Evidence for aggregation of endothelin 1 in water

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In this report it is shown by CD spectroscopy that endothelin 1, when dissolved in water, is able to present intermolecular interactions leading to formation of aggregates. Surface tension and conductivity measurements suggest that the aggregation occurs through formation of micelles with a CMC of about 2.2×10^{-5} M.

Endothelin; Aggregation; Circular dichroism; Surface tension; Conductivity

1. INTRODUCTION

In spite of numerous NMR investigations the conformation of the vasoconstrictor peptide, endothelin 1, is still matter of debate [1-5]. Indeed, beside the fact that the proposed conformations are based on measurements made at very high concentrations and in media containing organic solvents [1-4], the experimental conditions are far from those used in the biological assays [6]. Furthermore, it was suggested from HPLC size exclusion chromatography that endothelin 1 can exist as higher order aggregates [5]. In order to shed some light on the behaviour of this peptide in water, we have examined some properties of endothelin 1 when dissolved in water at various concentrations. As the experiments which will be reported below require quite large amounts of material, we have synthesized endothelin 1. We describe here this synthesis and report circular dichroism data together with surface tension and conductivity measurements which show clearly that endothelin 1 form aggregates and that this molecule has a micelle-like behavior at concentrations higher than about 0.05 mg/ml.

2. EXPERIMENTAL

2.1. *Solid-phase synthesis of endothelin 1*

Endothelin 1 was synthesized using an amidated polyacrylic resin (Expansin) [7] and the continuous flow technique [8] on a Milligen 9050 Pepsynthesizer. The C-terminal tryptophan was linked to the amino group of the support through a glycolamidic ester handle [9]. Amino acids were used as their Fmoc α-amino derivatives with tBu as side-chains protecting group except for Cys and Lys which were tritylated and Boc protected, respectively. They were purchased from Novabiochem and their chemical and optical purity were checked by RP-HPLC and $[\alpha]_D$ measurements.

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Activation of the carboxyl groups was performed with TBTU [10]. The endothelin 1 sequence was built using the standard protocol of the Milligen 9050 Pepsynthesizer. After the completion of the synthesis, side-chains were deprotected by treating the peptide still attached to the resin by TFA (90), H_2O (35), phenol (2.5) and ethanedithiol (2.5).

The peptide was removed from the resin by hydrolysis carried out with 5 equiv. of NaOH in isopropanol-H_2O (70:30) [7] and was prepurified by chromatography on Sephadex G10. For disulfide bridge formation, the crude peptide was dissolved in water (30 mmol/ml) and subjected overnight to air oxidation at pH 8.2. After freeze-drying, endothelin 1 was purified by semipreparative RP-HPLC (Fig. 1) and the compound thus obtained was identical to a commercial sample purchased from Novabiochem. The amino acid analysis was in full agreement with that expected on the basis of the sequence.

2.2. *Physicochemical investigations*

Circular dichroism measurements were made on a Jobin-Yvon Mark V dichrograph using 1, 2, 5 and 10 mm thick cells. Concentrations are given in the legends of the figures. Solutions were prepared as follows: the peptide is previously dissolved in pure water and aliquots of the required amount are added to the medium: pure water, NaF or NaCl 0.1 M phosphate buffer (10 mM) at pH 6.9.

Surface tension measurements were made at 25°C using a Prolabo tensiometer with a Wilhelmy platinum plate. Solutions were prepared by dissolving the peptide in water which was distilled over $KMnO_4$. The surface tension of this pure water was 72 mN/m. The starting concentration was 0.56 mg/ml and the various concentrations were obtained by dilution of the starting solution.

Conductivity measurements were made with a Tacussel conductimeter at a frequency of 1000 Hz. The solutions were prepared using the same procedure as above with a starting concentration of 0.44 mg/ml.

3. RESULTS AND DISCUSSION

The CD spectra of water solutions of endothelin 1 in the peptide absorption region (200-240 nm) at two different concentrations (0.15 and 0.0075 mg/ml) are shown in Fig. 2. Although some very small differences can be noticed in their intensities, they are all characterized by a negative band at 205 nm. The

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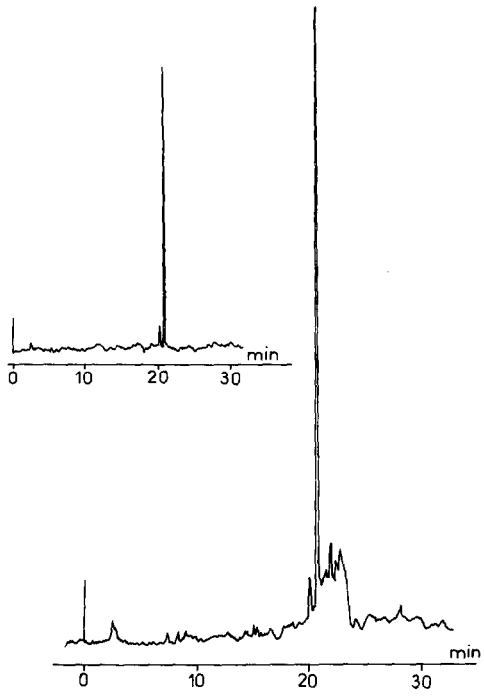

Fig. 1. RP-HPLC of crude and purified (insert) endothelin 1. Buffer A: TFA 0.1% in water; Buffer B: 60% acetonitrile in water containing 0.1% TFA. Linear gradient from 100% of A to 100% of B in 30 min. Detection wavelength 220 nm; flow rate 1 ml/min.

absence of any shoulder at higher wavelength rules out the possibility of presence of either α or β structures which would require at least the presence of shoulders at 222 or 215 nm, respectively [11], and owing to its strong similarity with spectra obtained for 19 residue long oligopeptides [12], we suggest the existence of an unordered structure. Further information was obtained by examination of the CD spectrum recorded at a higher wavelength, i.e. in the side-chain absorption region. Indeed, in this wavelength range the spectrum also depends on the peptide concentration (see Fig. 3). In Fig. 3 we have reported the spectra obtained at two different concentrations (3 and 0.3 mg/ml). Spectra recorded at 1.5 and 0.6 mg/ml are not shown as they are identical to that at 3 mg/ml. Clearly, such a behaviour indicates that a transition occurs around 1 mg/ml and also that endothelin 1 can selfassociate. In order to determine the type of association which can occur we used conventional techniques usually applied for the identification and characterization of aggregates such as micelles. These are surface tension and conductivity measurements which allow the determination of critical micelle concentration (CMC) [13]. Fig. 4 shows the variation of the surface tension as a function of the peptide concentration. It is characterized by a very strong decrease of the tension at low concentrations (<0.1 mg/ml) and then by a plateau at higher concentrations. Such a behavior is strongly reminiscent of that obtained with tensioactives. The first part of the curve which corresponds to the decrease in the tension is interpreted as the filling up of the air/water interface by the detergent molecules (the peptide in our case), while the second part of the curve corresponds to a domain where the micelles are formed. As usual, in such a situation when the detergent contains ionic groups or a net charge we tried to confirm the above conclusion by conductance measurements. The results are shown in the insert of

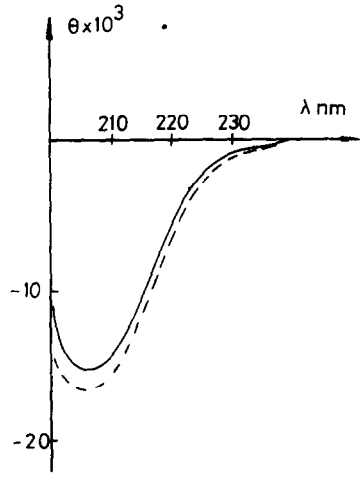

Fig. 2. CD spectra of endothelin 1 dissolved in water (—) 0.15 mg/ml, cell path 1 mm; (---) 0.0075 mg/ml, cell path 10 mm. The ellipticity θ is given in deg · cm^2/residue.

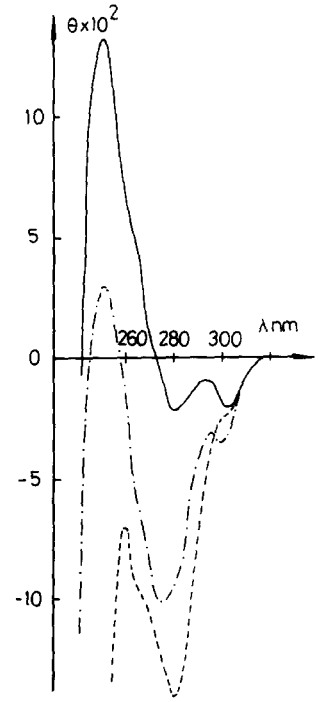

Fig. 3. CD spectra of endothelin 1. (—) conc. = 3 mg/ml in water, cell path 1 mm; (-.-) conc. = 0.3 mg/ml in water, cell path 10 mm; (---) conc. = 2 mg/ml in DMSO, cell path 1 mm. The ellipticity θ is given in deg · cm^2/mol.

Fig. 4 and are in full agreement with the surface tension measurements and indicate that the CMC is around 0.05 mg/ml (2.2×10^{-5} M). It must be mentioned here that another event occurs at higher concentrations (around 0.2 mg/ml) (see insert of Fig. 4) which can be related to the CD observations in the side-chain absorbtion region. The identification of this latter transition is underway. In order to try to determine the conformation of endothelin in the isolated form, we recorded CD spectra after addition of organic solvents such as ethanol, acetonitrile or acetic Mark to aqueous solutions of the peptide expecting that this operation will destroy the aggregates. No major changes could be detected when compared to solutions in pure water (spectra not shown). Only when endothelin 1 is dissolved in DMSO (2 mg/ml) some modifications occur around 260 nm (see Fig. 3). Unfortunately, owing to the spectroscopic properties of this latter solvent no information could be gained at lower wavelength.

As endothelin 1 is a biologically active peptide, it has also been examined under more physiological conditions by CD spectroscopy. In both media, NaCl and NaF 0.1 M at pH 6.9, in the side-chain absorption region (Fig. 5) although some small differences are noticeable around 245 nm when compared to the spectrum shown in Fig. 3 at 3 mg/ml, the same behavior is observed and resembles that obtained in pure water: a concentration dependence of the spectrum, indicating again that endothelin 1 can selfassociate. Only the spectra recorded at 3 and 0.3 mg/ml are shown in Fig. 5. The spectrum obtained at 0.2 mg/ml is not reported as it is very similar to that obtained in pure water at 0.3 mg/ml (see Fig. 3). The slight shift of the transition toward the lower concentrations (≈ 0.2 mg/ml) is probably due to a salting out effect. At lower wavelength, as the spectrum is obscured by Cl^- ions, the investigations were carried out only in the NaF medium and a typical spectrum is shown in Fig. 5. This spectrum remains identical whatever the concentration is between 0.15 and 0.015 mg/ml. Owing to the very strong similarity between these spectra and those obtained in pure water it can be concluded that the presence of NaF has no influence and no structuring effect on the backbone conformation of endothelin 1, at least under the experimental conditions used here. This probably holds true for NaCl, as at higher wavelength the nature of the anion (F^- or Cl^-) has very little influence.

Our investigations reveal that, except in DMSO which is very probably the most dispersive solvent, endothelin 1 forms aggregates and that the conformation of the peptide, both in the 'monomeric form' and when engaged in the aggregates, does not correspond to any classical ordered structure and is more probably close to a random coil form. This conclusion appears to conflict with previous proposals which suggested that the conformation of endothelin 1 could be, at least partly, α-helical [1–4]. Furthermore, our conclusion is supported by some experimental observations such as the HPLC behaviour [5]. Also the fact that the peptide can

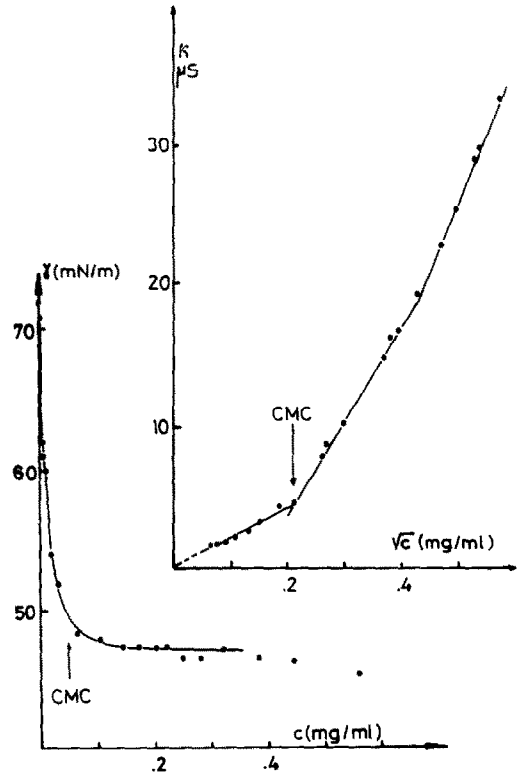

Fig. 4. Surface tension γ versus endothelin 1 concentration. (Insert) Specific conductance versus the square root of the endothelin 1 concentration. The CMC is indicated by the arrows.

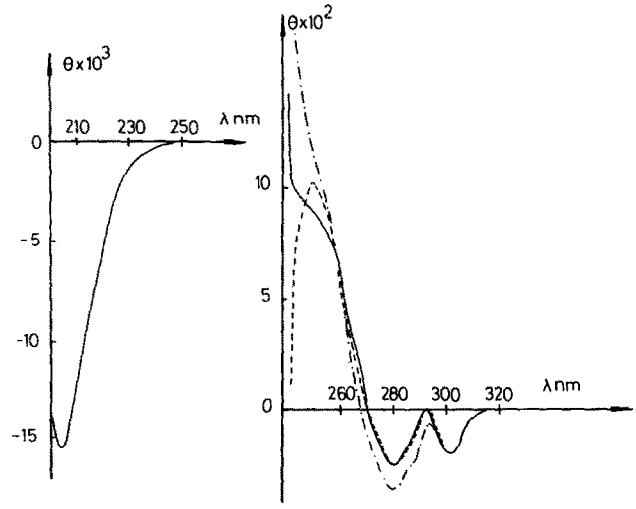

Fig. 5. (Left part) CD spectrum of endothelin 1; conc. = 0.075 mg/ml; NaF 0.1 M; pH 6.9. Cell path 1 mm. The ellipticity θ is given in deg · cm^2/residue. (Right part) CD spectra of endothelin 1. (—) NaCl 0.1 M, pH 6.9, conc. = 3 mg/ml, cell path 1 mm; (-.-) NaF 0.1 M, pH 6.9, conc. = 3 mg/ml, cell path 1 mm; (---) NaF and NaCl 0.1 M, pH 6.9, conc. = 0.3 mg/ml, cell path 10 mm. The ellipticity θ is given in deg · cm^2/mol.

selfassociate points out the importance of the experimental conditions and may explain some puzzling results which appear a priori contradictory.

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