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The Receptor Binding Affinity of Monocyclic [Ala^3,Xaa^{11}]Endothelin-1 Analogs Correlates with Inducible Helix Length

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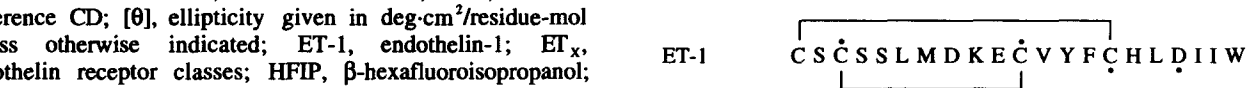
Abstract—Endothelin-1, a bicyclic 21-amino acid peptide with disulfide bridges between cysteines 1 and 15 as well as between cysteines 3 and 11, has been reported to be partially helical based on both CD and NMR data. However, this remains an area of controversy with some claims that CD data indicate no α-helical structure (Calas, B.; Harricane, M.-C.; Gulmard, L.; Heitz, F.; Mendre, C.; Chabrier, P. E.; Bennes, R. *Peptide Res.* **1992**, *5*, 97) and a recent X-ray crystal structure placing the helix at a different locus (Janes, R. W.; Peapus, D. H.; Wallace, B. A. *Structural Biology* **1994**, *1*, 311). The CD studies reported herein indicate that the helical structures reported in NMR studies (e.g. Andersen, N. H.; Chen, C.; Marschner, T. M.; Krystek, Jr. S. R.; Bassolino, D. A. *Biochemistry* **1992**, *31*, 1280) apply to pure aqueous media as well. The helix located from Lys^9 to the Cys^{15}/His^{16} juncture is *ca* 75% populated in pH 4 aqueous buffer. Titration difference CDs reveal that the helix extent increases by one to two residues and that the 'helical conformation' is more completely populated upon addition of TFE to 50+ volume-%. Comparison with a more helical analog suggests that the helix propagates towards (but not to the end of) the C-terminus upon fluoroalcohol addition. A variety of monocyclic derivatives of [Nle^7] ET-1 lacking the 3,11-disulfide were evaluated for biological activity and examined by TFE titration difference CD. The series included an Aib^{11} and a Pro^{11} analog. The helix promoting Aib analog was the most active while the Pro analog exhibited significantly lower vasoconstrictor activity and binding affinity for the ET_A receptor. All of the monocyclic analogs become significantly more helical upon addition of fluoroalcohols. The inclusion of a proline residue at position 11 does not preclude helix formation upon addition of fluoroalcohols. Rather, helix formation is relatively easily induced but limited to a 5 residue span. Apparently this is insufficient to orient required side chains optimally for interaction with the ET_A receptor. For the 1,15-monocyclic analogs differing only at position 11, ET_A binding affinity and vasoconstrictor potency correlate with the facility with which a 7–8 residue long helix can be induced. This presumably includes the segment Glu^{10} → Cys^{15} in all cases and may represent the full sequence from Lys^9 → His^{16}. CD studies also reveal that the C-terminal fragment of endothelins is not a fully disordered 'random coil' either alone or attached to the endothelin core.

Introduction

NMR† spectroscopy and X-ray crystallography can provide detailed information on the solution or solid state structure, respectively, of a peptide hormone. However, the relevance of either of these structures to the active conformation of a peptide when bound to its receptor requires additional proof. The introduction of residues or constraints whose effects upon conformation are understood should provide a probe of the receptor-bound peptide conformation through the correlation of structural preferences and biological activity and/or receptor affinity in the resulting analogs.

The potent vasoconstrictor peptide endothelin-1 (ET-1) is a bicyclic 21-amino acid peptide with disulfide bridges between cysteines 1 and 15 as well as between cysteines 3 and 11.[1]



Modifications at the indicated positions (3,11,15 and 17) were made in the analogs examined in this report.

Two classes of receptors, ET_A and ET_B, which mediate the biological effects of endothelins are well characterized[2,3] and some evidence for subtypes and an additional class have appeared.[4] The conformational requirements for binding to the ET_A receptor appear to be stringent in that acyclic analogs of endothelin

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†Abbreviations used: CD, circular dichroism; ΔCD, titration difference CD; [θ], ellipticity given in deg·cm^2/residue-mol unless otherwise indicated; ET-1, endothelin-1; ET_X, endothelin receptor classes; HFIP, β-hexafluoroisopropanol; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance (spectroscopy); NOESY, nuclear Overhauser effect spectroscopy; TFA, CF_3CO_2H; TFE, β-trifluoroethanol; 3,15-bisPen-ET [Pen^{3,15}, Nle^7] ET-1; $f_H(n)$, fractional helicity based on a helix length of n residues. The standard three- and one-letter abbreviations for amino acid residues and protecting groups are employed throughout; additional, less common ones used are: Aib, α-aminoisobutyric acid; Nle, norleucine = α-aminopentanoic acid; Pen, penicillamine.

exhibit minimal activity.[5] However, requirements for binding to the ET_B receptor are less demanding in that shortened acyclic peptides such as [Ala[11,15]]ET-1 (8–21) bind very tightly.[6]

From the onset, NMR structures of ET-1 in acidic aqueous media[7] suggested that two structural features of this peptide, a turn region from residues 5 to 8 and a somewhat irregular α-helix from residues 9 to 15 (or 16), were present in the dominantly populated conformers. In further studies,[8] we located families of conformers that were consistent with the NOESY data at pH 3.2–5.8. All acceptable conformers had the same (backbone rmsd ≤ 0.57 Å) α-helical conformation from residues 9 to 15, resulting in the alignment of Cys^{11} and Cys^{15} on one face of the helix while the residues Glu^{10}, Tyr^{13} and Phe^{14} which are important for binding and functional activity at the ET_A receptor subtype[9] occupy the opposite face. This conformational feature is retained[10] in [Pen[3,15],Nle[7]]ET-1 (3,15-bisPen-ET), a potent ET_A selective agonist,[11] and in most published NMR structures derived for endothelin analogs and sarafotoxins in largely aqueous media. The features reported by the different laboratories have been summarized and compared recently.[12] We[9a] and others[13] have reported that the monocyclic [Ala[3,11]]ET-1 exhibits about 10% of the activity of native ET-1 in preparations which contain predominantly ET_A receptor subtypes. One explanation for the lower activity of this peptide may be its increased flexibility, with the helix-containing structure being accessible only at a significant energy cost which would be reflected in a decreased binding constant. However, there have been reports that in some preparations [Ala[3,11]]ET-1 displays qualitatively as well as quantitatively different biological activity from endothelin-1, suggesting that other effects may be involved.[14] Using the $Ala^{3,11}$ monocyclic framework, we have prepared analogs which contain helix-inducing and helix-breaking residues at position 11 in order to study the relevance of the observed 9 to 15 helical region to the biologically active conformation of ET-1. In the present study, we report the vasoconstrictor activity and ET_A receptor binding affinity of these peptides as well as their conformational behavior as deduced from circular dichroism studies. The CD spectra are compared to those of native ET-1, which is analyzed in detail. Fluoroalcohol titration difference CD spectra (ΔCDs) are compared to those of other systems[15,16] with short helices which we employ as diagnostics for helix domain length.

Materials and Methods

Peptides

ET-1 was purchased from Peninsula Laboratories. Boc-Amino acids were purchased from Bachem (Torrance, CA). All monocyclic ET-1 analogs contained norleucine in place of Met^7. [Ala[3,11], Nle[7]]ET-1 and [Ala[3,11,17], Nle[7]]ET-1 were prepared as previously described.[9a] All other peptides were prepared by automated solid phase synthesis on a Biosearch 9600 peptide synthesizer using the standard manufacturer recommended *t*-Boc protocols. Peptides containing Pro^{11} and $D\text{-}Ala^{11}$ were prepared simultaneously using the technique of multiple peptide synthesis on a single support.[17] For this synthesis, position 11 was double coupled with a 2.5 : 1 molar ratio of Boc-proline:Boc-D-alanine and a 2 : 1 ratio of the Pro^{11} and $D\text{-}Ala^{11}$ peptides was obtained. Peptides were deprotected using anhydrous HF/anisole. The mixture of Pro^{11} and $D\text{-}Ala^{11}$ peptides was air oxidized in 8 M urea/0.1% NH_4OH and desalted on an HP-20 column (water, then 50% aqueous acetonitrile). The Aib^{11} peptide was air oxidized in 0.3% NH_4OH and sized on Sephadex LH-60 (80% aq. CH_3CN containing 0.3% ammonium hydroxide). All peptides were purified to ≥ 95% homogeneity by gradient preparative HPLC (acetonitrile:water:0.1% TFA) and characterized by amino acid analysis and fast atom bombardment mass spectrometry [Aib^{11}, $(M + H)^+$ 2425.1; $L\text{-}Ala^{11}$, $(M + H)^+$ 2411.4; $L\text{-}Ala^{11,17}$ $(M + H)^+$ 2368.8; $D\text{-}Ala^{11}$, $(M + H)^+$ 2411.0; Pro^{11}, $(M + H)^+$ 2437.3]. The $D\text{-}Ala^{11}$ preparation also provided, as a by-product, a sample of a 20 residue peptide with one of the two adjacent serines deleted. The sample of [Pen[3,15], Nle[7]]ET-1 came from the previously described synthesis.[11]

Vasoconstrictor and receptor binding assays

Vasoconstrictor assays were performed using isolated rings of rabbit carotid artery[18] and receptor binding assays using membranes prepared from A10 (rat thoracic aorta) vascular smooth muscle cells as previously described.[9] Despite the fact that the slope factors for several analogs were much less than unity, we believe that our binding assay contains only the ET_A receptor subtype. Sakurai *et al.*[3] have shown by northern blot analysis that A10 rat aortic smooth muscle cells do not contain ET_B receptors, and we (Dr Maria Webb, personal communication) have shown that there is no high affinity specific binding for iodinated endothelin-3 in this cell type. It is possible that the shallow slopes are due to two different G-protein coupled states for the receptor, as has been shown for agonists of the angiotensin II receptor.[19]

Circular dichroic spectroscopy

All CD spectra were recorded at ambient temperature (*ca* 25 °C) in 10 mM phosphate buffer (pH = 3.6, 4.0, 4.6, 6.0, 7.2 and 7.5) using a Jasco model J720 as described previously.[15,16,20] Peptide concentrations, 12–60 μM in the CD cells (typically of 0.5 mm path length), were based on UV assays of a stock solution of each peptide from which the samples were prepared by quantitative serial dilution. UV studies of native endothelin and a variety of monocyclic analogs, assuming that the alkali induced increase in absorbance at 242 nm could be ascribed to tyrosine titration and displays the same $\Delta\varepsilon_{242}$ (= 11100 cm^2 $mmol^{-1}$) as that

reported for an isolated tyrosine,[21] indicated the following absorptivities for all endothelins examined: at pH 4.5–7.5, ε_{278}–ε_{310} = 6980 ± 290 [versus 6810 estimated based only on the aryl residue transitions; or ε_{278} = 7250 (for bis-disulfides), 7125 (for monocyclic analogs) calculated by the method of Gill and vonHippel[22]]; at pH > 10, ε_{288}–ε_{315} = 7040 ± 430. The reported value for ET-1 based on quantitative amino acid analysis is ε_{279} = 7250.[23] The concentrations used for calculating mean residue molar ellipticities were based on the maximum observed values of ε_{278}–ε_{310} in the stock solution (120–300 μM in pH 7.5 aq. 2 mM phosphate buffer) and assumed a Δε-value of 7100 for all monocyclic analogs and 7200 for ET-1, and a residue count of 21 (even though there are only 20 backbone amide carbonyls). Fluoroalcohol titrations employed 1 to 10 dilutions of the stock into 10 mM buffers containing various vol-% levels of TFE or HFIP. Titration ΔCD curves were generated as CD (fluoroalcohol media) minus CD (at zero or lower per cent fluoroalcohol content) and are presented in a common 'normalized' format with the minimum in the 220–226 nm span set to a molar ellipticity of -10000°. UV spectra were recorded with a Perkin–Elmer Lambda 3A spectrophotometer.

Results and Discussion

As reported previously,[9a,13] the monocyclic ET analog containing norleucine at position 7 and alanine at positions 3 and 11 maintains about 10–30% of the activity of ET-1. In order to probe the structural changes which the Ala^{11} substitution causes in endothelin, we prepared several other 11-substituted monocyclic analogs. Replacement of the α-hydrogen of alanine with a methyl group to form aminoisobutyric acid (Aib) restricts the allowable φ and ψ angles to values near –60, –45 and +60, +45° regions corresponding to a right- or left-handed α- or 3_{10}-helices. In fact, incorporation of Aib into small and medium sized peptides has almost invariably resulted in local helix formation in the region around this residue (for a review, see Karle and Balaram[24]). On the other hand, proline is a well known helix dissuader (in C-peptide analogs as an example[25]), particularly beyond the first N-terminal turn since it lacks the amide NH required for forming the helix stabilizing H-bond.[26] We also studied the conformation of the monocyclic analog in which Leu^{17} is replaced with alanine, a substitution which causes a large decrease in binding affinity and an even larger decrease in vasoconstrictor activity.[9a] The NMR structural analysis of ET-1 had suggested that a hydrophobic cluster comprising Leu^{17} as well as Leu^6 and Val^{12} was a consensus feature of the allowed conformers and that this cluster might contribute to the stabilization of the helical region.[8] CD studies of the Ala^{3,11,17}-analog were performed in order to ascertain whether its reduced efficacy has an obvious conformational rationale. The receptor affinity and vasoconstrictor activity of ET-1 and the monocyclic analogs are shown in Table 1. The Aib^{11} analog had very high receptor affinity and was a potent vasoconstrictor peptide. In fact, it appears to be the most potent monocyclic analog reported to date. On the other hand, the Pro^{11} substitution produced a hundred-fold loss of receptor affinity and a similar loss of vasoconstrictor potency. The slight improvement in potency caused by the helix-inducing Aib residue and the marked loss of activity observed with the helix-breaking Pro residue provide strong suggestive evidence for the biological importance of a helical conformation in the vicinity of residue 11 in endothelins. The D-Ala^{11} analog displayed less biological activity than the L-Ala^{11} form, perhaps because of an unfavorable steric interaction between D-Ala^{11} and Phe^{14} across the face of the helix. Similar local steric interactions were postulated to cause activity differences between D- and L-isomers in a constrained helical analog of parathyroid hormone.[27] In order to more convincingly correlate the biological activity with structural information, spectroscopic studies were undertaken to determine the effect of the 11-position substitutions on the helicity of the endothelin analogs.

Endothelin-1, CD analysis

Although NMR has been the primary tool applied in structural studies of endothelins, CD spectroscopy figured in the structural studies on this biologically intriguing compound essentially from the onset.[28] Figure 1 shows representative results for ET-1 (and 3,15-bisPen-ET) in aqueous media, over the concentration range (12–250 μM) and pH range examined, and upon addition of HFIP, TFE, and ethylene glycol. The precisely refined NMR structures generated from

Table 1. Receptor affinity and agonist potency of ET-1 and 11-substituted analogs

Peptide	EC_{50} (nM)	k_d (nM)	Slope Factor
ET-1*	0.94 ± 0.31	0.15 ± 0.07	0.91 ± 0.23
[Pen^{3,15}, Nle^7] ET-1§	0.89 ± 0.22	0.70 ± 0.60	0.60 ± 0.10
[Ala^3, Nle^7, **Aib^{11}**] ET-1	1.5 ± 0.7	2.0 ± 0.9	0.60 ± 0.05
[**Ala^{3,11}**,Nle^7] ET-1*	3.3 ± 0.9	2.1 ± 2	0.54 ± 0.03
[Ala^3, Nle^7, **D-Ala^{11}**] ET-1	6.0 ± 1.0	76 ± 4	0.58 ± 0.08
[Ala^3, Nle^7, **Pro^{11}**] ET-1	170 ± 32	730 ± 100	0.85 ± 0.10
[**Ala^{3,11,17}**,Nle^7] ET-1*	>> 1000	930 ± 270	0.66 ± 0.04

*Previously reported by Hunt *et al.* [9]
§ Previously reported by Hunt *et al.* [11]

aqueous ethylene glycol data will be used in our comparisons. In that report, CDs were given over the 12–1400 μM range; no concentration effects were noted.[8] The ellipticity extremum at 205–208 nm for ET-1 has been noted by all groups ($[\theta] = -15{,}950 \pm 1140°$ reported,[23,28–31] versus our value of −15,450° at pH 7.5). The systematic increase in helicity (water → aq. glycol → aq. fluoroalcohol) is quite apparent in these spectra (Fig. 1), with the major change occurring upon fluoroalcohol addition. Several other groups[23,29,30] have also noted the 222 nm shoulder and its increased intensity upon addition of TFE. However, Perkins *et al.*,[28] who estimated 35% helicity based on analogy to apamin,[32] indicated that TFE did *not* cause a significant increase in helicity. In some contrast to this, Bennes *et al.*[31] noted no shoulders at either 222 or 215 nm and thus concluded the absence of significant amounts of both α- and β-structure for ET-1 under non-aggregating conditions (3 μM) and at 66 μM, the latter well above the CMC which they reported as 22 μM. Our data also indicate no change over this concentration range. Based on NMR studies, another group[33] reported that [Nle^7]ET-1 is helical from Lys^9 → His^{16} in 50% aqueous acetonitrile, conditions under which aggregation does not occur. Calas *et al.* have since reiterated[34] their conclusion that the CD of ET-1 "rules out the presence of any α-helical contribution" and proposed a structure made up of β turns. The data presented in this paper stands in clear conflict to that interpretation; a significant degree of helicity is present without the addition of alcohol co-solvents. The data for the bisPen-ET analog clearly indicates a more extensively (and presumably, less dynamic) helical conformation. The ellipticity data are given in Table 2.

Returning to our CD data for native ET-1 (Fig. 1), a small, but significant, pH effect was observed. In purely aqueous media the minimum is red-shifted upon acidification, with even greater relative changes at *ca* 225 and 193 nm. In all mixed aqueous alcohol media the dichroic extrema increase in intensity at pH ≤ 5.6 (versus pH ≥ 7.2). Changes in extrema location were

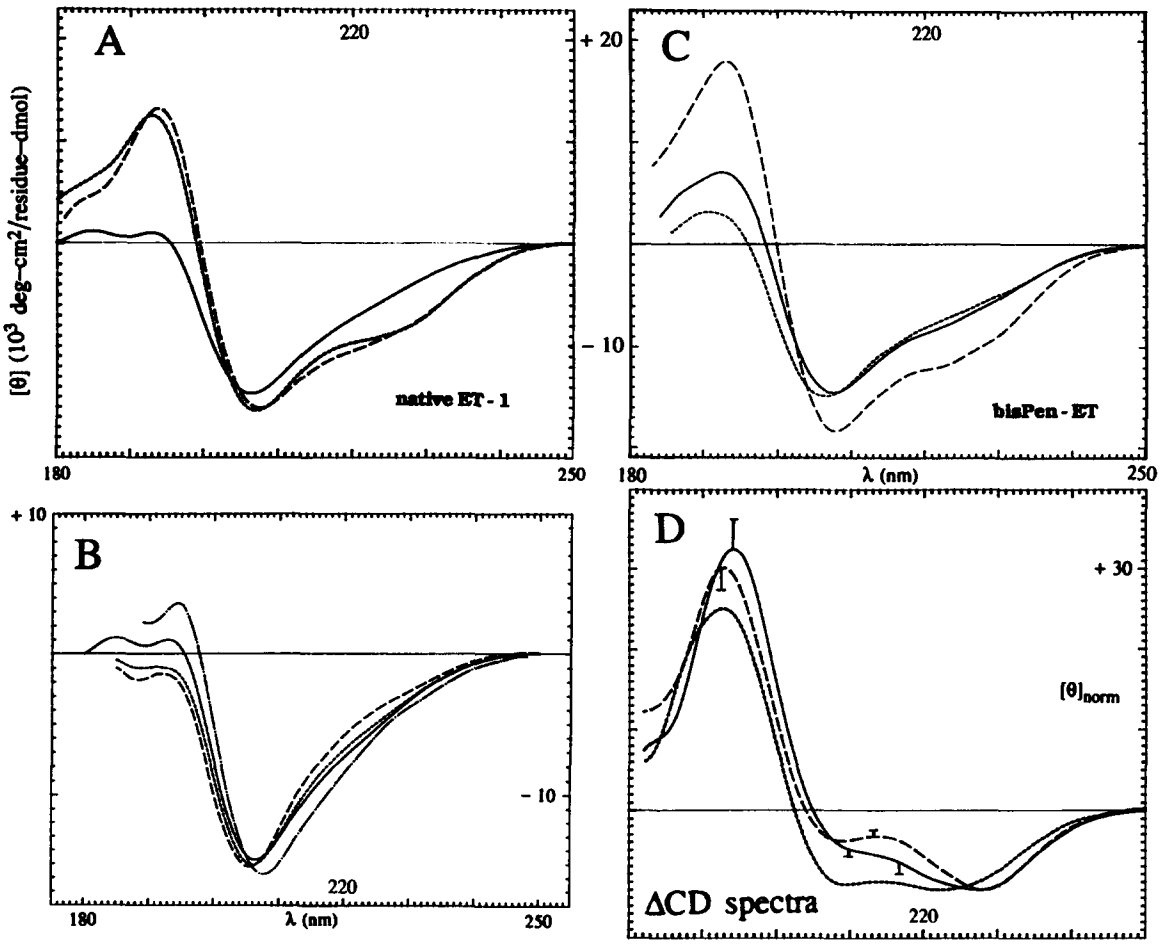

Figure 1. CD spectra of bis-disulfide endothelins. Native endothelin-1: Panel **A** compares the pH 4.6 spectra showing the effects of TFE (- - - -) and HFIP (·····) addition to 56 and 40 vol-%, respectively; the solid line trace is in the absence of fluoroalcohol. Panel **B** shows the changes for ET-1 with pH in aqueous phosphate buffer at pH 4.6 (—), pH 6 (·····) and pH 7.5 (- - - -) and upon addition of glycol to 55 vol-%, also at pH 4.6 (-·-·-·). Panel **C** shows spectra of 3,15-bisPen-ET: pH 4.6 (—), pH 8 (·····) and pH 4.6 with 62 vol-% TFE (- - - -). Panel **D** shows averages of normalized ΔCD spectra for TFE (——) and HFIP (- - -) addition to aqueous buffer (pH 4–6) solutions of ET-1. The extremum at 225–229 nm was set to −10000°, error bars indicate the standard errors at the other extrema and inflection points. The (·····) curve shows a representative TFE titration ΔCD curve for the 3,15-bisPen analog (62%–0% TFE at pH 4.6).

Table 2. Key CD data for ET-1 and its 1,15-monocyclic analogs

Peptide	pH	θ_{225}	$\theta_{221.5}$	θ_{min} (λ)	Other[a]
ET-1 (values from a single experiment are given, those used in the calculations are averages)					
	@ 7.5	–4000	–5190	–15480 (206.4)	~ –1400
@ pH 4.6		–5360	–6770	–14960 (206.4)	+1000(193.6)
glycol → 55%		–5860	–7700	–16000 (207)	+3660 (194.4)
TFE → 53%		–9000	–10500	–16100 (207.6)	+13100 (193.6)
HFIP → 40%		–8950	–9590	–16400 (207)	+12630 (193)
[**Pen^{3,15}**, Nle^7] ET-1					
@ pH 8.0		–6550	–7590	–14860 (206.2)	~+3200 (190.6)
TFE → 42%		–10200	–11300	–17440 (207.8)	+15300 (192.8)
@ pH 4.6		–7160	–8270	–14500 (207.4)	+7070 (192.4)
glycol → 42%		–9360	–11630	–16100 (208.6)	+12700 (194.6)
TFE → 62%		–11050	–12050	–18340 (207.8)	+17900 (193.0)
HFIP → 42% (λ_{iso} = 201.8)		–11960	–12790	–19100 (207.8)	+20600 (193.6)
Monocyclic [Nle^7] ET-1 Analogs					
[Ala^3, **Aib^{11}**]-	@ 7.5	–60	–720	–11890 (201)	
@ 4.6		–15	–460	–10150 (201.8)	–7300
TFE → 20%		–4500	–5120	–11580 (207.6)	+10300 (194.4)
TFE → 44%		–4880	–5290	–11700 (207.2)	+11080 (194)
HFIP → 33%		–4140	–4600	–12300 (206.8)	+11880 (193.8)
[**Ala^{3,11}**]-	@ 7.2	–2620	–3300	–13100 (199)	
TFE → 66%		–6310	–6700	–11660 (206)	~ +1000 (194)
[**Ala^{3,11,17}**]-	@ 7.5	–1580	–2110	–14000 (199.8)	
@ 4.6		–1410	–2030	–13100 (200.6)	–8500
Glycol → 40%		–2720	–3760	–13220 (203.2)	
TFE → 44%		–4290	–5290	–12360 (205.4)	~ +5800 (193)
[Ala^3, **D-Ala^{11}**]-	@ 8.0	–510	–1020	–9080 (198)	
	@ 4.6	–230	–820	–8600 (198.4)	–6300
TFE → 54%		–2580	–3100	–7450 (205)	+2040
HFIP → 40%		–2620	–3120	–7440 (205)	+3370
[Ala^3, **Pro^{11}**]-	@ 4.6	–360	–550	–14180 (197.2)	–11300
TFE → 60%		–2380	–2430	–10410 (202.8)	–2870
HFIP → 44%		–2840	–2900	–10390 (203.8)	+1220
[Ala^3, des-Ser^4, **D-Ala^{11}**] ET-1					
	@ 8.0	+410	–510	–16070 (198)	+680 (227.4)
	@ 4.6	+675	+10	–14540 (197.8)	–11600, +975 (227.6)
TFE → 24%		–3580	–4800	–12010 (205.8)	+4010 (193.4)
TFE → 48%		–4410	–5370	–12700 (206.0)	+7200 (192.8)
HFIP → 36%		–5670	-6230	–13020 (206.2)	+10780 (192.8)

[a]Other extrema and the value observed at 193 nm are recorded in this column. In the case of extrema the observed location is given in parentheses.

less noticeable in spectra recorded at 30–50% TFE or 40% HFIP, but displayed a *ca* 8% decrease in amplitude at pH 7.5 versus pH 4.6 (data not shown). The results can be rationalized as a small (≈ 6%, net) increase in helicity in acidic media with an isodichroic at 207.4 nm. NMR studies reveal that the N-terminus *and* the His sidechain titrate in this pH range. The latter being the more likely change responsible for an increase in helicity.

In order to isolate the effect of bulk solvent changes, all ΔCD spectra were obtained at constant buffer ratios. Independent of pH, the titration isodichroic for fluoroalcohol addition was observed at 203.4–205.5 nm,

red-shifted from its position (202 nm) in reference peptides which form helices of 11–12 residue length. The position of the isodichroic corresponds to the formation of a 7 residue long helix (205 nm, observed for [Pro^5]-C-peptide analogs, see panel B of Figure 3, *vide infra*).[16] This would imply a maximum helix domain length of 8–9 residues. Isodichroic values ≤ 204 nm were observed only with HFIP. We have determined the ΔCDs using three different lots of ET-1 (5 determinations for HFIP, 9 determinations for TFE), the average traces (normalized to −10,000° at the minimum) appear in panel D of Figure 1. The ΔCD curves display some anomalous features. Most notably the minima are at 225–229 nm, far to the red of the location expected for the helix $n \rightarrow \pi^*$ transition (221 ± 2nm). This suggests that either CD changes for the Tyr/Trp sidechain chromophores which occur during the titration and/or a $\beta \rightarrow \alpha$ transition is also occurring. (Including a $\beta \rightarrow \alpha$ change modifies the helix domain length estimate to 6–8.) The titration ΔCDs for the 3,15-bisPen analog was a better match to expectations: λ_{iso} = 202.2 ± 0.4 nm with the minimum at 223 nm; suggesting a longer helix and lessened contributions from solvent induced conformational changes in sidechain chromophore contributions.

The absence of any concentration effects, noted above, justifies the use of NMR structural conclusions concerning ET-1 to rationalize the CD data. The NMR data for aqueous glycol[7c,8] provide compelling evidence for a seven residue helix (from Lys^9 to Cys^{15}) and similar conclusions have been reached based on data collected in aqueous acetic acid[7a] and acetonitrile.[7b,12] Only a single spectrum for ET-1 in TFE has been figured;[30] it shows i/i+3 connectivities only from residues 9 through 16. Thus, it appears reasonable to ascribe a unit helix population (with some terminal fraying) and a helix length of n = 8 to the CD data recorded at high fluoroalcohol ratios. An estimate of helix population (f_H) in the absence of fluoroalcohol can then be calculated from the maximum observed value of $\Delta[\theta]_{225}$ upon fluoroalcohol addition. A 100% change in population, would be given by:

$$\Delta\,[\theta]_{225}(100\%\ \text{change}) = (8/21) \cdot \{[\,\theta\,]_{225}(\text{infinite})\} \cdot [(1-2.53/8)]$$

using Yang's value for $[\theta]_{225}$(infinite) (= −35,500°)[35] and the usual correction for the length dependence of helical ellipticities. If we assume that the Lys^9 to His^{16} (n = 8) helix population approaches 1.0 at high vol-% fluoroalcohol, the $\Delta[\theta]_{225}$ value (−3600 ± 200° going

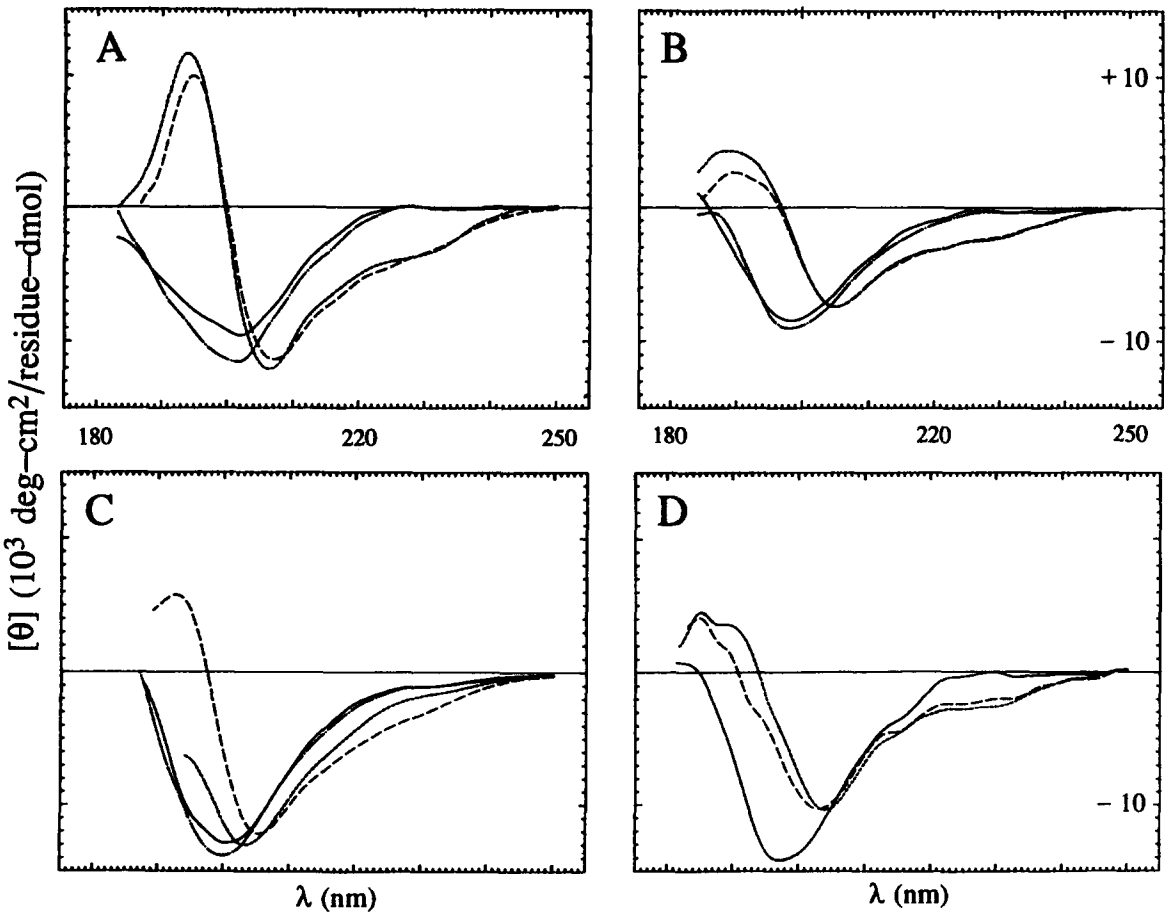

Figure 2. CD spectra of four [Ala^3, Nle^7, Xaa^{11,17}]ET-1 analogs. In each case the scales are [θ] = −15000 – +15000°, λ = 175–255nm. Throughout (——) indicates pH 4.6 aqueous buffer and (·-·-·) indicates pH 7.8 ± 0.2 aqueous conditions; (- - - -) is used for aqueous TFE at pH 4.6 [vol-% TFE as indicated], and (·····) corresponds to 33–50 vol-% HFIP (panels **A, B, D**): **A**, Aib^{11} [18%], **B**, D-Ala^{11} [54 %], **C**, Ala^{11,17} [44 %], **D**, Pro^{11} [66%]. In panel **C**, the dotted curve is for 40% glycol.

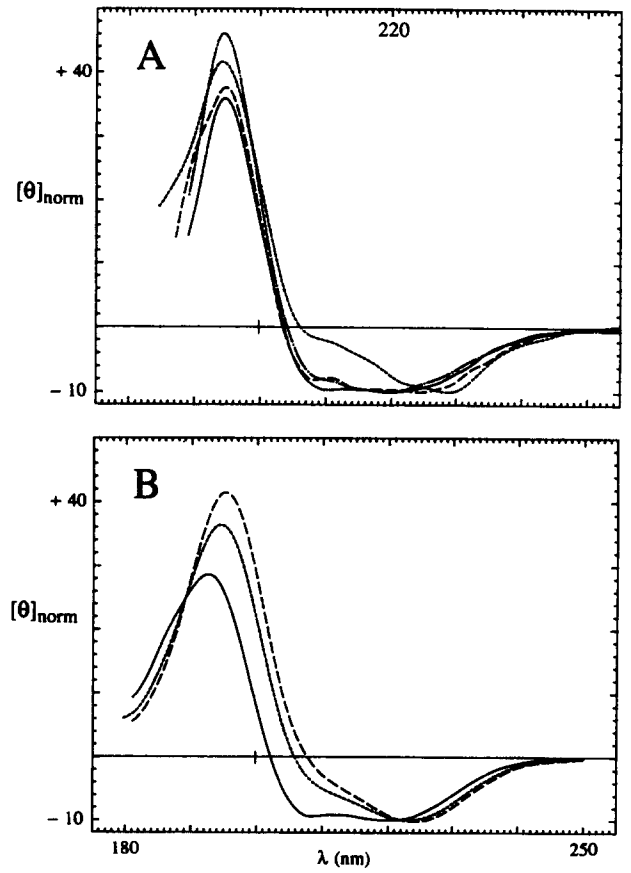

Figure 3. Panel **A**, Normalized ΔCD spectra for TFE addition to aqueous buffer (pH 4) solutions of [Ala^3, Nle^7, Xaa^{11,17}]ET-1 analogs: Aib^{11} (——), Ala^{11,17} (-·-·-), D-Ala^{11} (- - - -), and Pro^{11} (·····). Panel **B**, Reference titration ΔCD spectra for helix lengths of 11 (——), 7 (·····) and 5 (- - - -). The latter is an extrapolation based on the first two curves, which are averages of experimental curves.[16]

from 0 → 53% TFE) implies a helical state population of 0.74 (n = 7) or 0.61 (n = 8) in aqueous buffer free of added TFE. A similar CD analysis of the effect of ethylene glycol indicates a *ca* 0.04 increase in f_H occurs upon addition of ethylene glycol to 55 vol-%, fully consistent with the NMR data recorded in those conditions.[8] The CD data thus indicates some disorder within the helical domain.

NMR data also provides evidence for disorder within the helix. In the case of ET-3, Mills[36] used dynamics course time-averaged NOE constraints to conclude that the helical domain is not as highly populated as that in sarafotoxins. The evidence, in the case of native ET-1, deserves mention as it has not been fully explicated previously. The three-dimensional details of the helix in the region from Glu^{10} through Phe^{14} are very well-defined in the NMR structure ensemble for ET-1 in aqueous glycol medium, with the valine restricted to a single rotamer about χ^1 while in the helical form.[8] However, a population analysis based on either the value of $J_{\alpha\beta}$ or the HN/γ_1,γ_2 NOE ratios within the valine residue indicates that the major conformer has a 0.70 ± 0.06 fractional population. Thus, both the CD and NMR data provide support for the use of an incompletely populated helix (n = 7) in water becoming longer, n = 8 (or 9), and more fully populated upon addition of fluoroalcohol as a model for native ET-1. As further support of this analysis, we note that for the bisPen analog, with a longer (Lys^9 → Asp^{18}) helix which is less frayed, a similar analysis of NOE ratios and coupling constants indicates an essentially exclusive population of the χ^1 = 180° form for Val^{12}. In that analog, backbone NH exchange half-lives for residues 12–15 increase to 10$^+$ h under conditions where native ET-1 displays values of 1–3 h.[10]

Monocyclic analogs, CD analysis

CD spectra were recorded for each monocyclic analog in aqueous phosphate buffer at pH 7.2–8 and also at one or more acidic pH values 6.0, 4.6, 4.0 and/or 3.6. In general, we observed more consistent values at pH ≤ 4.6 and these were used for the analysis of helix propagation. Preliminary NMR studies of several of these species reveal aggregation phenomena at pH values of 5.0–6.8. The ease with which helix formation or extension occurred was assessed by adding trifluoroethanol (TFE) to 20–67 volume per cent. HFIP was also examined in some instances. Each analog was examined at ≥ 44% TFE. Representative spectra are shown in Figure 2. Table 2 summarizes the molar ellipticity values which were observed for the analogs, 3,15-bisPen-ET and native ET-1. Due to the low solubility of the D-Ala^{11} analog, we also examined the more soluble des-Ser^4 deletion by-product from the synthesis. In the absence of fluoroalcohols, all of the monocyclic analogs displayed less helicity than native ET-1. In general, %-helicity as indicated by the intensity of the negative band or shoulder at 221–225 nm was, in the purely aqueous media, somewhat greater at pH 4–6. The Aib^{11}-analog was an exception to the latter generalization displaying, as did the Pro^{11}-analog, no detectable helicity (as judged by the $[\theta]_{225}$ value) in the absence of added TFE. The red-shifting of the minimum, an alternate measure of helicity, suggests the following order of helicity for pH 4.6 aqueous media in the absence of added fluoroalcohol:

most helical ← bisPen-ET ET-1 Aib^{11} Ala^{11} D-Ala^{11} Pro^{11} (least helical)

λ (min) = 207.2 206.4 201.8 199.8–199 198 197 nm

All of the monocyclic analogs become significantly more helical upon addition of fluoroalcohols. The resulting titration ΔCDs appear in panel A of Figure 3. These were used to assess the maximum lengths of the resulting helices. Per cent helicities for these species were calculated in a number of ways. The results are collected in Table 3. The initial assessments used the observed ellipticities at 221.5 and 225 nm. Given that both the Trp and Tyr residues present can display positive sidechain chromophore ellipticities at these wavelengths, and that [Ala^{1,15}]ET-1 displays a $[\theta]_{max}$ of +1720 at 230 nm,[23] we calculated minimum and maximum %-helicity estimates as:

$$\text{minimum \%-helicity} = 100\ \{[\theta](\text{obs})_\lambda + 600\}/\ [\theta](\text{std})_\lambda$$

$$\text{maximum \%-helicity} = 100\ \{[\theta](\text{obs})_\lambda - 1600\}/\ [\theta](\text{std})_\lambda$$

Table 3. Calculated per cent helicities

	%-Helicity		Est'd helix length (n) at high	%-Helicity [f_H (n)][a]	
	water	+ TFE	vol-% TFE	water	+ TFE
ET-1	22 ± 5	32 ± 4 (7)	6–8[b]	29 [0.76 (8)]	40 [0.94 (9)][c]
3,15-bisPen	28 ± 4	39 ± 5 (9)	≥9	35 [0.81 (9)] or 40 [0.85 (10)]	42 [0.98 (9)]
[Ala^3,Nle^7,Xaa^{11}]-analogs					
Aib^{11}	< 7	18 ± 4 (4)	≥ 9	< 9	27 [0.62 (9)]
Ala^{11}	11 ± 4	23 ± 5 (5)	8	18 [0.48 (8)]	33 [0.86 (8)]
Ala^{11,17}	7 ± 4	17 ± 4 (4)	7	9 [0.43 (7)]	26 [0.80 (7)]
D-Ala^{11}	< 8	11 ± 4 (3)	8	≤ 10	18 [0.48 (8)]
des[Ser^4]	< 5	17 ± 4 (4)	8	< 2	26 [0.67 (8)]
Pro^{11}	< 6	9 ± 4 (2)	5	< 8	22 [0.90* (5)] 24 [1.00 (5)] in HFIP

[a]The percentage of helicity is calculated as [100(f_H • n)/21] where f_H is fractional population of the helix and n is the length.
[b]The isodichroic indicates n = 8–9, however at least a 1 nm blue shift is expected in the apparent isodichroic if the helix propagation occurs through a residue with a β conformational preference in aqueous media (see text).
[c]Given the assumptions concerning the contribution from the non-helical portion of the structure, a fully populated helix with n = 8 is also consistent with these data.

The values of $[\theta](\text{std})_\lambda$ used are: λ = 225 nm, –28,690; λ = 221.5 nm, –32,200.[16] For the latter, similar values have been used in recent work on 12-21 residue partially helical peptides.[37] The results appear in the first two columns of Table 3. From these data, the minimum length of helix required to explain the $[\theta]_{221-225}$ shoulder in TFE-containing media can be calculated (parenthetic value in column #2). For ET-1 this procedure gives a helical extent estimate (in non-fluoroalcohol containing media) less than that required by the NMR data; this is presumably the case for the monocyclic analogs as well and demonstrates the need to take into account the extensive fraying and reduced ellipticities associated with even shorter helices. If we had employed $[\theta]_{222}(\text{helix}) \approx -38{,}000 \rightarrow -40{,}000°$, values commonly used for longer helices and as the unfrayed reference value[38,39] [the first used in a study of RNAse S-peptide in aqueous TFE[40]], the under-estimation would have been more severe. We also calculated %-helicity values for the monocyclic analogs (and for ET-1) taking into account the expected change in per residue molar ellipticities associated with short helices.[35,39,41]

Our method for assessing "est'd helix length" in the high vol-% TFE media requires some comment. Fluoro-alcohol titration ΔCD have been measured for a series of C-peptide analogs for which NMR studies reveal a fluoroalcohol-induced increase in the population of an n = 11–12 helix. A [Pro^5]-analog of RNAse C-peptide, AETAPAKYQRAHANH$_2$, provided an experimental confirmation of this prediction and a helix length n = 7 titration difference curve. An extrapolated curve for n = 5 was also generated from the experimental data. These 'normalized' expectation ΔCD curves appear in panel B of Figure 3.

The helix length estimate for each analog (in the more helical state present at high TFE ratios) comes from the comparison of the CD difference plots associated with the addition of TFE to the curves in Figure 3 (panel B). The key observations are the isodichroic, shown as the point where λ crosses zero in the Δplot, and the relative intensities at *ca* 208 and 193 nm. With the exception of the Pro^{11} analog, the monocyclic analogs examined displayed minima at 220–223 nm consistent with a helix/coil transition with no complications due to changes in aryl side chain chromophore contributions during the titration. We therefore expected this method to yield more dependable estimates than that for native ET-1. None of the monocyclic analogs display a 215 nm maximum or a ΔCD spectral feature at that location (which would have reflected the presence of β structure and a change in β structure contribution, respectively). A simple two-state equilibrium could thus be assumed at each residue that becomes helical at high vol-% fluoroalcohol.

The per cent helicity in columns 4 and 5 takes into account the experimental estimate of helix length (n). The fractional population of the helical state, f_H, is calculated from the observed value of $[\theta]_{225}$ assuming that a unit population would afford a *molar* ellipticity of:

$$[\theta]_{225} \text{ (due to the helical portion of structure)} = 36780n\,(1 - 2.53/n).$$

A value of $[\theta]_{225}$ set to +2000° was used for the remaining portion and includes potential aryl side-chain contributions. The per cent helicity is then equated with (100/21) (n • f_H). This method should give the maximal extent of the helical segment and thus provide the largest %-helicities that are allowed by the data. The calculated helix populations for ET-1 were in agreement with the conclusions reached from the NMR data and suggest that the helix can extend, in a frayed form, to Leu^{17}. The data for the Pro^{11} analog suggests

that the helical domain contains, at most, five residues, presumably spanning either 11–15 or 12–16. The remaining analogs can all form a helix with at least a 7 residue span based on the TFE titration ΔCDs and the absolute value of $[\theta]_{-222}$ upon fluoroalcohol addition.

The titration behavior of the Ala^{11} and Aib^{11} analogs were examined in greater detail, since they displayed substantial pharmacological potency. The Aib^{11} analog differs in the ease with which helix propagation can be induced. The measures of %-helicity increase slowly but steadily for the Ala^{11} analog from 20–55 vol-% TFE. For the Aib^{11} analog, the initial helicity at 0% TFE *appears* to be less (the location of λ_{min} suggests that some helicity may be masked by other CD contributions of opposite sign at 220–225 nm), but at titration to 18 vol-% TFE helix propagation to nearly the maximum extent observed has occurred (see panel A, Fig. 2). The normalized ΔCDs recorded throughout the titration are within experimental error of each other (Fig. 4). They indicate either the partial formation of a rather long helix ($n \geq 9$) or a steady increase in helix length rather than population during the titration. The latter seems less likely; helix formation is expected to be a cooperative phenomenon. The Pro^{11} analog was strictly limited to a short helix which is fully populated at *ca* 40% TFE, no significant increases in $[\theta]_{225}$ occurred up to 66% TFE levels.

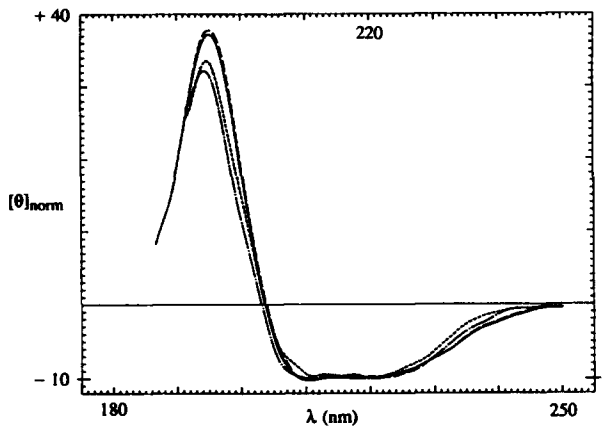

Figure 4. Normalized ΔCD spectra for TFE addition to aqueous buffer (pH 6) solutions of [Ala^3, Nle^7, Aib^{11}]ET-1: 0 → 18% (·····) and 0 → 62% (·–·–·–), taken from a single titration experiment. The solid line trace is 0 → 44% from another experiment at pH 4.6 and (- - - -) is 0 → 20% at pH 4.6.

Conformational studies of the C-terminal fragment

It is well-established that Trp^{21} and Tyr^{13} both represent binding requirements,[5,9a,42] with the C-terminus as the singularly most significant unit[5] for avid binding; however, there remains considerable dispute concerning the degree of structure in this unit and whether it bears a fixed relationship to the disulfide-linked core of ETs.[12,43] Our NMR studies of ET-1 indicate that the C-terminus is conformationally averaged,[8,12b] but not to the extent (or with the rapid segmental motion) seen in the loop region[8] and cannot be regarded as a fully disordered 'random coil' even though it's conformation is not affected by changes in the structuring within the disulfide-linked core.[12b] CD appeared to offer a probe of this as well. Both of the noted aryl sidechains are known to yield a positive CD peak at 223–228 nm when they are in an unstructured form[44] (confirmed in this laboratory). A positive CD band at > 220 nm (in aqueous media) was observed only for the [D-Ala^{11},desSer^4] analog; this maximum is masked by increasing helicity as fluoroalcohols are added. It remains to be established whether this feature is due to Trp^{21} or Tyr^{13}. CD spectra recorded for the C-terminal endothelin fragment, HLDIIW, provide some insight (Fig. 5). The spectra of HLDIIW do not resemble that expected for a random coil with a C-terminal Trp, which we model as the experimental CD of GHKW with two added units of the standard signature[16] for a disordered residue. In panel A of Figure 5, the spectra of HLDIIW and GHKW (without the addition of added units of disorder) appear with an ellipticity scale in molar, not residue-molar, units. It is also evident, by

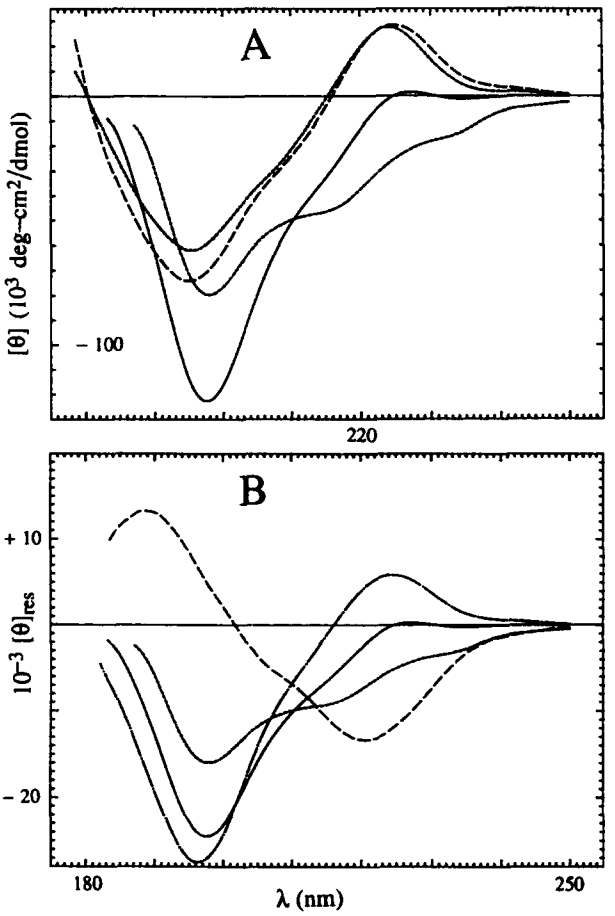

Figure 5. Comparisons of HDLIIW and GHKW. Panel **A**, spectra (in molar ellipticity units, from the far UV cut-off to 250 nm) of HDLIIW were recorded at pH 4 with no TFE (——) and with 40 vol-% TFE (·····); those of GHKW were at pH 4.6 with no TFE (- - - -) and with 60 vol-% TFE (·····). In panel **B**, the ellipticity scale is deg-cm^2/residue-dmol or, for (- - - -) deg-cm^2/turncore-dmol. The HDLIIW spectra in panel A are reproduced with the same symbols. Aqueous GHKW plus two residues of 'disorder' is shown (·–·–·). The difference curve, HDLIIW less (GHKW + 2'disorder'), is shown as a dashed line and is recast at a scale corresponding to that used by Perczel and Fasman.[45] A *ca* 60% population of a type I β turn is indicated.

CD changes, that the HLDIIW fragment undergoes a conformational change upon addition of TFE, while unstructured GHKW displays only a small blue-shift for the low energy aryl band. The difference spectra, HLDIIW less 'XXGHKW', is very similar to the p_1 component spectrum ascribed to a type I β-turn by Perczel and Fasman,[45] indicating an increased population of a compact structure in the fluoroalcohol enriched medium. Additional studies of C-terminal fragments by both CD and NMR are in progress.

Conclusions

With regard to CD analysis of the helix content of medium sized peptides, this study provides additional indications of the importance of using reference ellipticity values that are corrected for the length of the helices present in the system under study. Equations based on the work of the Yang group, $[\theta](\text{length } n)_\lambda = [\theta](\infty)_\lambda \cdot [1 - (n/k_\lambda)]$, such as that used by Baldwin and co-workers,[39] appear to work quite well for this purpose even though the basis for the correction (increased dynamic end-fraying or an inherent decrease in rotatory power for shorter chain lengths for a static model) remains obscure. Experiments designed to probe the latter question are in progress.

With regard to endothelin analogs, for the 1,15-monocyclic analogs differing only at position 11, binding at the ET_A receptor subtype as well as vasoconstrictor potency in rabbit carotid artery correlate with the facility with which a helix of 7–8 residue length can be induced. This presumably corresponds to the helical segment $Lys^9 \rightarrow His^{16}$ observed in NMR solution structure studies, with some minor end-effects associated with imperfect helical alignments at the C-terminal end of the domain. Therefore, it is probable that the ET_A receptor bound conformation of endothelin is helical in this region and that the poorer activity of monocyclic endothelins in ET_A preparations is largely due to lower conformer populations of helix-containing structures.

Our conclusion contrasts with a report from Panek *et al.*,[46] who argued that ". . . the helical region between residues 9 and 15 of ET-1 does not appear to be critical for receptor binding and functional activity." Their conclusion was based on the fact that [Pro^{12}]ET-1 bound to membranes from rat aorta with only a 2-fold higher IC_{50} than ET-1 and that this peptide contracted rat aorta with 30-fold lower potency, suggesting, according to the authors, that the insertion of Pro for Val^{12} affects the conformation important for receptor activation to a larger degree than the conformation necessary for receptor binding. No spectroscopic data were presented to define the conformational effects of the Pro^{12} substitution on the native bicyclic endothelin. However, the authors reference unpublished CD studies, stating that the Pro^{12} substitution disrupts the helix. The helical region of endothelin from residues 9 to 15 is enforced, at least in part, by a framework consisting of the Cys^{11}-X-X-X-Cys^{15} sequence crosslinked to the Cys^1-X-Cys^3 sequence. This has been called the CSH-motif, for cysteine-stabilized α-helical motif.[47] The propensity of this framework to enforce helicity is apparent from the fact that in endothelin, the sequence Val^{12}-Tyr^{13}-Phe^{14} within the 9–15 helix is comprised of residues which are predominantly helix-destabilizing.[48] The Pro^{12} analog, looked at as a stratagem to disrupt helical secondary structure, may not produce a major helix destabilization within the cysteine-stabilized α-helical motif. Located at position 12 in the first turn of the helix, where the NH groups of the residues are not hydrogen bonded if the helix is an α helix, the absence of a free NH group in Pro^{12} may not present a problem. Although this latter effect is also present in our analogs, our monocyclic framework is more conformationally mobile, maintains the helix-destabilizing valine residue and does not contain the cysteine-stabilized α-helical motif. Therefore, the helix-stabilizing or destabilizing effect of substituted residues should be transmitted more effectively to the overall peptide conformation. For these reasons, we believe that the structure–function correlation reported in this study provides strong evidence for the involvement of partially helical peptides at the ET_A receptor. The vasoconstrictor potency of 3,15-bisPen-ET and its high affinity binding to the ET_A receptor provide confirmatory evidence. In the 3,15-bisPen analog, the NHs of residues 12–15 display D_2O exchange protection factors greater than 20-fold. This degree of sequestration of an NH from exchange by the formation of a helical i/i–4 H-bond requires that the three intervening residues have f_H values greater than 0.92.[49] It seems unlikely that the high receptor affinity of this analog is concordant with the disruption of a structural preference of this magnitude.

In our monocyclic analogs, the inclusion of a proline residue at position 11 does not preclude helix formation upon the addition of fluoroalcohols. Helix formation is relatively easily induced but is limited to a 5 residue span, which is apparently insufficient to orient the side chains for optimal binding to the ET_A receptor. The Glu^{10} and/or Asp^8 side chains are thus implicated as binding requisites. Point mutation at the latter has also been shown to change the conformational preference of ET-1;[50] this has been rationalized[12b] as Asp involvement in a helix N-cap. Now that a correlation between inducible helicity and receptor affinity has been established for the ET_A receptor, the structural features elucidated by NMR for this helical region can be used with greater confidence for pharmacophore definition and analog design.

The poor receptor affinity and lack of vasoconstrictor activity of the $Ala^{3,11,17}$-analog does not appear to be associated with a divergent backbone conformational preference in the 9–16 region. The analog displays a marginally smaller helix population than the more active analogs, but the required helix length does occur.

Instead, it is probable that Leu^{17} either provides a necessary hydrophobic interaction with the receptor or that it plays a role in favoring a conformation of the endothelin tail (particularly at residues Asp^{18}, Trp^{21}) which is critical to receptor binding and activation. Additional information on the structure of the tail is required to evaluate these possibilities and to provide strategies for the design of endothelin antagonists. The CD studies focused on the C-terminus suggest a turn preference for the isolated fragment which may be retained or modified in endothelin structures. The high potency and the experimentally demonstrated C-terminal extension of the helix in 3,15-bisPen-ET, together with the recent report that native ET-1 is helical nearly to the C-terminus in a solid state structure,[43] raises the question of the possible significance of compact structures in this pharmacophore region. Ascertaining the solution state conformational preferences in the C-terminus of intact endothelins and the 3-D details of this pharmacophore region are clearly high priority goals at this juncture. Analogs designed to test the alternate hypotheses are now under study.

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