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Biophysical characterization of the isolated C-terminal region of the transient receptor potential vanilloid 1

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ABSTRACT

Transient receptor potential (TRP) proteins are sensory-related cation channels. TRPV subfamily responds to vanilloids, generating a Ca^{2+} current. TRPV1, a thermal-sensitive non-selective ion channel, possesses six transmembrane helices and the intracellular N- and C-terminal domains. The latter contains the PIP_2 and calmodulin binding sites, the TRP domain and a temperature-responding flexible region. Although the function of C-TRPV1 is known, there are no experimental reports on its structural features. Here, we describe the conformational features of C-TRVP1, by using spectroscopic and biophysical approaches. Our results show that C-TRVP1 is an oligomeric protein, which shows features of natively unfolded proteins.

Structured summary of protein interactions:
C-TRPV1 and **C-TRPV1** bind by dynamic light scattering (View interaction)
C-TRPV1 and **C-TRPV1** bind by static light scattering (View interaction)
C-TRPV1 and **C-TRPV1** bind by cross-linking study (View interaction)
C-TRPV1 and **C-TRPV1** bind by comigration in non-denaturing gel electrophoresis (View interaction)

1. Introduction

Many membrane proteins are signal integrators evolved to respond to extracellular ligands, intracellular signal transduction, transmembrane voltage changes or environmental modifications. Human sensory nerves use specialized ion-channel proteins to report environmental temperatures, and among those, we find the transient receptor potential (TRP) ion channels [1,2]. TRP proteins are a large family of cation receptor-operated channels [3], characterized for the presence of a highly conserved TRP-like domain [4,5]. This family is composed of seven subfamilies [6], responding to diverse physical stimuli such as heat, cold, osmotic stress or pH changes [7]. One of these subfamilies is the vanilloid receptor subfamily, named TRPV [5,8]. The TRPV family includes six members (TRPV1-TRPV6): the TRPV1-TRPV4 members have thermal sensibility; and TRPV5 and TRPV6 play a role in Ca^{2+} uptake in the kidney and intestine [8].

TRPV1 is a ~95 kDa integral plasma membrane protein, composed of 838 amino acids, with a transmembrane region formed by residues 433–684 and organized in six transmembrane helical segments, S1–S6, plus a "P-loop". This loop, together with helices S5 and S6, have been predicted to form the ion pore and selectivity filter, based on the homology with other ion channels [9,10]. The rest of the protein, along with the N- and C-terminal sequences, forms the large cytoplasmic region. TRPV1 functions as a non-selective tetrameric cation channel with a high permeability to calcium [11,12]. Vanilloids, and noxious chemical and thermal stimuli (>43 °C) activate TRPV1, which exhibits a time- and Ca^{2+}-dependent outward rectification. Mild acidic conditions (pH ~6) could potentiate the noxious heat and vanilloid-mediated TRPV1 activation, whereas acidic pH (<6) directly activates the channel [13]. TRPV1 has a role in acute thermal nociception, hyperalgesia and inflammation [14,15].

Abbreviations: ANS, 1-anilino-8-naphtalene sulfonate; CD, circular dichroism; DLS, dynamic light scattering; IB, inclusion body; TRP, transient receptor potential; TRPV, transient receptor potential vanilloid receptor; SLS, static light scattering; TRPV1, transient receptor potential vanilloid receptor subtype 1; C-TRPV1, the C-terminal domain of TRPV1

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The cytosolic C-terminal domain of the TRPV1 (C-TRPV1) possesses two distinct regions, namely, the TRP domain, comprising residues 684–721, and a regulatory region, consisting of amino acids 767–810, being both connected by a flexible linker [13,16,17]. The C terminus has been proposed to include the temperature sensor activity (specifically in the linker segment (amino acids 727–752) [18,19]), tubulin-interacting modules [20] and phosphorylation sites [21]. The TRP domain of the C-TRPV1 has been defined as an association domain involved in the tetramerization of the TRPV1 channel subunits into functional channels [13]. This domain is thought to form a tetrameric coiled-coil structure [22], as the ones found in the K^+-selective voltage gated channels [23]; this segment also contains the interacting region with PIP_2. The regulatory region of C-TRPV1 has significant similarities with the HCN2 cyclic nucleotide binding domain [24] and it comprises another PIP_2 interacting region (residues 777–810) and a calmodulin binding site (residues 767–801) [25,26].

There are several studies on the C-TRPV1 functional features and their structure–activity characteristics, but the knowledge about its biophysical and structural behavior is scarce, although, recently, a modeled structure has been proposed [22]. In this work, we report the first structural characterization of C-TRVP1, by using spectroscopic and biophysical techniques.

2. Materials and methods

Further details are provided in the Supplementary information.

2.1. Fluorescence

Fluorescence spectra for C-TRPV1 were collected on a Cary Eclipse spectrofluorometer (Varian, USA) interfaced with a Peltier temperature-controlling system. Sample concentration was 2 μM, and the final concentration of the buffer was, in all cases, 100 mM. A 1-cm-path-length quartz cell (Hellma) was used.

(a) *Steady state fluorescence measurements*–Samples of C-TRPV1 were excited at 280 and at 295 nm at 25 °C in the pH range 2.0–13.0 to characterize for a putative different behavior of either tryptophan or tyrosine residues. The slit width was 5 nm for the excitation and emission lights. The fluorescence spectra were recorded between 300 and 400 nm. The signal was averaged for 1 s and the wavelength increment was 1 nm. Blank corrections were made in all spectra. The salts and acids used in buffer preparation were: pH 2.0–3.0, phosphoric acid; pH 3.0–4.0, formic acid; pH 4.0–5.5, acetic acid; pH 6.0–7.0, NaH_2PO_4; pH 7.5–9.0, Tris acid; pH 9.5–11.0, Na_2CO_3; pH 11.5–13.0, Na_3PO_4. The pH was measured with an ultra-thin Aldrich electrode in a Radiometer (Copenhagen) pH-meter.

(b) *ANS-binding*–The pH-titrations of C-TRPV1, followed by ANS-binding, were acquired at 25 °C. Excitation wavelength was 380 nm, and emission was measured from 400 to 600 nm. Slit widths were 5 nm for excitation and emission. Stock solutions of ANS were prepared in water and diluted into the samples to yield a final 100 μM dye concentration. Dye concentrations were determined using an extinction coefficient of 8000 M^{-1} cm^{-1} at 372 nm. In all cases, blank solutions were subtracted from the corresponding spectra.

(c) *Thermal-denaturations*–Thermal-denaturations were monitored by following the changes at 315 nm, 330 nm and 350 nm, after excitation at 280 and 295 nm. The scan rate was 60 °C/h, with an average time of 1 s, and collecting data every 0.2 °C. Protein concentration was 2 μM. Experiments were carried out at several pHs.

(d) *Urea denaturation*–Urea titration of C-TRPV1 was carried out at pH 7.0; the proper amount of the denaturant from an 8 M stock solution was used and samples were left overnight to equilibrate at 4 °C. Chemical-denaturations were repeated three times with new samples.

The chemical- and pH-variations were monitored by the changes in fluorescence intensity and/or in the average energy, which is defined as: $\langle\lambda\rangle = \sum_1^n \left(\frac{1}{\lambda_i} I_i\right) \Big/ \sum_1^n I_i$ [27].

2.2. Circular dichroism

Circular dichroism spectra of C-TRPV1 were collected on a Jasco J810 spectropolarimeter (Japan) fitted with a thermostated cell holder and interfaced with a Neslab RTE-111 water bath. The instrument was periodically calibrated with (+) 10-camphorsulphonic acid.

Isothermal wavelength spectra were acquired at a scan speed of 50 nm/min with a response time of 4 s and averaged over ten scans at 25 °C. Far-UV measurements were performed using 6 μM protein in 50 mM buffer, in a 0.5-cm-path-length quartz cells (Hellma). Spectra were corrected by subtracting the proper baseline. The molar ellipticity, $[\Theta]$, was calculated according to: $[\Theta] = \frac{\Theta}{10lcN}$, where Θ is the measured ellipticity, l is the pathlength cell in cm, c is the protein concentration and N is the number of amino acids (162 for C-TRPV1).

3. Results

Further details are provided in the Supplementary information.

3.1. C-TRPV1 is an oligomeric protein, which seems to be unfolded

We first characterized experimentally the conformational features of C-TRPV1.

3.1.1. Fluorescence

(a) *Steady-state fluorescence*–We recorded fluorescence spectra to monitor the environment around the tryptophan and tyrosines of the domain [28]. The C-TRPV1 has five tryptophan residues at positions 18, 61, 70, 73 and 108 (in the numbering of the domain) and two tyrosines at positions 59 and 138. The emission fluorescence spectrum of the domain at pH 7.0, either by excitation at 280 or 295 nm has a maximum at 350 nm, indicating solvent-exposure of the tryptophan residues (in an aqueous environment, the maximum should be at *c.a.* 350 nm [28]).
The pH-dependence of <λ> showed a bell-shaped behavior, with a plateau between pH 4.0 and 10.0 (Fig. 1A), suggesting that the conformation of the protein was not modified in that pH range. At high and low pHs, the <λ> decreased because of protein hydrolysis (Fig. 1A, red circles), as indicated by SDS-gels (data not shown).

(b) *ANS-binding*–ANS is used as a fluorescence probe, which binds to spatially close solvent-exposed hydrophobic patches [29]; it is interesting to note that random-coils do not bind to the probe, despite the large amount of solvent-exposed hydrophobic surface, because the solvent-exposed hydrophobic patches are not close enough within the ensemble of populations [29]. At low pH values, there was a large increase in the fluorescence intensity, suggesting binding of ANS to the protein (Fig. 1A, blue squares).

(c) *Thermal-denaturations*–Several pHs were explored by following the changes in the emission fluorescence at 315, 330 and 350 nm. We chose pHs at each of the three regions

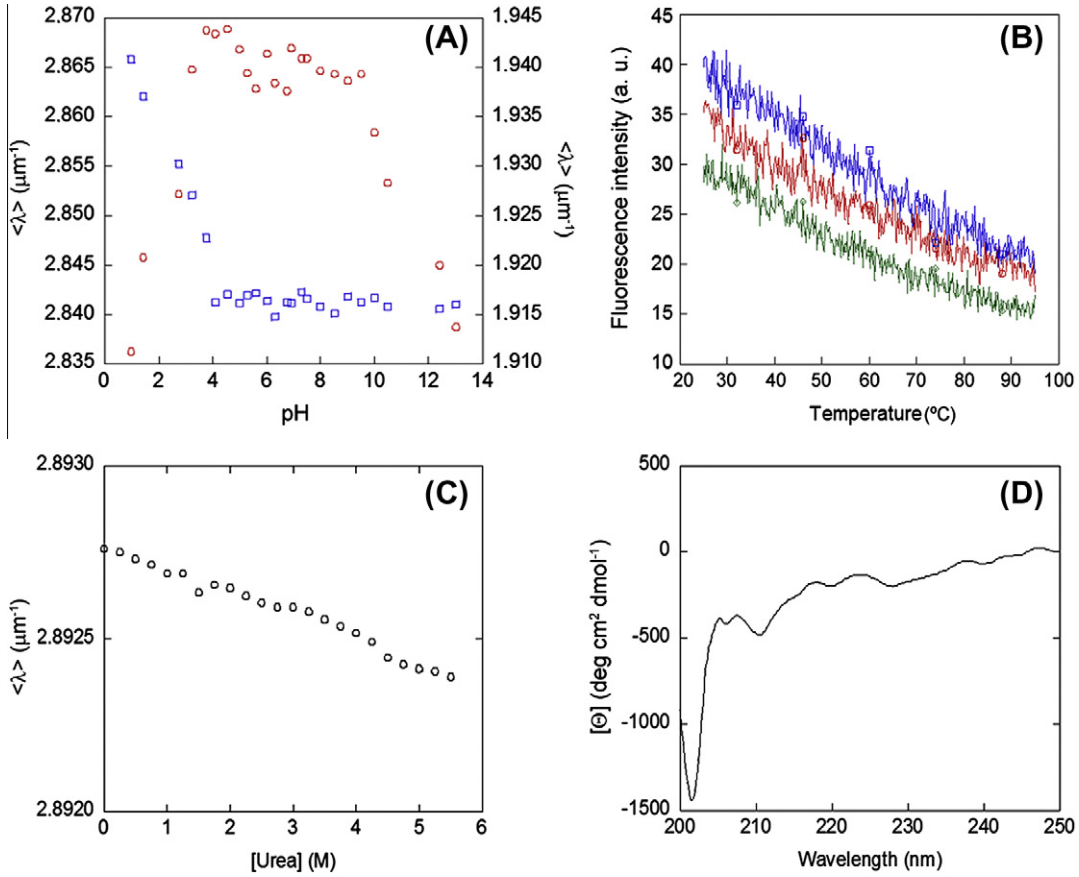

Fig. 1. Spectroscopy of C-TRPV1: (A) pH denaturation of C-TRPV1 followed by intrinsic fluorescence (red circles, left axis) and ANS (blue squares, right axis) followed by the changes in <λ>. (B) Thermal denaturations followed by fluorescence at pH 4.0 (green), pH 7.0 (red) and pH 10.0 (blue). (C) Changes in the <λ> during the urea-denaturation. The conditions were: 2 μM of protein, at 25 °C; buffer concentration was 100 mM in all cases. (D) Far UV-CD spectrum in acetate buffer pH 5.5. Experiments were made at 6 μM of protein, 25 °C; buffer concentration was 50 mM.

defined by the intrinsic fluorescence experiments (Fig. 1A, red circles): acidic, basic and in the plateau region. In all cases, no sigmoidal behavior was observed (Fig. 1B).

(d) *Chemical denaturations*–In the presence of urea, a non-sigmoidal behavior was observed in the <λ> (Fig. 1C) (or at other wavelength), which suggests that the protein does not contain any type of stable structure, as also indicated by the thermal-denaturations (Fig. 1B).

3.1.2. Circular dichroism

We used far-UV CD in the structural analysis of C-TRPV1 as a spectroscopic probe sensitive to protein secondary structure [30]. The CD spectrum was very weak at a protein concentration of 6 μM and in a 0.5-cm-pathlength quartz cell (Fig. 1D). It showed a minimum negative ellipticity at *c.a.* 200 nm, which is characteristic of intrinsically disordered proteins.

We also acquired near-UV spectra in a cell of 0.5 cm pathlength. No signal was observed, probably due to the lack of a well-fixed asymmetric environment [30] for the aromatic residues of the protein (data not shown).

Attempts to use 1D-NMR spectroscopy were unsuccessful due to the broadness of the peaks, and only some protons at the methyl region were observed which appeared clustered at the random-coil values [31].

Then, the spectroscopic probes suggest that the protein is devoid of a well-fixed secondary structure.

3.1.3. Light scattering measurements

As we describe in SI during the purification protocol, we could not perform size exclusion chromatography on C-TRPV1 to obtain information about its shape or molecular weight due to binding to the column; then we tried a light-scattering and native electrophoresis approaches. The DLS experiments allow obtaining the molecular weight estimation of a protein if we know its shape, or alternatively we assume a spherical shape. DLS experiments with C-TRPV1 showed two peaks with volumes of 65% (27.3 nm) and 35% (145 nm) (Fig. 2). These data pinpoint to the presence of a large self-associated species. To allow for a comparison, it is interesting to calculate the predicted radius for a well-folded protein or for a random-coil. The theoretical radius of a well-folded molecule is given by: $R = (4.75 \pm 1.11)N^{0.29\pm0.02}$ [32], where N is the number of residues; this expression yields a value of 21 ± 3 Å for a monomeric C-TRPV1. The theoretically predicted R for a monomeric random-coil with the same number of residues as C-TRPV1, according to Flory's theory, was 38 ± 3 Å. Thus, from these values we can conclude that C-TRPV1 is not a well-folded monomer, but neither is it a completely random-coil protein.

We also performed SLS experiments to determine the molecular weight from the Rayleigh scattering. In most cases, if the molecular weight of the protein is lower than 4000 kDa, the intensity of scattered light that these particles produce is proportional to the product of the weight-average molecular weight and the concentration (C, in g ml^{-1}) of the solute: $\frac{KC}{R_\Theta} = \left(\frac{1}{M} + 2A_2C\right)$, where R_Θ is the

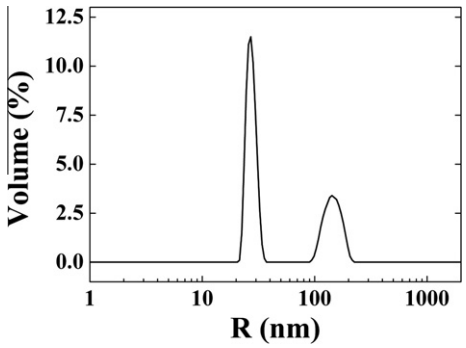

Fig. 2. Light scattering experiments: DLS experiment performed with 5 μM of C-TRPV1 in acetate buffer pH 4.5; buffer concentration was 50 mM and 1 M NaCl. Experiments were made in 0.2-cm-pathlength cells. Experiments were acquired at pH 4.5 (50 mM acetic/acetate buffer) at 25 °C.

Rayleigh ratio; M is the molecular weight of the solute, A_2 is the second virial coefficient (ml mol g^{-2}); and K, an optical constant (which depends of the refractive index of the solvent (1.33 for aqueous solutions), and the wavelength of the incident light used in the experiment) [33]. A plot of $\frac{KC}{R_\Theta}$ versus C yields a straight line (the so-called Debye plot), where the molecular weight is determined from the x-axis intercept, and the second virial coefficient (A_2) from the slope of the line. However, the x-axis intercept for C-TRPV1 yielded a value very small, close to the measurement uncertainty, which results in a large molecular mass, according to the above expression. A similar behavior has been observed in proteins with a high tendency to aggregate, since at high concentrations, we are observing the effect of the protein concentration on the shift of the self-association equilibrium [34]. By using the approach described (which fits the data to a second-order polynomial [34]) (Fig. 1 SI), the estimated molecular weight is 500 kDa.

Finally, to further confirm the presence of those aggregated species detected by DLS and SLS, we run 10% native acrylamide gels. The gels reveal a sole band above the highest molecular weight marker band, corresponding to bovine serum albumin (66 kDa) (Fig. 2A SI). Cross-linking assays performed with 0.5% glutaraldehyde show also a high molecular weight band in standard denaturing conditions (Fig. 2B SI). These results suggest that C-TRPV1 is forming high molecular weight aggregates in solution.

Unsuccessfully, we further tried testing the presence of those aggregated species, by using measurements of the proton T_2-relaxation time in NMR spectroscopy; however, for the residual weak signal of the methyl groups the calculated molecular weight was close to 34.0 kDa. This is probably due to the fact that the signals, from the aggregated species, are broadened beyond detection, and we are observing the weighted-average T_2-relaxation time of all self-associated species of C-TRPV1.

Thus, the light scattering findings, the cross-linking experiments and the native electrophoresis suggest that C-TRPV1 is a self-associated species.

4. Discussion

Our results suggest that the C-TRPV1 behaves as a protein with a non-fixed structure, and a tendency to oligomerize (as suggested by scattering measurements and native electrophoresis). It could be thought that, due to the results of the theoretical predictions (see SI), the experimental absence of a well-fixed structure is due to the refolding protocol. Several pieces of evidence suggest that this is not the case. Firstly, a similar refolding protocol has been used by others [35,36] and for ourselves (JLN, JB, AC, unpublished results) to refold several proteins (which also accumulate as IBs), and the refolded proteins were biologically active. Second, there are several evidences in the literature where the unfolded predictors predict a folded conformation (Fig. 3 SI), which is not rigid in solution, and then, it is not observable under most of the current spectroscopic techniques (see [37] and references therein). And finally, the fact that there is ANS-binding at low pHs suggests the existence of at least partially folded conformations in C-TRPV1 at low pH, since fully random-coil proteins do not bind ANS [29]. Thus, we favor that C-TRPV1 behaves as a protein, which has a non-fixed structure.

Natively unfolded proteins (or intrinsically disordered proteins) are proteins without a rigid secondary and/or tertiary structure under physiological conditions. Despite their lack of structure, they carry out their function in their cell environment [38,39]. Our results show experimentally that C-TRPV1 in solution behaves as a self-associated natively unfolded protein. No co-operativity was observed during the thermal- and chemical-denaturations of the protein, indicating that the tertiary structure of the C-TRPV1 is very weak, if it exists; this non-cooperative behavior is observed in denaturations of intrinsically disordered proteins lacking persistent long-range tertiary contacts [40]. However, the modeled structure of C-TRPV1 [22] shows the presence of secondary and tertiary). The difference between the experimental and the modeled structure might be rationalized as the modeled structure employed well-folded templates, namely, the X-ray structure of HCN2 [24]. Interactions in crystal structures confer rigidity to the proteins, even causing disorder-to-order transitions in protein regions with a great functional importance [41,42]. Thus, the modeled structure, and even the results from the in silico studies in this work, might be reflecting a more rigid protein structure, than that observed in solution, as it has been observed in other natively unfolded proteins [38], and tested when compared the crystal and solution studies in a few proteins (see [43] and references therein).

Light scattering experiments, native electrophoresis and cross-linking with glutaraldehyde show that C-TRPV1 had a very high hydrodynamic radius and molecular weight, suggesting self-association. The protein oligomerization of C-TRPV1 was suggested on the basis of their modeled structure [22], and if in the whole protein, this self-association, detected in this work for the isolated domain, also occurs, it implies that not only the transmembrane region of TRPV1 is involved in intermolecular association, but also the rest of the regions of the intact protein. The TRP domain of the C-terminus of the whole protein was pointed to form coiled-coils [13] and to be essential for oligomerization of the channel, forming a four-helix bundle. It has been demonstrated that disrupting mutations in the TRP domain might have a high influence on the activation energy of channel gating, suggesting that TRP domain interactions between monomers are critical for channel gating [16]. Despite the intrinsically disordered structure of some proteins, they are still able of forming oligomers, such as the dynamic dimer formed by the small disordered ribonuclease reductase Sm11 [44], HIV-1 Vif [45], the disordered inhibitory domains of the coiled-coil dimeric kinases [46], the termini of coiled-coil dimeric proteins involved in signaling pathways [47], or the cytoplasmic domain of the ζ-chain of the T-cell receptor. This latter domain has been demonstrated to form homodimers without a disorder–order transition [48]. Interestingly enough, the T-cell receptor belongs to a group of receptors whose signaling depends on a two-step monomer–dimer–tetramer fast dynamic equilibrium [49]; this oligomerization of the intrinsically disordered region is essential to the receptor signaling [50,51]. We hypothesize that the C-TRPV1 self-associated species might be originated by suffering a very similar process, being the domain oligomerization the essential step for an effective signaling of the whole TRPV1.

To sum up, we can conclude that the first characterization of isolated C-TRPV1 in vitro suggest that the protein is oligomeric and it has a non-fixed structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.03.030.

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