potential bioactive structure. A broad spectrum of structures have been proposed which differ in the spatial array of the side chains, but more importantly in the peptide backbone, too. We have recently performed an ^{1}H NMR analysis of the fully active CCK analog (Thr, Nle)-CCK-9 [18] in the cryomixture dimethylsulfoxide/water at 278 K [19,20]. At this low temperature and high viscosity a predominant conformation was determined consisting of a γ-turn centered on threonine and separated by the glycine residue from an α-helix involving the C-terminus, whilst the N-terminus is flexible and saltbridged between the tyrosine-O-sulfate and the arginine guanido function.

In order to investigate systematically a possible stabilizing effect of a gradual peptide chain elongation on the conformationale state of the bioactive CCK-core and its biological relevance, a set of peptides synthesized previously [18, 21–23] and listed in Table I was analyzed in this context in the present study. Although CD measurements have found only limited attention in the conformational analyses of CCK-peptides [10], in the present study we made use of this spectroscopic technique as it has already provided useful information about predominant conformers at equilibrium under different physicochemical conditions in a previous study on the homologuous gastrin molecule [24,25].

Experimental

Materials

The CCK- and gastrin-peptides used in the present study are listed in Table I. Their synthesis and complete characterization have been reported elsewhere: (Thr, Nle)-CCK-9 [18], CCK-10 [21], Ser(dGal)-CCK-11 [22], CCK-12 [22], CCK-13 [23] and CCK-15 [23]; (Nle^{15})-human little gastrin [26]; sulfated (Nle^{15})-human little gastrin [27]; non-sulfated (Thr, Nle)-CCK-9 has been prepared in our laboratory (unpublished synthesis). CCK-8 was purchased from Neosystem (Strasbourg), SDS from Serva (Heidelberg) and OGP from Fluka (Ulm); CTAH was obtained from the corresponding hydrobromide by ion exchange on DOWEX 1X8 (OH form), TFE (UVASOL grade) from Merck AG (Darmstadt) was used in the spectroscopic measurements.

Spectroscopic methods

CD spectra were recorded on a Yobin-Yvon Dichrograph Mark IV equipped with a thermostated

cell holder and connected to a data station for signal averaging and processing. All data are averages of 10 scans and the spectra were recorded at 20 °C employing quartz cells of 1 mm optical path length. The spectra are original computer-drawn CD spectra reported in terms of ellipticity units per mole of peptide residues, $[\theta]_R$ (deg·cm^2·dmol^{-1}). The computed difference spectra were obtained by subtracting the CD spectra of the peptides expressed in ellipticity units per mole of peptide ($[\theta]_M$).

Stock solutions for spectroscopic measurements were prepared by dissolving weighed samples of peptides in a minimum amount of 0.1% ammonia and diluting to the desired volume with the buffers. The following buffers were used: 10 mM phosphate buffer (pH 7.0), 20 mM phosphate buffer containing 0.1 M NaCl (pH 7.0) and 50 mM TRIS·H_3PO_4 (pH 7.0). The concentrations were determined by weight and peptide contents of the lyophilisates (determined by quantitative amino acid analysis of the acid hydrolysates) and were additionally controlled by absorbance of the aqueous solutions at pH 7.0 in the near-uv (ε = 5760 at 280 nm). The peptide content of Ser(dGal)-CCK-11 was corrected for the carbohydrate moiety, since this does not contribute by itself to the CD in the wavelength range examined. Unless differently statet, the peptide concentrations used in the CD measurements were in the range of $1 \cdot 10^{-5}$ to $5 \cdot 10^{-5}$ M: Solutions containing the surfactants SDS, OGP and CTAH were prepared by mixing peptide and surfactant solutions in 50 mM TRIS·H_3PO_4 (pH 7.0) at 1:1 (v/v) ratios. The surfactant concentrations were twice their cmc values, i.e. 16.6 mM for SDS (cmc = 8.3 mM), 50 mM for OGP (cmc = 25 mM) and 1.84 mM for CTAH (cmc = 0.92 mM).

Biological assays

The tracer used for the competitive binding assays on CCK-receptor containing systems was ^{125}I-BH-(Thr, Nle)-CCK-9; this was prepared following the procedure previously described [28]. The CCK-peptides were analyzed for their binding affinities to intact AR4–2J cells [29] as well as to membrane preparations of these cells and to membranes from normal pancreas as described previously [30]. Binding to intact rat pancreatic acini was determined following known protocols [31]. Moreover, the CCK-peptides were analyzed for their adenylate cyclase activation potency in plasma membranes from rat pancreas, again according to known procedures [32].

Results

Dichroic properties of CCK-peptides in aqueous solution

In 10 mM phosphate buffer (pH 7.0) noisy CD spectra of unexpectedly weak intensities were recorded for CCK-8, (Thr, Nle)-CCK-9 and CCK-10 in the far-uv region as shown representatively in Fig. 1 for (Thr, Nle)-CCK-9 (curve 1). Further N-terminal elongation to the CCK-12, CCK-13 and CCK-15 was found to slightly improve the quality of the spectra, but the signal to noise ratios remained too low. On the other hand, desulfation of (Thr, Nle)-CCK-9 led to a CD spectrum (curve 2 of Fig. 1) of normal signal to noise ratio, which strongly reminds that of non-sulfated gastrin peptides of similar size in aqueous solution [25]. It is typical both in shape and dichroic intensities for aperiodic structures as expected for these short linear peptides in water. This would imply that sulfation of the tyrosine residue is responsible for the

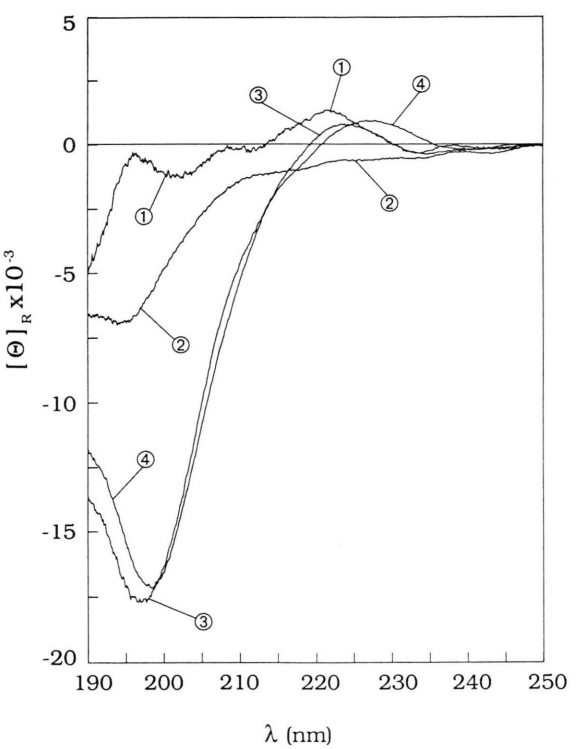

Fig. 1. CD spectra of (Thr, Nle)-CCK-9 (curve 1), non-sulfated (Thr, Nle)-CCK-9 (curve 2), sulfated (Nle^{15})-HG-17 (curve 3) and non-sulfated (Nle^{15})-HG-17 in 10 mM phosphate buffer (pH 7.0).

observed anomalous CD of CCK; however, a similar effect could not be observed in the case of gastrin despite the strong sequence homology, where sulfation (curve 3) was even found to enhance the dichroic intensities of the negative maximum. Among the aromatic residues present in the C-terminal CCK sequence, the contributions to the optical activity of phenylalanine and possibly of tyrosine-O-sulfate (in agreement with the known tyrosine effect) are positive in the region of 240 to 193 nm, whereas tryptophan has a positive contribution at 230 nm, but a negative CD below 213 nm [33]. However, the weak CD intensities of the CCK-peptides cannot be attributed to the opposite contributions of the aromatic chromophores as these are present in the gastrin sequence, too, where normal CD spectra could be recorded. Thus, the anomalous effect of sulfation of the tyrosine residue on the CD properties represents a peculiar feature of CCK for which, so far, a rational explanation could not be found.

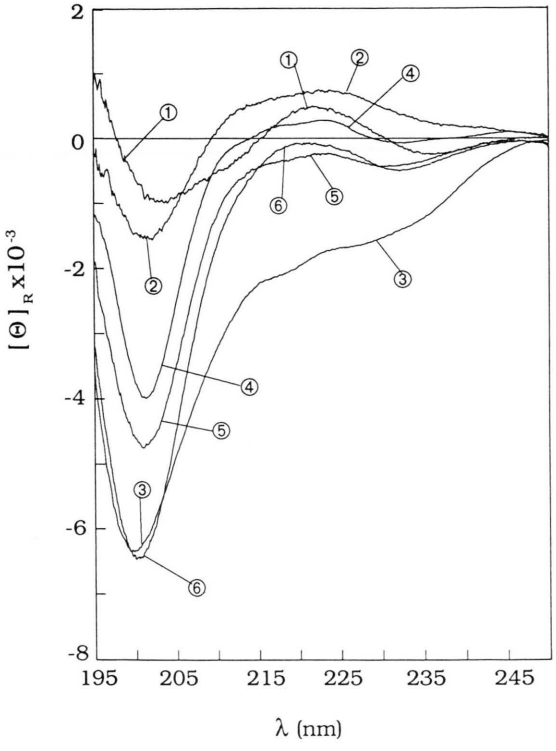

Fig. 2. CD spectra of (Thr, Nle)-CCK-9 (curve 1), CCK-10 (curve 2), Ser(dGal)-CCK-11 (curve 3), CCK-12 (curve 4), CCK-13 (curve 5) and CCK-15 (curve 6) in 20 mM phosphate buffer containing 100 mM NaCl (pH 7.0).

Increasing the ionic strenght to more physiological values, *i.e.* in 20 mM phosphate containing 100 mM NaCl (pH 7.0), more reliable CD spectra could be recorded for (Thr, Nle)-CCK-9 and CCK-10 and particularly, for the higher molecular weight CCK-peptides as shown in Fig. 2; however, the signal to noise ratio of the CD spectrum of CCK-8 was still unacceptable. As the spectra were found to be concentration-independent in the $1 \cdot 10^{-4} - 5 \cdot 10^{-5}$ M range, intermolecular aggregation at extents capable of affecting the CD properties can be excluded. The spectra clearly reveal that N-terminal extension of CCK-10 induces remarkable changes in the dichroic properties with an abrupt increase of the negative maximum around 200 nm whose intensity increases as a function of the chain length, except for Ser(dGal)-CCK-11; this effect may reasonably be attributed to weakened endgroup effects. The overall CD patterns are consistent with predominantly aperiodic structures. Not in line with this general trend is the CD spectrum of Ser(dGal)-CCK-11 which exhibits the negative maximum at 200 nm of nearly identical intensity as that recorded for CCK-15, but additionally a broad negative shoulder above 215 nm. Thus, incorporation of a carbohydrate moiety at the N-terminal serine residue leads to dichroic properties which are not reflecting merely random coil structures. Stabilization of ordered conformations by built-in carbohydrates has recently been observed in the case of model peptides [34].

Similarly to what was observed in phosphate buffered NaCl, acceptable signal to noise ratios are characterizing the CD spectra of (Thr, Nle)-CCK-9 and CCK-10 in 50 mM TRIS · H_3PO_4 (pH 7.0) as shown in Fig. 3; but again a reliable spectrum could not be recorded for CCK-8 because of the excessive noise. Upon elongation of the peptide chain a gradual increase of the intensity at 200 nm to values similar to those measured in phosphate buffered NaCl is observed. However, a broad negative shoulder is observed above 210 nm which does not exclude the presence of periodic structures at least to some extent in this buffer.

Dichroic properties of CCK-peptides in aqueous TFE

TFE has routinely been used in conformational analysis of peptides in order to lower the dielectric

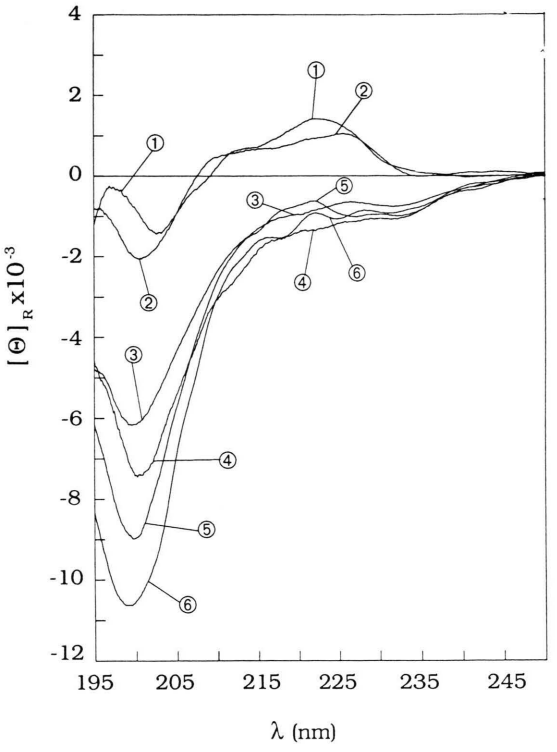

Fig. 3. CD spectra of (Thr, Nle)-CCK-9 (curve 1), CCK-10 (curve 2), Ser(dGal)-CCK-11 (curve 3), CCK-12 (curve 4), CCK-13 (curve 5) and CCK-15 (curve 6) in 50 mM TRIS · H_3PO_4 (pH 7.0).

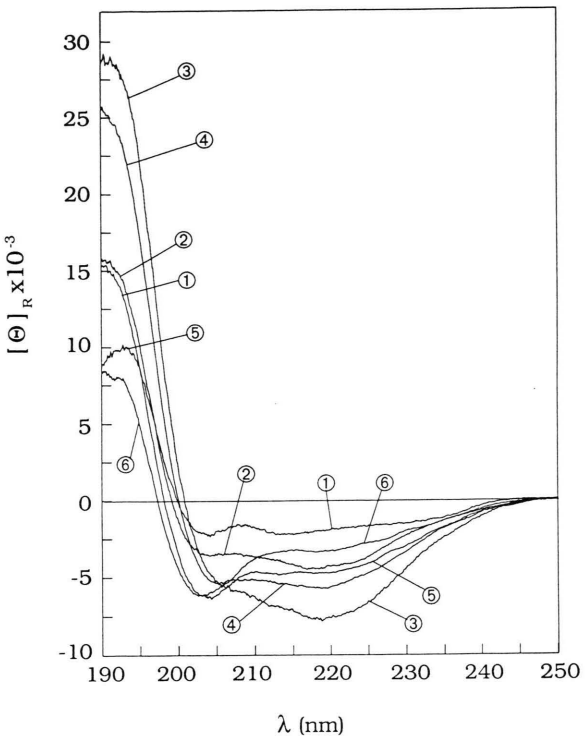

Fig. 4. CD spectra of (Thr, Nle)-CCK-9 (curve 1), CCK-10 (curve 2), Ser(dGal)-CCK-11 (curve 3), CCK-12 (curve 4), CCK-13 (curve 5) and CCK-15 (curve 6) in TFE/50 mM TRIS · H_3PO_4 (pH 7.0), 90:10 (v/v).

constant of the medium and thus, to mimic the environment of membrane-bound receptors. Recently, it has been reported that TFE may not be as appropriate for this purpose as generally believed, since it is a too strong α-helix inducer [35, 36]. Nevertheless we have analyzed the CCK-peptides by titrating their aqueous solutions with TFE in order to allow for a direct comparison of the resulting dichroic properties with those previously determined for the homologous gastrin-peptides [25].

TFE titration of (Thr, Nle)-CCK-9 in 50 mM TRIS · H_3PO_4 (pH 7.0) was not accompanied by well expressed isodichroic points and the final spectrum in 90% TFE (Fig. 4, curve 1) was found to exhibit a broad weak negative band with a maximum at 212–214 nm followed by a second weak negative maximum at 204 nm, a crossover point at 200 nm to positive CD at shorter wavelengths. In the case of CCK-10 two successive isodichroic points, *i.e.* at 204 nm and at 200 nm, were detected on increasing the TFE concentration to reach a final CD-spectrum in 90% TFE (curve 2) which differs from that of the nonapeptide mainly by enhanced intensities and more pronounced extrema at 216–218 nm and 204 nm. Although the dichroic intensities are still very weak, the location of the maxima seem to reflect an increased content of ordered structures in the less polar environment. The spectra are similar to those recorded for short gastrin peptides in aqueous TFE [25]. These were attributed to the onset of a 3_{10}-helix on the basis of known CD data [38, 39].

TFE titrations of the buffered aqueous solutions of the larger-size CCK-peptides are characterized by single isodichroic points located at 204 nm for Ser(dGal)-CCK-11 and CCK-12, slightly red-shifted for CCK-13 and at 207 nm for CCK-15. The resulting CD spectra in 90% TFE are shown in Fig. 4 (curves 3–6). The negative CD at 218 nm and 206 nm and the positive maximum are of

higher intensities than those recorded for (Thr, Nle)-CCK-9 and CCK-10, but are decreasing, particularly from CCK-12 on, in function of chain length forming an isodichroic point at 206 nm (Fig. 4). This should indicate that the N-terminal chain elongations beyond CCK-11 lead to an increase of one conformational state, in the present case of aperiodic structures, and that correspondingly these N-terminal extensions are mainly randomly coiled even in presence of TFE. A comparison of the difference spectra obtained by subtracting the CD of CCK-10 from those of the larger-size CCK-peptides (Fig. 5) shows an increase in the content of ordered structures upon elongation of CCK-10 to the undecapeptide, whereas further extension to CCK-12, CCK-13 and CCK-15 leads to difference spectra which clearly support an enhanced contribution of random coil structures. Although the difference spectra do not allow to localize the ordered conformations in the peptide chains, it is reasonable to assume that the inherent conformational preferences of the CCK-active site portion of the molecule, if existing, are unaffected in the higher mass CCK-peptides. Thereby elongation of CCK-8 to CCK-11 is apparently stabilizing to some extent ordered structures at conformational equilibrium possibly *via* suppression of endgroup effects. Further elongation of the CCK molecule is simply increasing the percentage of random coil related to the extension itself.

The TFE titration of the CCK-peptides in phosphate buffered NaCl (pH 7.0) led to very similar CD results whereby only 70% TFE concentrations could be obtained because of salt precipitation at higher TFE/water ratios. The CD spectra strongly remind those reported and discussed above and thus, allow similar conclusions to be drawn (data not shown). Interestingly, at high TFE concentrations CD spectra of the CCK-peptides can also be recorded at low ionic strength. The CD spectrum

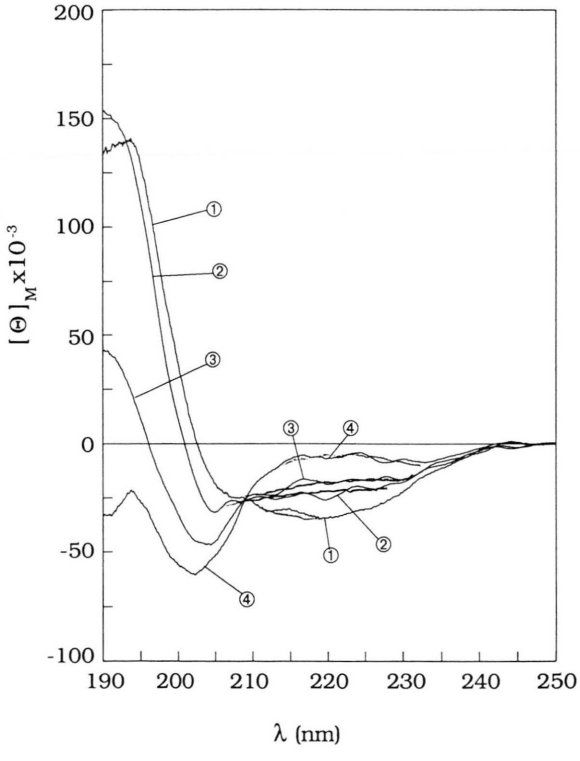

Fig. 5. Difference CD spectra obtained by subtracting the CD spectrum of CCK-10 from the CD spectrum of Ser(dGal)-CCK-11 (curve 1) CCK-12 (curve 2), CCK-13 (curve 3) and CCK-15 (curve 4).

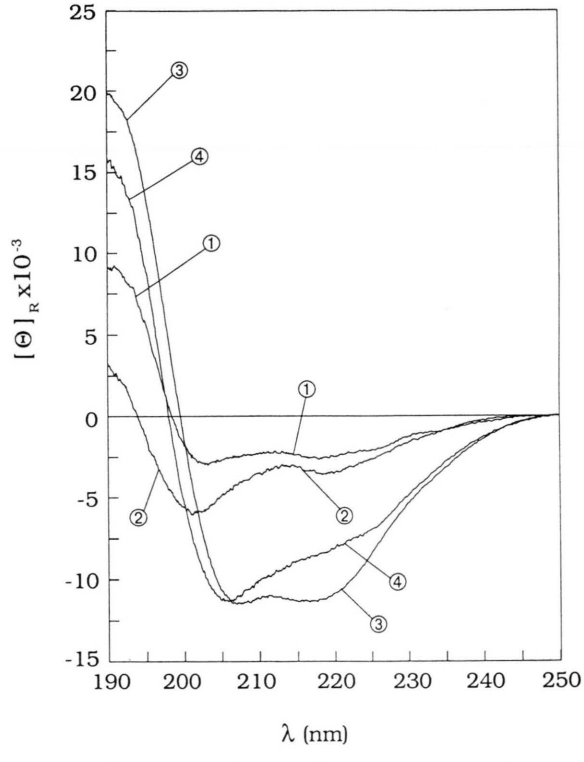

Fig. 6. CD spectra of (Thr, Nle)-CCK-9 (curve 1), non-sulfated (Thr, Nle)-CCK-9 (curve 2), sulfated (Nle^{15})-HG-17 (curve 3) and non-sulfated (Nle^{15})-HG-17 (curve 4) in TFE/10 mM phosphate buffer (pH 7.0), 90:10 (v/v).

of (Thr, Nle)-CCK-9 in TFE/10 mM phosphate buffer, 9:1 (pH 7.0) is shown in Fig. 6; it is practically identical to that recorded in TFE/50 mM TRIS·H_3PO_4, 9:1 (pH 7.0). Again desulfation of the CCK-9 analog was found to induce significant changes in the dichroic properties with a slight blue shift of the $\pi-\pi^*$ transition band from 203 to 201 nm and a concomitant increase in intensity from a $[\theta]_R$ value of −3000 to −6000 as shown in Fig. 6. An opposite effect is observed in the case of gastrin where sulfation is remarkably decreasing the CD of the $n-\pi^*$ transition band at 217 nm from a $[\theta]_R$ value of −11300 to −8500.

Dichroic properties of CCK-peptides in surfactant solution

Interaction of peptide hormones with surfactant micelles has widely been used for an alternative, possibly more proper mimicry of membrane-bound receptor environments than organic solvents. Previous studies on gastrin peptides [40] have shown that only upon protonation of the carboxyl functions of this molecule, *i.e.* at pH 2.0, embedment and thus, interaction with SDS micelles is occurring. This was well assessed by the blue shift of the tryptophan fluorescence emission maximum as well as by the CD spectra which were found to be surprisingly similar to those determined in TFE.

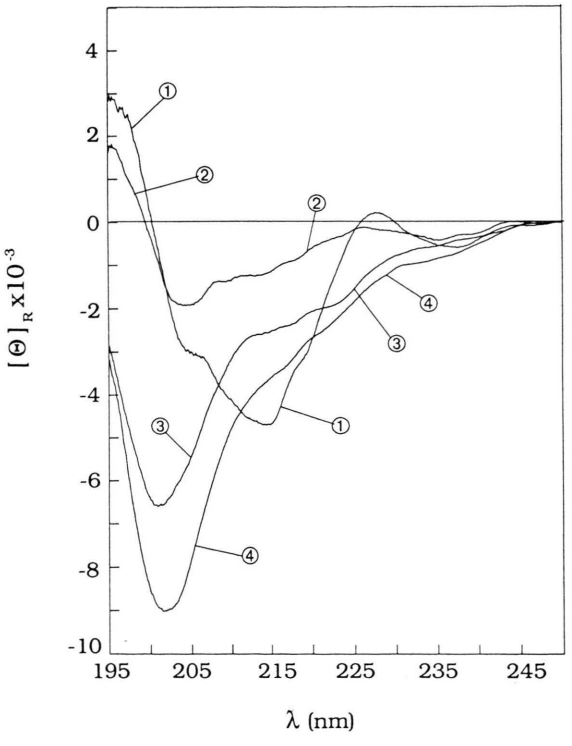

Fig. 7. CD spectra of (Thr, Nle)-CCK-9 (curve 1), CCK-10 (curve 2), CCK-13 (curve 3) and CCK-15 (curve 4) in 50 mM TRIS·H_3PO_4 (pH 7.0) containing 16.6 mM SDS.

Despite the presence of the negatively charged side chain groups strong CD changes were observed for the CCK-peptides upon addition of SDS micelles even at pH 7.0. The CD spectra of (Thr, Nle)-CCK-9, CCK-10, CCK-13 and CCK-15 in 50 mM TRIS·H_3PO_3 (pH 7.0) containing SDS at a 2-fold cmc concentration are reported in Fig. 7. The CD spectrum of the CCK-9 analog is characterized by a relatively strong negative band centered at 215 nm with a shoulder in the 204–208 nm range and by a positive maximum at 195 nm. Remarkable CD effects were induced by extending this CCK molecule to CCK-10, *i.e.* by incorporation of an additional negative charge which apparently is strongly weakening the embedment of the peptide into the micelles. The CD spectrum exhibits an overall reduced dichroic intensity in comparison to that of the CCK-9 analog with a blue shift of the negative band from 215 nm to 204 nm, whilst the positive band remains centered at 195 nm. Further N-terminal extension of the molecule to CCK-13 and CCK-15 causes an additional blue shift of the negative band to 202 nm with a concomitant strong increase of the intensity to $[\theta]_R$ values of −6600 and −9000, respectively, whereby a broad shoulder above 210 nm is retained. This negative CD above 210 nm, which is absent in the CD spectra of the peptides in aqueous buffers, is reflecting even in the larger size CCK-peptides the presence of ordered structure which may be located in the micelle-inserted C-terminal portion, whereas the charged hydrophilic extensions are most probably exposed to the aqueous phase in randomly coiled structures. An embedment of at least part of the CCK molecule into more hydrophobic compartments of the SDS micelles agrees well with previous studies on C-terminal CCK/gastrin pentapeptide derivatives [41,42]. Despite the presence of a carboxylate group, their interaction with lipid bilayers was well documented by fluorescence and microcalorimetry techniques. This is further confirmed in the present study by

the observation that the CD spectrum of (Thr, Nle)-CCK-9 in presence of SDS does not significantly change upon acidification of the solution to pH 2.0.

In the presence of uncharged OGP micelles again the most striking effect is related to peptide-size induced CD differences. As shown in Fig. 8 the CD spectrum of (Thr, Nle)-CCK-9 exhibits a negative band at 215 nm followed by a second one at 204 nm and a crossover at 200 nm to a positive band at 198 nm; besides the additional relatively strong positive band at 225 nm the spectrum reminds both in shape and weak intensity that obtained at high TFE concentrations. The CD spectrum of CCK-10 differs again remarkably from that of the CCK-9 analogue with a negative maximum located at 203 nm. Upon further size-increase of the CCK molecule to the tri- and pentadecapeptide remarkably increased dichroic intensities were observed for the negative maximum at 202 nm with a large shoulder above 210 nm. The latter spectra strongly remind those recorded for CCK-13 and -15 in SDS micelles.

Interesting changes in the CD spectra were induced upon addition of CTAH micelles with their positively charged head groups to the aqueous peptide solutions (Fig. 9). The CD spectrum of (Thr, Nle)-CCK-9 is characterized by a surprising strong positive band at 226 nm, a negative maximum centered at 215 nm with a weak shoulder at 209 nm and finally a maximum at 200 nm. In the case of CCK-10 the latter band is sensibly enhanced to positive values ($[\theta]_R = +600$), the negative band is again located at 215 nm, but the shoulder observed for CCK-9 is better expressed at 204 nm, whereas the positive maximum at longer wavelengths is of weaker intensity and red-shifted to 228 nm. In the CD spectra of CCK-13 and CCK-15 the negative dichroism at 215 nm is retained, but the second negative band is blue-shifted to 206

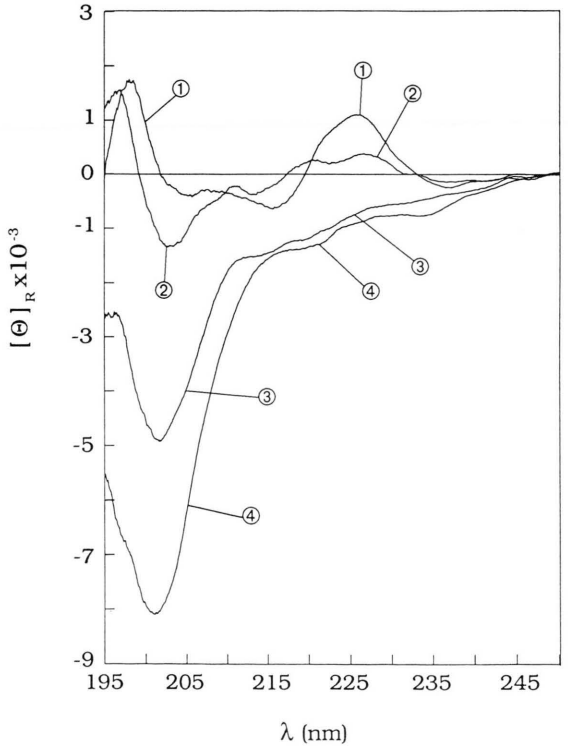

Fig. 8. CD spectra of (Thr, Nle)-CCK-9 (curve 1), CCK-10 (curve 2), CCK-13 (curve 3) and CCK-15 (curve 4) in 50 mM TRIS·H_3PO_4 (pH 7.0) containing 50 mM OGP.

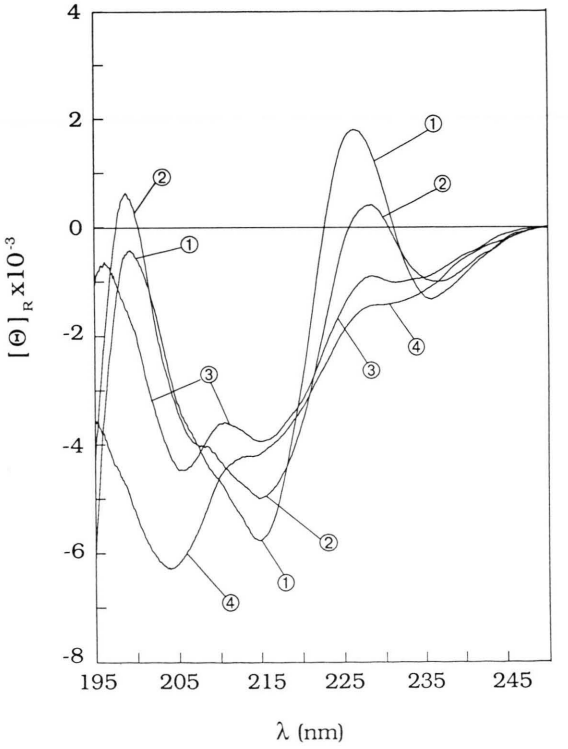

Fig. 9. CD spectra of (Thr, Nle)-CCK-9 (curve 1), CCK-10 (curve 2), CCK-13 (curve 3) and CCK-15 (curve 4) in 50 mM TRIS·H_3PO_4 (pH 7.0) containing 1.89 mM CTAH.

Tab. II. Biological properties of CCK-peptides of increasing chain length.

CCK-peptides	Binding to intact AR4–2J cells	Bindung to AR4–2J cell membranes	Binding to dispersed intact rat pancreatic acini	Binding to pancreatic plasma membranes	Stimulation of adenylate cyclase of pancreatic plasma membranes
	IC_{50}	IC_{50}	IC_{50}	IC_{50}	ED_{50}
CCK-8	n.d.	$1.96 \cdot 10^{-10}$	$4.03 \cdot 10^{-9}$	$1.64 \cdot 10^{-10}$	$5.99 \cdot 10^{-9}$
(Thr, Nle)-CCK-9	$3.71 \cdot 10^{-10}$	$1.54 \cdot 10^{-10}$	$3.95 \cdot 10^{-9}$	$1.26 \cdot 10^{-10}$	$8.15 \cdot 10^{-9}$
CCK-10	$3.71 \cdot 10^{-10}$	$2.26 \cdot 10^{-10}$	$4.92 \cdot 10^{-9}$	$1.26 \cdot 10^{-10}$	$8.15 \cdot 10^{-9}$
Ser(dGal)-CCK-11	$5.62 \cdot 10^{-10}$	$2.16 \cdot 10^{-10}$	$2.91 \cdot 10^{-9}$	$1.91 \cdot 10^{-10}$	$4.88 \cdot 10^{-9}$
CCK-12	$4.27 \cdot 10^{-10}$	$2.34 \cdot 10^{-10}$	$3.93 \cdot 10^{-9}$	$1.50 \cdot 10^{-10}$	$5.99 \cdot 10^{-9}$

nm and 204 nm, respectively, and characterized by a great enhancement in intensity as a function of the peptide size. Thus, N-terminal elongation of the CCK-molecule leads to changes of the conformers at equilibrium, although the location of the maxima related to $\pi-\pi^*$ and $n-\pi^*$ transitions strongly suggest a folding into ordered structures at larger extents than in the negatively charged and uncharged surfactants.

Biological properties of the CCK-peptides

The dichroic properties of the CCK-peptides of increasing chain length under various physicochemical conditions led us to conclude that N-terminal extensions of CCK-8 beyond the dodecapeptide sequence are not affecting the overall content of ordered structure of the bioactive portion of the CCK molecule. Therefore, a correlation between preference for ordered conformations and bioactivity as observed previously in the case of the gastrin peptides [25], should be detectable only upon extending CCK-8 to CCK-12. Correspondingly, the biological properties of this series of CCK-peptides were analyzed in different CCK characteristic assay systems and compared with those of the naturally occurring CCK-8 form.

The cancerous rat pancreatic acinar cell line AR4–2J is known to contain 80% CCK/gastrin preferring and 20% CCK preferring receptors [43]. As summarized in Table II the binding affinities of Ser(dGal)-CCK-11 and CCK-12 to the intact AR4–2J cells are apparently slightly inferior to those determined for (Thr, Nle)-CCK-9 and CCK-10. However, these differences may not be significant, since the specific binding affinities determined for the CCK-peptides in the case of AR4–2J cell membrane preparations are identical within the limits of error of the assay system for all peptides from CCK-8 to CCK-12. As shown in Table II similar identical results were obtained for this set of CCK-peptides in terms of their binding to dispersed intact rat pancreatic acini known to contain high and low affinity CCK receptors [44], and to pancreatic plasma membranes from normal rat pancreas which contain low affinity CCK receptors. Even the potency and efficacy of the peptides to activate adenylate cyclase of rat pancreatic

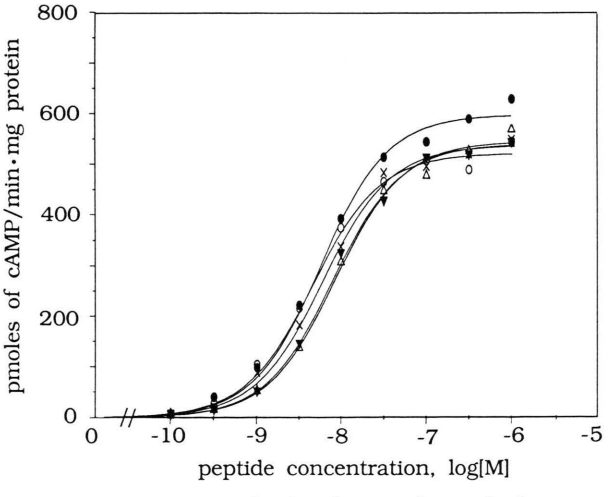

Fig. 10. Stimulation of adenylate cyclase of plasma membranes from normal rat pancreas by CCK-peptides: CCK-8 (●–●), [Thr, Nle]-CCK-9 (△–△), CCK-10 (▼–▼), Ser(dGal)-CCK-11 (○–○) and CCK-12 (×–×).

plasma membrane preparations, an effect which is related to occupancy of low affinity CCK receptors [32], was not affected by the N-terminal elongation of CCK-8 as clearly shown by the ED_{50} values reported in Table II and by the dose response curves shown in Fig. 10. In full agreement with these findings are the potencies of the CCK-peptides in stimulating amylase secretion from dispersed rat pancreatic acini as reported previously [22]. The results fully confirm that with the CCK-octapeptide the minimum size for full hormonal potency is reached, despite the fact that higher molecular weight CCK-peptides were identified and isolated as circulating forms in various tissues. This conclusion agrees with previous reports on differently sized CCK-peptides [6–7], but it contrasts others [8–9], particularly the observation of Yanaihara *et al.* [9] that synthetic CCK-12 represents the most active peptide in terms of pancreatic secretion in anaesthetized rats among a similar set of peptides, *i.e.* CCK-8, CCK-10, CCK-12 and CCK-14.

Discussion

N-Terminal elongation of the minimum hormone-active portion of gastrin, *i.e.* of the C-terminal tetrapeptide amide, in sequence mode was accompanied by a sigmoidal transition to full potency upon incorporation of most of the pentaglutamic acid cluster [45]. This transition was paralleled by a similar one related to the onset of the N-terminal α-helix in aqueous TFE, a fact which led to propose the CD- and NMR-derived 3D structure as the potential bioactive conformation [46,47]. It consists of a hairpin structure with an N-terminal α-helix followed by a β-turn-mediated chain reversal and a 3_{10}-helix in the C-terminus. The C-terminal pentapeptide amide which is shared by both CCK and gastrin, is part of the β-turn and for the rest constrained in a series of successive γ-turns, *i.e.* in a 3_{10}-helix.

Taking into account that the tyrosine-O-sulfate is essential for recognition of CCK-peptides by the peripheral sulfate-dependent type A receptors and that elongation of the minimal sequence, *i.e.* of CCK-7 to CCK-8 leads to a noticeable increase in potency [48], we could definitely confirm in the present study that further N-terminal extension of CCK-8 in sequence mode to the pentadecapeptide does not augment the binding affinity to high and low affinity CCK receptors and correspondingly, does not affect any of the CCK characteristic bioactivities examined. This confirms that with CCK-8 the optimal size is reached for maximum biopotency. By extending N-terminally the CCK-8 the incorporated arginine allows for an intramolecular salt bridge to be established between its guanido function and the tyrosine-O-sulfate group. It is reasonable to assume that a similar strong electrostatic interaction involving the tyrosine-O-sulfate is occurring at the level of the type A receptor-bound CCK-peptides. Since induction of such interaction already at the level of the ligand molecule was not found to alter its receptor binding potency, an exchange of the salt bridge partner in the binding process has to occur.

In the present study we could also confirm that N-terminal extensions of the CCK molecule are affecting only marginally the conformational states of the bioactive core under different conditions. As expected for linear flexible peptides the CD spectra of the CCK-peptides in aqueous solution were consistent in shape and intensity with predominantly random coil structures at conformational equilibrium. However, titration of the aqueous solutions of the CCK-peptides with TFE was found to induce ordered conformations which are apparently stabilized to some extent by N-terminal sequence-extensions up to the undecapeptide. On the other side, the biological properties were insensitive towards increase of the peptide size. It seems therefore reasonable to assume that the biologically relevant conformational states of the bioactive core, *i.e.* of CCK-8, are not affected by the peptide size, but that this conformation is stabilized by weakening endgroup effects.

The CD spectra recorded for this set of CCK-peptides at high TFE concentrations strongly remind those measured under similar conditions for gastrin peptides of similar size both regarding CD intensities and location of maxima. The latter spectra were attributed to γ-type turns and a folding of the C-terminus of gastrin in this conformation was later confirmed by ^1H NMR analysis of the gastrin in aqueous TFE [46,47]. The presence of the identical C-terminal pentapeptide in the CCK molecule makes it most reasonable to assume that the observed CD properties in aqueous TFE reflect a similar conformational state of this

sequence portion in CCK, too. This, however, would mean that the flexible N-terminus, salt-bridged in the larger-size CCK-peptides, is not conformationally constrained and does not exert significant effects on the conformational space of the C-terminus. This would fully agree with the observation that sulfate-independent central type-B receptors recognize both CCK and gastrin with identical affinity [4]. On the other hand, a gastrin-type folding of CCK-peptides could not be assessed by ^{1}H NMR analysis in aqueous dimethylsulfoxide. Moreover, even more importantly, the dichroic properties of the CCK-peptides in presence of surfactant micelles are completely different from those recorded in TFE, whereas in the case of gastrin identical spectra were measured in SDS micelles and aqueous TFE. The CD spectra of the CCK-peptides in charged surfactant solutions suggest the presence of β-type structures in full agreement with the CD properties of a double-tailed lipophilic (Thr, Nle)-CCK-9 derivative embedded into phosphatidylcholine bilayers [49]. This type of extended conformation contrasts also the 3D structure in dimethylsulfoxide/water as determined by NMR and would therefore suggest that differently from what was observed for gastrin, the CCK-peptides exhibit a pronounced tendency to fold into various ordered conformations depending upon the physicochemical invironment. This is fully consistent with the ability of this hormone to interact specifically with different receptors.

The present study was supported by the European Community grant No. SC 1/CT 91–0632.

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