Calcium-induced calmodulin conformational change. Electrochemical evaluation

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Calmodulin (CaM) is an essential protein present in all eukaryote cells, ranging from vertebrates to unicellular organisms. CaM is the most important 
Ca2+ signalling protein, composed of two domains, N- and C-terminal domains, linked by a flexible central α-helix, and is responsible for the regulation of numerous calcium-mediated signalling pathways. Four calcium ions bind to CaM, changing its conformation and determining how it recognizes and regulates its cellular targets. The oxidation mechanism of native and denatured CaM, at a glassy carbon electrode, was investigated using differential pulse voltammetry and electrochemical impedance spectroscopy. Native and denatured CaM presented only one oxidation peak, related to the tyrosine amino acid residue oxidation. Calcium-induced calmodulin conformational change and the influence of Ca2+ concentration on the electrochemical behaviour of CaM were evaluated, and significant differences, in the tyrosine amino acid residue peak potential and current, in the absence and in the presence of calcium ions, were observed. Gravimetric measurements were performed with a graphite coated piezoelectric quartz crystal with adsorbed CaM, and calcium aggregation by CaM was demonstrated.

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### 1. Introduction

Calmodulin (CaM) is a small protein present in all eukaryote cells, ranging from unicellular organisms to vertebrates. CaM is the most important calcium signalling protein, involved in the regulation of numerous 
Ca2+ dependent pathways [1,2].

CaM is essential in all organisms due to the broad spectrum of functions in the control of numerous physiological processes: synthesis and release of neurotransmitters, regulation of intracellular calcium concentration, cell motility and proliferation, apoptosis, autophagy, metabolic homeostasis, protein folding, ions transport, osmotic control, reproductive processes, and gene expression, among others [2,3].

CaM is composed of two domains, N- and C-terminal domains, linked by a flexible central α-helix (Scheme 1) [4]. The N-terminal domain contains EF-hands, EF-1 and EF-2, called CaM₁, and the C-terminal domain contains EF-hands EF-3 and EF-4, called CaM₂ [5]. The EF-hand structural motif was devised in 1973 and is the most common calcium-binding motif found in proteins [6].

The EF-hand motif contains a helix-loop-helix topology, much like the thumb and forefinger of the human hand, each representing a helix and the middle finger bent to suggest the octahedral loop in which the 
Ca2+ ions are coordinated by ligands within the loop region (usually about 12 amino acids). The EF-hand motif contains approximately 40 residues and is involved in binding intracellular calcium [6].

The CaM can bind to four calcium ions (two Ca2+ per domain) and exists in two forms: without calcium (ApoCaM) and in the calcium saturated form (HoloCaM) [3,5]. Increasing calcium intracellular levels, Ca2+ binds to CaM and undergoes an “open form” conformational change with the hydrophilic structure exposed [5]. Each domain of the Ca2+ saturated HoloCaM contains a methionine-rich cavity. The flexibility and polarizability of the methionine amino acid residues, present on the hydrophilic surface and other hydrophobic amino acid residues, enable the occurrence of adjustable interaction areas that can aggregate CaM targets [5,6]. CaM is able to bind to a large array of peptides, enzymes, protein myosin kinase, protein kinase II and others, with different sizes and shapes, and modulate their activity in many different ways, leading to biochemical and cellular changes [5,7].

The Ca2+ binding to CaM is not always essential for some of the vital roles and regulatory function of CaM. ApoCaM also binds and can regulate a variety of proteins, including neuromodulin, neurogranin, unconventional myosins and enzymes that can interact with CaM, in the absence of or for very low Ca2+ concentrations [1].

CaM-dependent signalling mechanisms involved in cell proliferation, programmed cell death or autophagy, are essential in tumour cell biology [2,8–10]. Some studies have demonstrated anomalous intracellular concentrations of CaM and other Ca2+ binding proteins in tumour cells, compared with cells from normal tissues [2].

CaM has also been considered a crucial molecule in the etiology of adolescent idiopathic scoliosis and some studies suggest that platelet
CaM levels may be a better biomarker for the disease curve progression [11,12].

The improvement of effective methods for the determination and quantification of CaM is essential for drug development, clinic diagnosis and disease etiology research due to the importance of CaM in the control of numerous physiological processes. There are some biochemical, thermodynamic and structural studies about CaM and the Ca\(^{2+}\)-induced CaM conformational changes [13–17].

Investigation of the electrochemical behaviour and the protein interactions with solid electrode surfaces is very important in drug discovery and a key to novel applications in biosensors, biotechnology and medical devices. The electrochemical study of proteins has been the main subject of many scientific papers over recent years, using gold and carbon electrodes [18–21]. From the 20 amino acids present in proteins, the oxidation only occurs in five electroactive amino acids: tyrosine (Tyr), at \(E_P \sim 0.65\) V, tryptophan (Trp), at \(E_P \sim 0.65\) V, histidine (His), at \(E_P \sim 1.1\) V, cysteine (Cys), at \(E_P \sim 0.55\) V, and methionine (Met), at \(E_P \sim 1.25\) V [22]. At carbon electrodes these amino acids are oxidized in an irreversible pH-dependent electron transfer mechanism [23–25].

The CaM structure is formed by a sequence of 148 amino acids, and the electroactive amino acids are methionine (10 residues), tyrosine (2 residues) and histidine (1 residue) (Scheme 2). Tyrosine undergoes oxidation at glassy carbon electrodes and at boron doped diamond electrodes, with one irreversible peak corresponding to the oxidation of the hydroxyl group [19]. Methionine oxidation occurs in two steps involving the adsorption and the protonation/deprotonation of the thioether group [19,23,24]. Histidine oxidation occurs at a higher positive potential in a single step [22,25].

In the present work, the influence of Ca\(^{2+}\) on the CaM conformational change, and the electrochemical oxidation behaviour of native and

<table>
<thead>
<tr>
<th>Nomenclature</th>
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<tbody>
<tr>
<td>CaM (calmodulin)</td>
</tr>
<tr>
<td>Tyr (tyrosine)</td>
</tr>
<tr>
<td>Trp (tryptophan)</td>
</tr>
<tr>
<td>His (histidine)</td>
</tr>
<tr>
<td>Cys (cysteine)</td>
</tr>
<tr>
<td>Met (methionine)</td>
</tr>
<tr>
<td>SDS (sodium dodecyl sulphate)</td>
</tr>
<tr>
<td>DTT (dithiothreitol)</td>
</tr>
<tr>
<td>TCEP (tris(2-carboxyethyl)phosphine)</td>
</tr>
<tr>
<td>GCE (glassy carbon electrode)</td>
</tr>
<tr>
<td>CV (cyclic voltammetry)</td>
</tr>
<tr>
<td>DP (differential pulse)</td>
</tr>
<tr>
<td>(P_{\text{tyr}}) (tyrosine oxidation peak)</td>
</tr>
<tr>
<td>EIS (electrochemical impedance spectroscopy)</td>
</tr>
<tr>
<td>EQCM (electrochemical quartz crystal microbalance)</td>
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<td>CPE (constant phase element)</td>
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Scheme 1. 3D Structure of Apo-Calmodulin (left) and Holo-Calmodulin (right) with calcium atoms (red) and the electroactive residues, methionine (yellow), tyrosine (green) and histidine (blue) [4]. (For interpretation of the references to color in this scheme legend, the reader is referred to the web version of this article.)
denature CaM were investigated, using differential pulse voltammetry and electrochemical impedance spectroscopy at a glassy carbon electrode, and a quartz crystal microbalance with a graphite coated quartz crystal. In order to unfold CaM tertiary and quaternary structures, the glassy carbon electrode surface modified by a thick multilayer CaM film was incubated for different time periods with urea, SDS and the reductants TCEP and DTT, and the behaviour of denatured CaM was investigated.

2. Experimental

2.1. Materials and reagents

Calmodulin (CaM), from bovine testicles, was obtained from Genaxxon bioscience (Sölingen, Germany) and used without further purification. A stock solution of 60 μM CaM was prepared in deionized water and stored at 4 °C.

Sodium dodecyl sulphate (SDS), dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), urea, calcium chloride hexahydrate (CaCl₂) and potassium hexacyanoferrate (II) (K₄[Fe(CN)₆]), were obtained from Sigma-Aldrich (Steinheim, Germany). Stock solutions of 0.2 M SDS, 0.2 M DTT, 0.1 M TCEP, 8 M urea and 0.2 M CaCl₂ were prepared in deionized water and stored at 4 °C. Solutions of different concentrations of SDS, DTT, TCEP, and urea were prepared by dilution in water and stored at 4 °C.

All supporting electrolyte solutions were prepared using analytical reagents (EMD Millipore, Massachusetts, USA) (Certificate ISO 9001 Quality systems standards). The supporting electrolyte buffer solutions were: 0.1 M HAcO/NaAcO acetate buffer pH 3.4, 4.5, and 5.4; 0.1 M Tris/HCl buffer pH = 7.0; 0.1 M NaH₂PO₄/Na₂HPO₄ phosphate buffer pH 6.08 to 8.05; and 0.1 M NaOH/Na₂B₂O₇ buffer pH = 9.25.

All supporting electrolyte solutions were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity ≤ 0.1 μS cm⁻¹) (EMD Millipore, Massachusetts, USA) (Certificate ISO 9001 Quality systems standards). The supporting electrolyte buffer solutions were: 0.1 M HAcO/NaAcO acetate buffer pH 3.4, 4.5, and 5.4; 0.1 M Tris/HCl buffer pH = 7.0; 0.1 M NaH₂PO₄/Na₂HPO₄ phosphate buffer pH 6.08 to 8.05; and 0.1 M NaOH/Na₂B₂O₇ buffer pH = 9.25.

Microvolts were measured using electronic pipettes (EP), EP-10 μM and EP-100 μM, Plus Motorized (Rainin Instrument Co. Inc., Woburn, USA). The pH measurements were performed with a CRISON 2001 (Crisom Instruments, Barcelona, Spain) micro pH-meter with an Ingold-combined glass electrode.

All experiments were done at room temperature (25 ± 1 °C).

2.2. Voltammetric parameters and electrochemical data

Voltammetric experiments were carried out using a Autolab Type III potentiostat-galvanostat running with GPES 4.9 software, Metrohm, Utrecht, The Netherlands. Measurements were carried out using a glassy carbon electrode (GCE) (d = 1.0 mm) as working electrode, a Pt wire counter electrode and an Ag/AgCl (3 M KCl) as reference electrode, in a 2 mL one-compartment electrochemical cell (eDAQ, Europe). The GCE was polished using diamond spray (particle size 1 μm) before every electrochemical assay. After polishing, the electrode was rinsed thoroughly with Milli-Q water. Following this mechanical treatment, the GCE was placed in buffer supporting electrolyte and various differential pulse voltammograms were recorded until a steady state baseline voltammogram was obtained. This procedure ensured very reproducible experimental results.

The experimental conditions for cyclic voltammetry (CV) were scan rate 100 mV s⁻¹, and for differential pulse (DP) voltammetry were pulse amplitude 50 mV, pulse width 70 ms and scan rate 5 mV s⁻¹. The electrochemical impedance spectroscopy (EIS) measurements were carried out using an Autolab PGSTAT 10, and performed using FRA software version 4.9. Metrohm, Utrecht, The Netherlands. A sinusoidal perturbation of root-mean-square (r.m.s.) amplitude 10 mV was applied over the frequency range 10 kHz to 0.1 Hz with seven frequency values per decade. The impedance spectra were analysed by fitting to a Randles-type equivalent electrical circuit using ZView software (Scribner Associates, USA).

The EIS measurements were obtained at the hexacyanoferrate (III)/ (II) midpoint potential, E₀ = +0.20 V, applied to the GCE, in 0.1 M (Tris/HCl) pH = 7.0, and for different concentrations of K₄[Fe(CN)₆].

Gravimetric measurements were performed with an electrochemical quartz crystal microbalance (EQCM) integrated in an AWS A20 platform with a graphite coated piezoelectric Au quartz crystal (AuQC_Gr) of 9 MHz central frequency, running with A20RPSoft software (AWSensors, Scientific Technology, Valencia, Spain). The cell integrated in the AWS A20 platform was coupled to computer-controlled SP-200 Research Grade Potentiostat/Galvanostat/FRA running with EC-Lab V10.40 software (Bio-Logic Science Instruments SAS, Clai, France).

The Au electrodes were cleaned with Milli-Q water and dried in a stream of pure N₂. After each measurement, the Au electrodes were cleaned with ethanol, then washed with Milli-Q water, and dried at room temperature.

2.3. Acquisition and presentation of voltammetric data

All the DP voltammograms presented were baseline corrected using the moving average application with a step window of 5 mV, included in GPES version 4.9 software. This mathematical treatment improved the visualization and identification of peaks over the baseline without introducing artefacts, although the peak current intensity is in some cases reduced (10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values for peak current presented...
in all graphs were determined from the original untreated voltammograms.

2.4. Electrochemical procedures

The oxidation of the native and denatured CaM was investigated using two experimental procedures.

2.4.1. Procedure 1

For the electrochemical oxidation of native CaM, 100 μL of 60 μM CaM stock solution, 1 mg/mL CaM in water, were placed in the electrochemical cell, containing 100 μL of the supporting electrolyte 0.1 M Tris/HCl buffer pH = 7.0, in order to obtain 30 μM CaM, and DP voltammetry was performed.

2.4.2. Procedure 2

For the electrochemical oxidation of the denatured CaM the GCE surface was modified with an immobilized CaM thick multilayer film, obtained by depositing one drop 5 μL of 60 μM CaM 4 times on the GCE surface and after each drop the GCE was dried under a N2 constant flux. Then the GCE modified with the CaM film was incubated in 0.1 M (Tris/HCl) buffer pH = 7.0, containing a denaturing agent, 6 M urea, 50 mM TCEP, 50 mM SDS or 10 mM DTT, during different time periods: 30, 60, 120 and 180 min. Afterwards, the CaM modified GCE was removed from the solution, washed with deionized water, to remove the excess of denaturing or reductant agent, and of native and denatured CaM, and placed in the electrochemical cell containing only the supporting electrolyte 0.1 M Tris/HCl buffer pH = 7.0, where DP voltammetry was performed.

3. Results and discussion

The binding of Ca2+ to CaM induces a significant conformational change in the protein structure. Consequently, the influence of Ca2+ concentration on CaM conformational change will lead to changes in the protein electrochemical behaviour. These changes were evaluated by DP voltammetry and EIS, using the GCE surface modified with a multilayer CaM film, and by the gravimetric measurements (QCM) performed with a graphite coated piezoelectric quartz crystal with adsorbed CaM. However, in order to clearly understand the electrochemical evaluation the Ca2+-induced CaM conformational changes, the electrochemical oxidation, of native and denature CaM, at the GCE, was also investigated.

3.1. Oxidation of native CaM. Effect of pH

DP voltammetric experiments in 30 μM CaM, in 0.1 M (Tris/HCl) buffer pH = 7.0, showed only one oxidation peak, PTyr at $E_p = +0.66 \ V$, corresponding to the Tyr99 or Tyr138 amino acid residue oxidation [26] (Fig. 1).

The electrochemical oxidation of native CaM was also investigated by DP voltammetry, over a wide pH range between 2 and 11. The DP voltammograms were all recorded in solutions of 30 μM CaM, in different buffer electrolytes with 0.1 M ionic strength, and only the Tyr amino acid residue oxidation peak, $P_{\text{Tyr}}$, occurred (Fig. 2). Increasing the pH, the potential shifted to more negative values, and the highest peak current was obtained in 0.1 M phosphate buffer pH = 6 (Fig. 2A).

The oxidation mechanism involved the same number of electrons and protons because the slope of the dotted line in the $E_p$ vs. pH plot was $-59 \ \text{mV per pH unit}$ (Fig. 2B). Taking into consideration that the peak width at half height was $W_{1/2} \approx 100 \ V$, it is concluded that the oxidation process in the native CaM involved the transfer of one electron and one proton, in agreement with the oxidation of Tyr amino acid residues [26].
3.2. CV and EIS characterization of the CaM modified GCE

The GCE surface modified with immobilized multilayer CaM film was characterized using [Fe(CN)₆]⁴⁻ as the redox probe, by CV and EIS.

The CV of [Fe(CN)₆]⁴⁻ recorded with a bare GCE surface presented one reversible peak, whereas the CV with the GCE modified with immobilized multilayer CaM film showed one irreversible peak (Fig. 3).

Electrochemical impedance spectroscopy (EIS) is an effective and convenient method for monitoring the interfacial characterization of electrodes, and [Fe(CN)₆]⁴⁻ was used as the redox probe in the EIS measurements at the CaM modified GCE surface [27,28].

A Randles-type electrical equivalent circuit (Scheme 3), was used to fit the experimental spectra. In the circuit, $R_{ct}$ represents the cell resistance (solution and bulk composite resistances). The constant phase element (CPE), defined as:

$$\text{CPE} = \frac{1}{(j\omega)^\alpha}$$

was modelled as a non-ideal capacitor where the capacitance, $C$, describes the double layer charge separation at the modified electrode-solution interface, $\alpha$ is the radial frequency in rad s⁻¹, the exponent $\alpha$ is due to the non-uniformity and heterogeneity of the surface. $R_{ct}$ is the charge-transfer resistance across the electrode-solution interface.

The Warburg element, $Z_w$, represents diffusion and is given by:

$$Z_w(W_o) = R_{diff}((j\omega)^{-\alpha}/(j\omega)^{\alpha})$$

where $R_{diff}$ is a diffusion resistance of electroactive species, $\tau$ is a time constant depending on the diffusion rate ($\tau = \rho / D$, where $\rho$ is the effective diffusion thickness, and $D$ is the effective diffusion coefficient of the species), the exponent $\alpha = 0.50$ for a perfect uniformly flat interface. Values of $0 < \alpha < 0.50$ correspond to a non-uniform interface.

The complex plane impedance plots were recorded at the hexacyanoferrate (III)/(II) midpoint potential, $E_{ap} = +0.20 \, V$, applied to the GCE, in 1, 3 and 5 mM [Fe(CN)₆]⁴⁻, in 0.1 M (Tris/HCl) buffer pH = 7.0 (Fig. 4).

The GCE modified with the immobilized multilayer CaM film showed a semi-circle corresponding to the occurrence of the charge transfer reaction, with a charge transfer resistance ($R_{ct}$), represented by the diameter of the semi-circle, larger than that at a bare GCE (Fig. 4). It was observed, as expected, for the GCE with and without immobilized multilayer CaM film, that the $R_{ct}$ values decreased with increasing [Fe(CN)₆]⁴⁻ concentration.

The [Fe(CN)₆]⁴⁻ redox process using the GCE surface modified with immobilized multilayer CaM film gave a $R_{ct} \approx 500 \, k\Omega$ (Fig. 4B) (curve (c)) for 1 mM [Fe(CN)₆]⁴⁻, while the [Fe(CN)₆]⁴⁻ redox process using the bare GCE, for 1 mM [Fe(CN)₆]⁴⁻, showed a much lower $R_{ct} \approx 50 \, k\Omega$ (Fig. 4A) (curve (a)).

The increase of $R_{ct}$ using the GCE modified with the multilayer CaM film was expected, since the immobilized CaM molecules on GCE surface blocked electron transfer between the redox probe and the GCE surface, leaving approximately 10% of the glassy carbon electrode surface uncovered.

**Scheme 3.** Electrical equivalent circuit for EIS data fitting.

**Fig. 3.** Cyclic voltammograms, in 0.1 M (Tris/HCl) buffer solution pH = 7.0: (○) bare GCE, (●) GCE in 3 mM K₄[Fe(CN)₆] and (□) GCE modified with a multilayer CaM film in 3 mM K₄[Fe(CN)₆]. Scan rate 100 mV s⁻¹. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 4.** Complex plane impedance spectra, in 0.1 M (Tris/HCl) buffer pH = 7.0, for (□) 1, (○) 3 and (●) 5 mM K₄[Fe(CN)₆], at ferrocyanide applied midpoint potential, $E_{ap} = +0.2 \, V$: (A) bare GCE and (B) GCE modified with a multilayer CaM film.
3.3. Effect of denaturing agents on CaM oxidation

Proteins are complex molecules that follow structural organization levels. The denaturing process causes morphological changes in the 3D structure which unfolds, covering the electrode surface and making the electrochemical oxidation of the electroactive amino acid residues easier.

The denaturing agent urea, the most widely used, affects the secondary and tertiary structure without affecting the primary structure, because of its high affinity for peptide bonds, breaking the hydrogen bonds and the salt bridges between positive and negative side chains, thereby disrupting the tertiary structure of the peptide chain.

The use of the denaturing detergent SDS, that dissolves hydrophobic proteins, and the reducing agents DTT or TCEP, that disrupt disulphide bonds, was also important for a qualitative and quantitative analysis in proteomic oxidation mechanisms investigation.

Both denaturing agents DTT and TCEP are electroactive on GCE, in phosphate buffer pH = 7.0, with one oxidation peak for DTT, at $E_p \approx +1.15$ V, and for TCEP, at $E_p \approx +0.9$ V [29]. SDS and urea are not electroactive.

The oxidation process at the GCE modified with immobilized multilayer CaM film showed one small oxidation peak (Fig. 5) (●), corresponding to the Tyr$^{99}$ or Tyr$^{138}$ amino acid residue oxidation, at $E_p = +0.66$ V, in agreement with the CaM oxidation DP voltammogram (Fig. 1).

The effect of the denaturing agents on CaM electrochemical oxidation was evaluated by comparing the behaviour of the GCE modified with immobilized multilayer native CaM film as control, with the changes observed after interaction with each denaturing agent. A new film was always prepared for each experiment. After incubation the GCE was washed with deionized water to remove the excess of denaturant and only then the DP voltammograms, in 0.1 M (Tris/HCl) buffer pH = 7.0, were recorded.

The multilayer native CaM film immobilized on GCE, Procedure 2, was denatured after incubation in the agents 50 mM SDS and TCEP, 10 mM DTT, and 6 M urea, in 0.1 M (Tris/HCl) buffer pH = 7.0, during different time periods: 30, 60, 120 and 180 min (Fig. 5). The oxidation mechanism of denatured CaM film immobilized on GCE was investigated using DP voltammetry. Care was taken for the GCE surface to be completely covered by the CaM molecules immobilized multilayer film, in order to avoid non-specific adsorption and binding of denaturant molecules.

After incubation of the GCE modified with immobilized multilayer CaM film in 6 M urea solution, for different time periods, an increase of the Tyr amino acid residue oxidation peak, $P_{Tyr}$, current, at $E_p = +0.66$ V, occurred but no new CaM oxidation peaks were observed (Fig. 5A).

![Fig. 5. DP voltammograms baseline corrected, at GCE modified with a multilayer CaM film, in 0.1 M (Tris/HCl) buffer pH = 7.0: (●) before and (●) after incubation in: (A) 6 M urea, (B) 50 mM SDS, (C) 10 mM DTT, and (D) 50 mM TCEP. Scan rate 5 mV s$^{-1}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)
DP voltammograms of GCE modified with immobilized multilayer CaM film after incubation in SDS showed a marked decrease of the Tyr amino acid residue oxidation peak, PTyr, current after 30 min, but no new oxidation peaks were detected when compared with the control (Fig. 5B). The peak current increased again after 180 min. This was due to the SDS affecting the protein structure. SDS is a denaturing agent that disrupts noncovalent interactions by electrostatic repulsion, transforming the protein into a linear polypeptidic chain.

DP voltammograms of GCE modified with immobilized multilayer CaM film after incubation in reductants DTT or TCEP presented no significant changes, besides the Tyr oxidation peak current, PTyr, decrease after 30 min but no new peaks occurred (Fig. 5C and D). DTT and TCEP have been applied to protein samples to cleave the disulphide bond of cysteine amino acid residues\cite{18}. The results are coherent because CaM has no cysteine group, so DTT did not cause any effect on CaM structure.

However, the DP voltammograms of GCE modified with immobilized multilayer CaM film after 60 min incubation in TCEP showed the Tyr amino acid residue oxidation, PTyr, peak, and a new peak, at $E_p = +0.95 \text{ V}$, corresponding to the His amino acid residue oxidation, PHis, also occurred. The influence of TCEP on CaM three-dimensional structure was more extensive than that of DTT.

Denaturation of CaM by urea, SDS, DTT and TCEP was performed using a GCE modified with immobilized multilayer CaM film for different incubation time periods. Urea caused an enhancement of the Tyr amino acid residue oxidation peak, PTyr, current, due to better access to the GCE surface. SDS and DTT caused a decrease of the Tyr amino acid residue oxidation peak current, due to their high reactivity to promote a total unfolding of the CaM structure, which made the interaction and subsequent adsorption of non-electroactive amino acid residues to the electrode surface easier, but no new peak appeared. TCEP after 60 min showed an enhancement of the Tyr amino acid residue oxidation peak, PTyr, current and the new His amino acid residue oxidation peak, PHis, occurred.

3.4. Electrochemical evaluation of the Ca$^{2+}$-induced CaM conformational changes

CaM is involved in the regulation of numerous Ca$^{2+}$-dependent pathways and undergoes a Ca$^{2+}$-induced change in conformation, leading to biochemical and cellular changes, being one of the most important signalling proteins. Structurally, the CaM calcium saturated form (HoloCaM) contains four Ca$^{2+}$ binding sites (Scheme 1).

The Ca$^{2+}$ ligand-binding site of CaM contains aspartic and glutamic acid; two negatively charged amino acids at physiological pH. Once Ca$^{2+}$ is bound to CaM there are many conformational changes in the protein secondary structure. The electroactive Tyr residues are situated in close proximity to the Ca$^{2+}$ binding sites, and the Ca$^{2+}$-CaM changes on secondary structure can affect the Tyr residues redox signal, i.e. oxidation peak potential and current. Therefore, the redox behaviour of CaM in the presence of different Ca$^{2+}$ concentrations, by DP voltammetry, at the GCE, in 0.1 M (Tris/HCl) buffer pH = 7.0, was investigated.

The DP voltammogram in 30 μM CaM without Ca$^{2+}$ showed an anodic peak $I_{P_Tyr}$ at $E_p = +0.66 \text{ V}$, corresponding to the Tyr$^{99}$ or Tyr$^{138}$ amino acid residue oxidation. Taking into consideration the CaM secondary structure (Scheme 1), it is likely that the Tyr$^{99}$ was more easily
oxidized than Tyr$^{38}$, since the last one is surrounded by three $\alpha$-helix regions.

In the DP voltammograms in 30 mM CaM containing micromolar Ca$^{2+}$ concentrations (30, 60 and 120 $\mu$M) (Fig. 6A), the Tyr amino acid residue oxidation potential was slightly shifted to a more positive potential and the oxidation peak current decreased and was inversely proportional with the increase of Ca$^{2+}$ concentration. At 120 $\mu$M Ca$^{2+}$ the Tyr amino acid residue oxidation peak current had the lowest value, corresponding to the 1 CaM:4 Ca$^{2+}$ ratio (Scheme 1).

In the DP voltammogram in 30 mM CaM containing 1 mM Ca$^{2+}$, the Tyr amino acid oxidation occurred at the same potential, and with the same current, as for 120 $\mu$M Ca$^{2+}$ (Fig. 6A). Increasing the Ca$^{2+}$ concentration up to 10 mM Ca$^{2+}$ the oxidation peak current remained the same but the Tyr amino acid residue oxidation peak potential was shifted to more positive potentials, at $E_p \approx +0.8$ V.

However, in 30 mM CaM containing higher Ca$^{2+}$ concentrations, >30 mM, the Tyr amino acid residue oxidation peak current, in the DP voltammogram, increased one fold and the peak potential remained constant, at $E_p \approx +0.8$ V (Fig. 6B). The current decrease and the positive potential shift are due to the Ca$^{2+}$-induced CaM conformational changes.

For high Ca$^{2+}$ concentrations, the Tyr$^{38}$ phenol moiety remained between the $\alpha$-helix regions being protected for oxidation, but the Tyr$^{38}$ phenol moiety became more close to the protein backbone (Scheme 1) (right), and the nearby presence of other non-electroactive amino acid residues made the Tyr amino acid residue oxidation more difficult.

3.5. EIS characterization of Ca$^{2+}$-induced CaM conformational changes

The influence of Ca$^{2+}$ concentration on the surface of GCE modified with immobilized multilayer CaM film was also probed by EIS, at the hexacyanoferrate (III)/(II) midpoint potential, $E_{ap} = +0.20$ V, applied to the GCE, in 0.1 M (Tris/HCl) buffer pH = 7.0. EIS were recorded for 1, 3 and 5 mM K$_4$[Fe(CN)$_6$] solutions. Complex plane plots, in 3 mM K$_4$[Fe(CN)$_6$], for different Ca$^{2+}$ concentrations, between 60 $\mu$M and 1 mM, are presented in Fig. 7.

The EIS were fitted using the Randles-type equivalent electrical circuit (Scheme 3), as previously, see Section 3.2.

The spectra showed that, increasing the Ca$^{2+}$ concentrations, both real and imaginary impedance decreased (Fig. 7). For a higher Ca$^{2+}$ concentration, 1 mM, it was possible to observe two regions: the semi-circular part at higher frequencies corresponding to the electron transfer process and a linear part at lower frequencies corresponding to diffusion (Fig. 7). At Ca$^{2+}$ concentrations below 240 $\mu$M only the electron transfer process region was observed.

Analysis of the spectra, Table 1, showed a decrease in $R_s$ with increase of Ca$^{2+}$ concentration in the solution leading to an increase of the available electrode surface area. The diffusion resistance increased with the Ca$^{2+}$ concentration due to the change in the CaM conformation at the electrode surface.

The values of $\alpha_1$ - 0.94, close to 1.0, are related with the electrode/CaM interface, and remained constant upon increasing the Ca$^{2+}$ concentration, indicating that there is a homogeneous non-porous interface, not influenced by Ca$^{2+}$ concentration. For a higher concentration of Ca$^{2+}$, the diffusional Warburg resistance was identified with a high diffusional time of 200 s, indicating a slow diffusion of Ca$^{2+}$ within the protein layer for the high Ca$^{2+}$ concentration. The value of $\alpha_2$ = 0.42 corresponds to Ca$^{2+}$ ions diffusion through a CaM protein uniform layer on the electrode surface.

Low frequency inductive loops appeared for lower Ca$^{2+}$ concentration because the CaM conformational change did not occur, the CaM structure was rigid and the protein was attached to the electrode surface in a conformation less favourable for charge transfer to take place.

In the presence of higher Ca$^{2+}$ concentrations, the protein underwent a Ca$^{2+}$-induced conformational change to an "open form", becoming more flexible at the electrode surface, enabling the [Fe(CN)$_6$]$^{3-}$ ions to reach the GCE surface more easily, leading to a lower value of charge transfer resistance. At the same time, the flexibility of the protein hinders the diffusion process. Moreover, the diffusion resistance increase can be associated with a competition between [Fe(CN)$_6$]$^{3-}$ and Ca$^{2+}$ ions, to reach the electrode surface.

3.6. Gravimetric study of Ca$^{2+}$-induced CaM conformational changes

The electrochemical quartz crystal microbalance (EQCM) is an essential tool that enables the monitoring of both mass changes and current during the potential cycling. The mass variation in time can be monitored on the basis of the relationship between the induced frequency variation and the deposited mass on the QCM crystal.

In the specific case of rigid films adsorbed at the QCM crystal surface, the relationship is described by the Sauerbrey equation:

$$\Delta f = -\frac{2f_0}{\sqrt{\mu_2 \rho_2}} \Delta m$$

where $f_0$ is the resonant frequency (for these crystals is 9 MHz), $\Delta f$ is the frequency change (Hz), $\Delta m$ is the mass change (g), $\mu_2$ is the piezoelectrically active crystal area, $\rho_2$ is the density of quartz (2.648 g cm$^{-3}$), and $\rho_2$ is the shear modulus of quartz for AT-cut crystals (2.947 × 10$^3$ g cm$^{-1}$ s$^{-2}$) which leads to $\Delta f/\Delta m = -894$ Hz g$^{-1}$.

The aggregation of Ca$^{2+}$ ions by CaM was observed using a quartz crystal microbalance (QCM). The gravimetric measurements were performed in a graphite coated piezoelectric quartz crystal (Au/QC-Gr) with the surface covered with a drop of 10 $\mu$L of 60 $\mu$M CaM, dried at room temperature. Once dried, the crystal was placed in the QCM and

![Fig. 7. Complex plane impedance spectra 3D plot of the GCE modified with a multilayer CaM film, in 3 mM K$_4$[Fe(CN)$_6$], in 0.1 M (Tris/HCl) buffer pH = 7.0, at ferrocyanide applied midpoint potential, $E_{ap} = +0.20$ V, for (□) 60 $\mu$M, (●) 120 $\mu$M, (▲) 240 $\mu$M, and (◇) 1 mM CaCl$_2$ concentrations.](image-url)
covered with 5 mL of buffer solution, 0.1 M (Tris/HCl) pH = 7.0. Afterwards, successive additions of CaCl₂ were performed, the frequency variation was recorded, and the mass changes in the modified electrode were determined. The frequency obtained after stabilization of the system was taken as the reference value, to make easier to follow the change in frequency, corresponding to each Ca²⁺ ion addition.

After each addition of 30 μL of 10 mM CaCl₂ the corresponding frequency was determined (Fig. 8). The frequency peaks that appear are due to the noise caused by each addition. It was observed that the aggregation of Ca²⁺ by CaM does not occur uniformly (Fig. 8A). In the first and second steps, the Ca²⁺ concentration in the solution was ~120 μM and the frequency did not change, remaining constant, which means that the mass change did not occur (Fig. 8B).

After the addition of 90 μL of CaCl₂, corresponding to 180 μM Ca²⁺, the mass in the electrode surface increased with increasing Ca²⁺ concentration. The frequency started decreasing successively until the Ca²⁺ concentration in the solution was ~350 μM Ca²⁺. These results were expected because, in the presence of Ca²⁺, the protein changes its conformation from Apo-CaM to Holo-CaM.

CaM can bind four Ca²⁺ ions, consequently the mass detected in the electrode surface increased. For Ca²⁺ concentrations higher than 350 μM the frequency continued to decrease but not linearly. In the presence of higher Ca²⁺ concentrations, the protein became more flexible on the electrode surface and this flexibility may make the aggregation of Ca²⁺ to the protein molecules absorbed in the innermost film layer at the electrode surface more difficult.

The total frequency variation detected for the successive additions of Ca²⁺ was 106 Hz, which corresponded to a mass increase at the electrode surface of 0.12 μg of Ca²⁺. Ideally the mass increase should have been ~0.1 μg (value calculated from the ratio 1:4 CaM/Ca²⁺) but the value obtained from the frequency was slightly higher which meant that at the same time some Ca²⁺ adsorption at the electrode surface also occurred.

4. Conclusions

Calmodulin (CaM) is an essential protein present in all organisms and is one of the most important Ca²⁺ signalling proteins, responsible for the regulation of numerous Ca²⁺-mediated signalling pathways.

The oxidation of native and denatured CaM was characterized by DP voltammetry. The native CaM oxidation showed a pH-dependent peak corresponding to the Tyr amino acid residue oxidation. The denaturing agent urea unfolded the CaM molecule, improved the electrochemical response and sensitivity, and enhanced the Tyr oxidation peak current. SDS and DTT unfolded the CaM molecule but caused a marked decrease of the Tyr oxidation peak current, due to their high reactivity, which caused CaM molecule conformational changes, enabling the interaction and the adsorption of CaM non-electroactive amino acid residues on the electrode surface easier. TCEP had a similar behaviour but an enhanced Tyr oxidation peak current and a new peak corresponding to His amino acid residue oxidation was observed.

The interaction of Ca²⁺ with CaM was evaluated by DP voltammetry and EIS, and significant changes of Tyr amino acid residue oxidation peak potential and current, decreasing for lower concentration and increasing for higher Ca²⁺ concentrations, were found. The Ca²⁺ bind with adsorbed CaM, on a graphite coated piezoelectric quartz crystal, was demonstrated, and the relationship between the induced frequency variation and the deposited mass in time, was monitored.

These results showed that the Ca²⁺-induced CaM conformational structural changes results in an alteration of Tyr amino acid residues electrochemical signal and may constitute the base for the development of a new generation of CaM based Ca²⁺ biosensors.

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