ChemPhysChem

# Identification of Calcium-Binding Sites in the Nucleotide Binding Domain of the Plasma Membrane H+-ATPase from Saccharomyces cerevisiae

--Manuscript Draft--

| | |
|---|---|
| **Manuscript Number:** | |
| **Article Type:** | Research Article |
| **Corresponding Author:** | JOSE G. SAMPEDRO, BSc, PhD<br>Universidad Autonoma de San Luis Potosi Instituto de Fisica Manuel Sandoval Vallarta<br>San Luis Potosí, SLP MEXICO |
| **Corresponding Author E-Mail:** | sampedro@dec1.ifisica.uaslp.mx |
| **Order of Authors (with Contributor Roles):** | Claudia L. Gómez-Flores, BS, MS, PhD (Formal analysis: Supporting; Investigation: Supporting; Methodology: Supporting) |
| | JOSE G. SAMPEDRO, BS, PhD (Conceptualization: Lead; Data curation: Lead; Formal analysis: Lead; Funding acquisition: Lead; Investigation: Equal; Methodology: Lead; Project administration: Lead; Resources: Lead; Software: Lead; Supervision: Lead; Validation: Lead; Visualization: Lead; Writing – original draft: Lead; Writing – review & editing: Lead) |
| **Keywords:** | Calcium-binding site; plasma membrane H+-ATPase; molecular dynamics simulation; terbium binding; energy transfer |
| **Manuscript Classifications:** | Biochemistry and Chemical Biology |
| **Suggested Reviewers:** | Hidetoshi Iida, PhD<br>Tokyo Gakugei University<br>iida@u-gakugei.ac.jp<br>Expertise in calcium ion in biology |
| | Joseph A. Cotruvo, PhD<br>The Pennsylvania State University<br>juc96@psu.edu<br>Expertise in biochemistry and chemical biology, metal (lantanides) selectivity in biological systems. |
| | Chih-Hau Lu, phD<br>Graduate Institute of Basic Medical Science, China Medical University<br>chlu@mail.cmu.edu.tw<br>Expertise in the prediction of metal binding sites in proteins |
| **Opposed Reviewers:** | |
| **Abstract:** | In Saccharomyces cerevisiae, calcium signaling mediates plasma membrane H+-ATPase activation by glucose. Proton pumping activity and calcium influx appear to be closely linked, as H+-ATPase interacts physically with Mid1, a subunit of the voltage-gated Ca2+ channel (VGCC). Thus, calcium finds immediately with the whole H+-ATPase cytoplasmic structure during influx, where potential interactions may occur. In this work, several calcium-binding sites were identified in the nucleotide-binding domain (N-domain) by three-dimensional (3D) structural analysis and molecular dynamics simulation (MDS); calcium-binding sites consisted mainly of Asp and Glu residues displaying bidentate coordination geometry. Calcium-binding was confirmed in vitro by intrinsic fluorescence quenching of a recombinant N-domain and by energy transfer sensitized Tb3+ luminescence. Tb3+ binding to N-domain was further tested under different experimental conditions confirming interaction. Calcium ion binds to the H+-ATPase N-domain and is probably involved directly in the modulation of enzyme activity |
| **Author Comments:** | This research paper has been revised and modified according to the requests of reviewer 2. The manuscript is being resubmitted to ChemPhysChem for review and eventual publication.<br><br>We would like to express our gratitude to the reviewers and are particularly grateful for |

| | |
|---|---|
| | the comments from reviewer 2. In this regard, all requested experiments and modifications were completed. The most significant of these was the use of a different buffer solution for the experiment described in Figure 8 (Tris buffer was suggested); the results are shown in SI 4. In addition, further molecular biology experiments were carried out, which confirmed the presence of one of the predicted calcium binding sites (please refer to the response to reviewers point 4).<br><br>The final manuscript, therefore, describes the prediction of the existence of calcium-binding sites in the nucleotide-binding domain (N-domain) of the plasma membrane H+-ATPase from Saccharomyces cerevisiae, that potentially modulate H+-ATPase activity. |
| **Section/Category:** | Invited Article (not for Special Collection) |
| **Additional Information:** | |
| **Question** | **Response** |
| Do you agree to comply with the legal and ethical responsibilities outlined in the journal's Notice to Authors? | Yes |
| Has a previous version of this manuscript been submitted to this journal? | Yes |
| If yes, please provide details as follow-up to "Has a previous version of this manuscript been submitted to this journal?" | Manuscript number: cphc.202200737, cphc.202200425<br>MS Type: Research Article<br>Title: "Identification of Calcium-Binding Sites in the Nucleotide Binding Domain of the Plasma Membrane H+-ATPase from<br>Saccharomyces cerevisiae"<br>Correspondence Author: Dr. JOSE G. SAMPEDRO |
| Is this manuscript, or part of it, currently under consideration elsewhere? | No |
| Is this manuscript, or part of it, published, posted, or in press? This includes content posted on preprint servers (preprint guidelines) or published as part of a thesis. | No |
| Please provide us with information about the history of your manuscript, including previous submissions, transfers, or prior versions: | Manuscript number: cphc.202200737, cphc.202200425<br>MS Type: Research Article<br>Title: "Identification of Calcium-Binding Sites in the Nucleotide Binding Domain of the Plasma Membrane H+-ATPase from<br>Saccharomyces cerevisiae"<br>Correspondence Author: Dr. JOSE G. SAMPEDRO. |
| Do you or any of your co-authors have a conflict of interest to declare? | No |
| Does the research described in this manuscript include animal experiments? | No |
| Does the research described in this manuscript include human research participants (including for experiments with sensors or wearable technologies) or tissue samples from human subjects (including blood or sweat)? | No |

# Identification of Calcium-Binding Sites in the Nucleotide Binding Domain of the Plasma Membrane H$^+$-ATPase from *Saccharomyces cerevisiae*

Claudia L. Gómez-Flores,[b] and José G. Sampedro*[a]

[a] Ph.D. C. L. Gómez-Flores
Instituto de Física, Universidad Autónoma de San Luis Potosí.
Avenida Chapultepec 1570, Privadas del Pedregal. San Luis Potosí. C.P. 78295, SLP, México
[b] Prof. J. G. Sampedro
Instituto de Física, Universidad Autónoma de San Luis Potosí.
Avenida Chapultepec 1570, Privadas del Pedregal. San Luis Potosí. C.P. 78295, SLP, México
E-mail: sampedro@dec1.ifisica.uaslp.mx

**Abstract:** In *Saccharomyces cerevisiae*, calcium signaling mediates plasma membrane H$^+$-ATPase activation by glucose. Proton pumping activity and calcium influx appear to be closely linked, as H$^+$-ATPase interacts physically with Mid1, a subunit of the voltage-gated $Ca^{2+}$ channel (VGCC). Thus, calcium finds immediately with the whole H$^+$-ATPase cytoplasmic structure during influx, where potential interactions may occur. In this work, several calcium-binding sites were identified in the nucleotide-binding domain (N-domain) by three-dimensional (3D) structural analysis and molecular dynamics simulation (MDS); calcium-binding sites consisted mainly of Asp and Glu residues displaying bidentate coordination geometry. Calcium-binding was confirmed *in vitro* by intrinsic fluorescence quenching of a recombinant N-domain and by energy transfer sensitized $Tb^{3+}$ luminescence. $Tb^{3+}$ binding to N-domain was further tested under different experimental conditions confirming interaction. Calcium ion binds to the H$^+$-ATPase N-domain and is probably involved directly in the modulation of enzyme activity.

## Introduction

In the cytoplasm of yeast, as in most eukaryotic cells, changes in calcium concentration modulate a large number of cellular processes.[1–3] Control of calcium concentration results in the tight regulation of calcium signaling.[1,2] In this regard, some of the enzyme/protein targets of calcium-mediated activity modulation are well known, but others remain to be identified.[3]
In yeast cells, monomers of the plasma membrane H$^+$-ATPase associate to form hexamers,[4,5] whose activity is required for nutrient transport, maintenance of ionic equilibrium and internal pH, and other processes.[6,7] Thus, control of the switch between the active/inactive state of H$^+$-ATPase is vital. In this regard, H$^+$-ATPase activation by glucose is known to be mediated by the entrance of calcium into the cytoplasm.[6,8–12] Nonetheless, the mechanism(s) by which H$^+$-ATPase becomes activated by calcium influx still are not completely known;[9,13–15] it has been found, that phosphorylation of the carboxy-terminal (C-term) domain by a calcium-dependent protein kinase (Ptk2) activates the H$^+$-ATPase by increasing ATP affinity,[9–11,16,17] but the protein kinase(s) involved in enzyme efficiency regulation ($V_{max}$) still is not known.[15] Further, the existence of other activity regulatory mechanisms has not been considered, e.g., during yeast morphogenesis.[18–22]
Calcium ion nonetheless is known to modulate directly the activity of some enzymes/proteins.[3,23–25] In this work, we hypothesize the existence of calcium-binding site(s) in the H$^+$-ATPase from *Saccharomyces cerevisiae*, specifically in the nucleotide (ATP) binding domain (N-domain). The N-domain contains multiple glutamic (Glu) and aspartic (Asp) amino acid residues which may participate in calcium-binding (Figure 1A). This hypothesis is further supported by recent experiments in *S. cerevisiae* demonstrating the physical and physiological coupling of the voltage-gated $Ca^{2+}$ channel (VGCC) with the plasma membrane H$^+$-ATPase,[26] thus, suggesting a potential direct interaction between calcium ion and the H$^+$-ATPase.
In this work, the prediction of potential calcium-binding site(s) in the N-domain of the plasma membrane H+-ATPase from S. cerevisiae was performed initially *in silico* by analyzing the amino acid sequence and three-dimensional (3D) structure using online software.[27] Then, molecular dynamics simulations (MDS) results suggested the presence of two calcium-binding sites. After that, calcium-binding was tested *in vitro* using a recombinant H$^+$-ATPase N-domain by monitoring intrinsic fluorescence intensity changes, while luminescence energy transfer aromatic amino acid-sensitized $Tb^{3+}$ supported the binding of calcium to the N-domain.[28]

## Results and Discussion

In calcium-protein complexes, the calcium ion is coordinated by oxygen atoms.[29] In this regard, there exist two classes of calcium-binding sites mainly (continuous and non-continuous).[30] The most studied continuous (canonical) calcium-binding sites are pockets formed by a stretch of sequential amino acid residues (~12 amino acid residues),[30] where the presence of either Glu and/or Asp residues chelates calcium with their carboxyl chemical groups; coordination of calcium ions occurs mainly with the mono- and bidentate chemical structures of these amino acids;[30,31] nonetheless other amino acids containing oxygen atoms and even the carbonyl group of the peptide backbone may participate in calcium binding.[23,30,32,33] In contrast, identification of a non-continuous calcium-binding site in proteins is more difficult because of amino acid sequence irregularity, i.e, protein folding leads to the formation of the calcium-binding pocket bringing together the oxygen atoms that participate in the chelation of calcium.[30,34] These calcium-binding sites are therefore highly complex and thus difficult to identify.[33,35] In this regard, the amino acid sequence of the H$^+$-ATPase N-domain shows a relatively large number of acidic amino acids (Glu and Asp; 14 and 9 residues, respectively)

(Figure 1A).[36] Nonetheless, other amino acids involved in calcium binding are also present in the N-domain significantly (Thr, Ser, and Asn; 5, 7, and 2 residues, respectively) (Figure 1A).[30] Most of the Glu and Asp residues are located at the protein surface as shown in the three-dimensional (3D) structure of the N-domain (Figure 1B). These amino acid residues therefore might potentially form a calcium-binding site. The identification of putative calcium-binding sites in the N-domain was performed using the online software MIB2 (Metal Ion-Binding site prediction and modeling server, http://combio.life.nctu.edu.tw/MIB2/).[27,37] Five calcium-binding sites were predicted in the N-domain and formed by the amino acid residues as follows: 1) Asp402, Asp403, Met405, Leu406, 2) Gly418, Asp420, Ile422, Asp423, 3) Ser464, Glu466, Glu468, 4) Val484, Glu486, Asp487, Asn488, Trp524, Glu425, and 5) Glu492, Asp493, Val494, Glu496, Asn497, Asn500 (SI Table 1, and SI 1A and 1B);[5] especially in the activated state of the $H^+$-ATPase, the scores for Glu492 and Asp493 were the highest (SI 1B), illustrating a dynamic behavior in the putative calcium binding sites. Analysis of the whole $H^+$-ATPase amino acid sequence predicted more additional calcium-binding sites located in other protein domains: 1) Glu620 and Leu622; 2) Asp634, Asp656, and Ala657; 3) Asp200 and Iso201; 4) Asp718, Asp720, and Leu721. However, the study of these is out of the scope of the present work but will eventually be performed in future work.

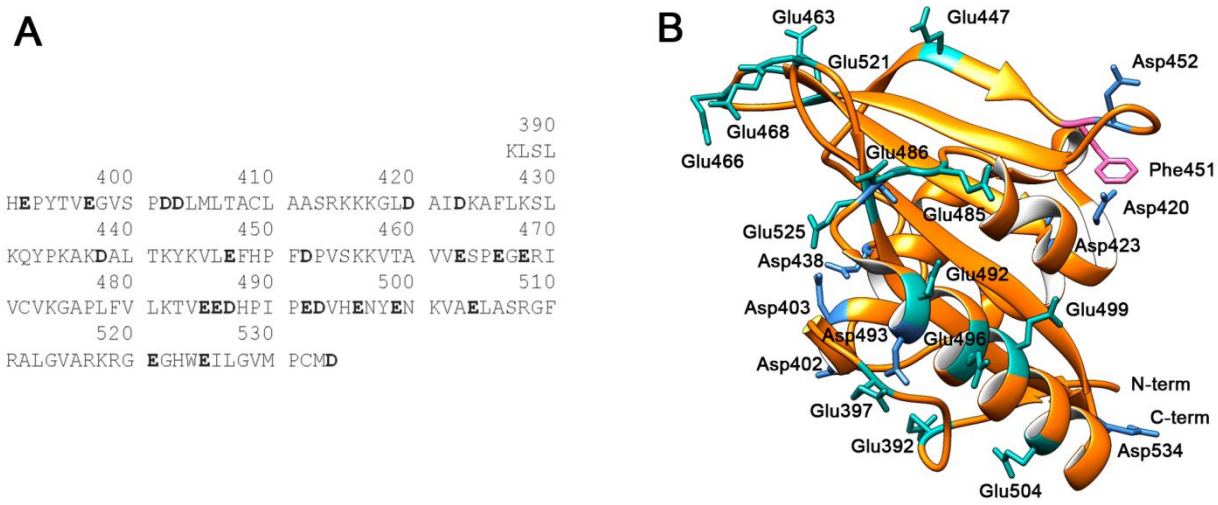



**Figure 1. A)** Amino acid sequence of the plasma membrane $H^+$-ATPase N-domain (387-534) from *S. cerevisiae*. Glu (E) and Asp (D) amino acid residues are in bold. **B)** Three-dimensional (3D) structure of the N-domain in the autoinhibited state. The 3D N-domain structure was separated from the cryo-EM structure of the $H^+$-ATPase (PDB ID 7VH5).[5] Glu and Asp amino acid residues are colored in cyan and blue, respectively. Phe451 residue that interacts with the adenine group of ATP in nucleotide-binding site is shown in pink as a reference.

MIB2, as the state-of-the-art software for predicting cation binding sites in proteins, has a prediction sensitivity of 61% for the calcium ion; which is 12% higher than its predecessor MIB, but far from the case for other ions such as $Fe^{2+}$ with 92.5%,[27,37] indicating the need for other analytical techniques.

Molecular dynamics simulation (MDS) of N-domain was performed using first the autoinhibited structural state of the $H^+$-ATPase (PDB ID 7VH5, Figure 1B) and in the presence of calcium ions, the above to test *in silico* the predicted calcium binding sites.[31,38,39] The number of oxygen atoms from amino acid residues in proteins required for calcium binding is four to eight,[33,40] although, calcium coordination by three or two oxygen atoms from amino acids does occur, albeit less commonly.[33,40] Two calcium binding sites were identified by MDS (Figure 2A). As depicted in Figure 2A, amino acid residues Asp402 and Asp403, and Glu496 and Glu499 formed binding sites A and B, respectively. Both calcium binding sites (A and B) appears to be of the continuous type. Calcium-N-domain complex in calcium binding site B was the first to form at an early stage (5-6 ns) of the simulation and was kept stable during the 50 ns simulation time. Notably the stability of the complex was kept even after extending the simulation by 200 ns. In this regard, calcium-N-domain complex in calcium binding site A was formed after 9-10 ns of simulation and remained stable during the whole 50 ns simulation time. When using the N-domain structure of $H^+$-ATPase in active state (PDB ID 7VH6), calcium-binding site A was formed with Asp438 and Asp402 (Figure 2B), while calcium-binding site B did with Glu496 and Glu499; similar formation times and stabilities for the complexes were observed as above. Hence, prediction of calcium-binding sites differs depending on the initial state of the structure.[5] Further, quality of the 3D structure seems to be an important factor for calcium-binding site prediction by MDS, as calcium binding site A was different (Glu486 and Glu525) (not shown) when using an online generated N-domain 3D model,[41] Importantly, when using magnesium ions (instead of calcium) in MDS no binding sites were observed (not shown).

The *in silico* (MDS) determination of the calcium-binding sites A and B (Figure 2) in the N-domain was validated by using the same experimental setup with both the apo state of troponin C (PDB ID 1TN4),[24,32,34] and horse milk lysozyme (PDB ID 2EQL);[42] both are well-known calcium-binding proteins. Identification of the four calcium-binding sites (EF-hand) in troponin was performed (SI 2A), i.e., calcium was coordinated with oxygen atoms of the expected amino acid residues (SI 2A). In the calcium-binding site of horse milk lysozyme, coordination of two calcium ions stabilized by water molecules was observed (SI 2B). In both proteins, calcium ions interact with Asp and Glu residues (SI 2A and 2B). Nonetheless, slight differences are observed in the number of coordinating amino acid residues and stability of the complexes, which are probably due to the conditions of the 3D structure; i.e., one is in the crystalline state and the other is in virtual solution.

Therefore, MDS seems to be a useful experimental approach to identifying calcium (and probably other cations/anions) binding sites in proteins, as described above.[24,34]

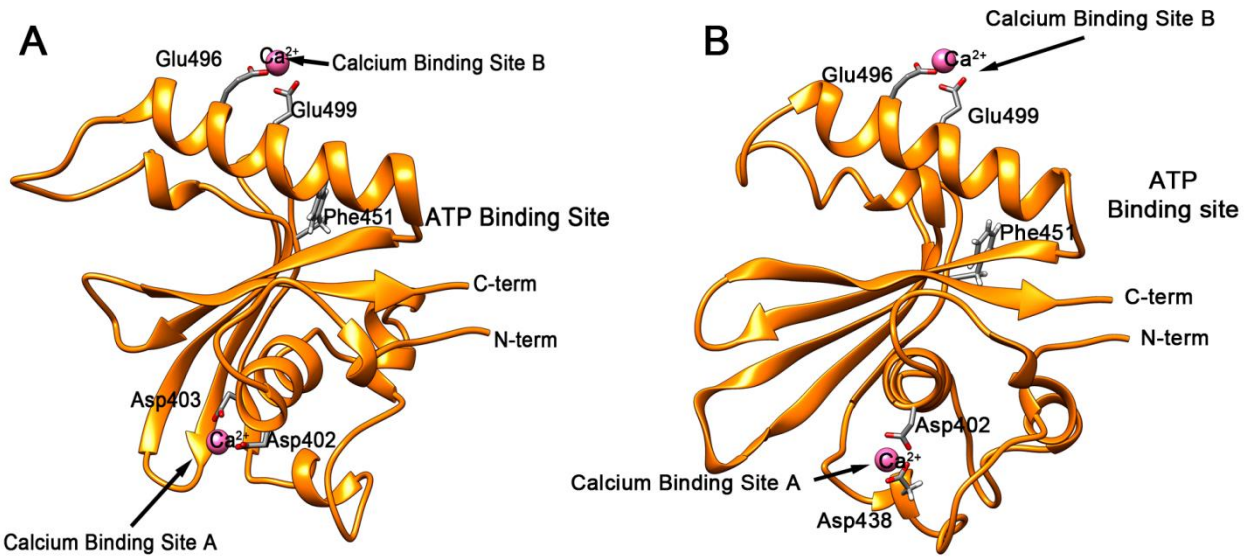



**Figure 2.** Prediction of calcium binding sites in the H⁺-ATPase N-domain by molecular dynamics simulation (MDS). MDS was performed using the software GROMACS (http://www.gromacs.org) and a standard simulation setup.[43,44] The initial structures used for simulation were those from the **A)** autoinhibited (PDB ID 7VH5) and **B)** active (PDB ID 7VH6) states of the H⁺-ATPase.[5] The systems were equilibrated using the canonical NVT ensemble and the isobaric-isothermal ensemble (NPT) for 0.1 ns for position restraint, then MDS was performed (50 ns). Calcium binding site A is formed by Asp402 and Asp403 residues in **A**, and Asp438 and Asp402 in **B**. Calcium binding site B is formed by Glu496 and Glu499 residues in both figures. Asp and Glu residues display bidentate coordination geometry for calcium; that is, a coordination index of four, with water molecules (Not shown) complete the eight oxygens required for total calcium ion coordination index (VIII). Calcium ions are colored in pink. The Phe451 residue that interacts with the adenine group of ATP during binding is shown as reference.

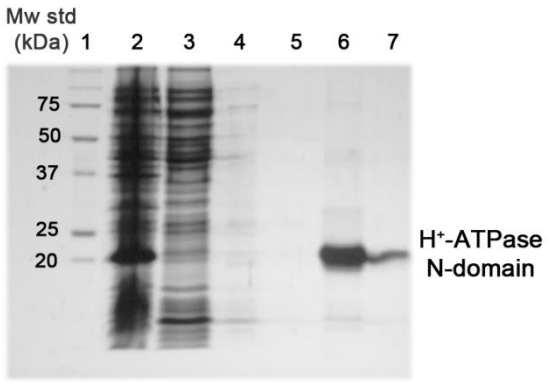



**Figure 3.** SDS-PAGE of the recombinant H⁺-ATPase N-domain. The coding sequence for the N-domain was cloned into the pET28a expression vector. His-tag N-domain (~20 kDa) was purified by chromatographic affinity using a nickel Sepharose™ fast flow column chromatography. Protein samples at different purification steps were subjected to electrophoresis and stained with Coomassie Brilliant Blue G-250. Lanes: 1) protein molecular weight standards; 2) cell homogenate after lysis; 3) unbound protein to nickel Sepharose™ Fast Flow column; 4) elution with 20 mM imidazole; 5) elution with 50 mM imidazole; 6) elution with 350 mM imidazole; and 7) elution with 500 mM imidazole. Fractions displaying high N-domain purity were pooled and then, cleaned, buffer exchanged, and desalted using PD10 chromatography columns. N-domain purity was above 95% as determined by densitometry.

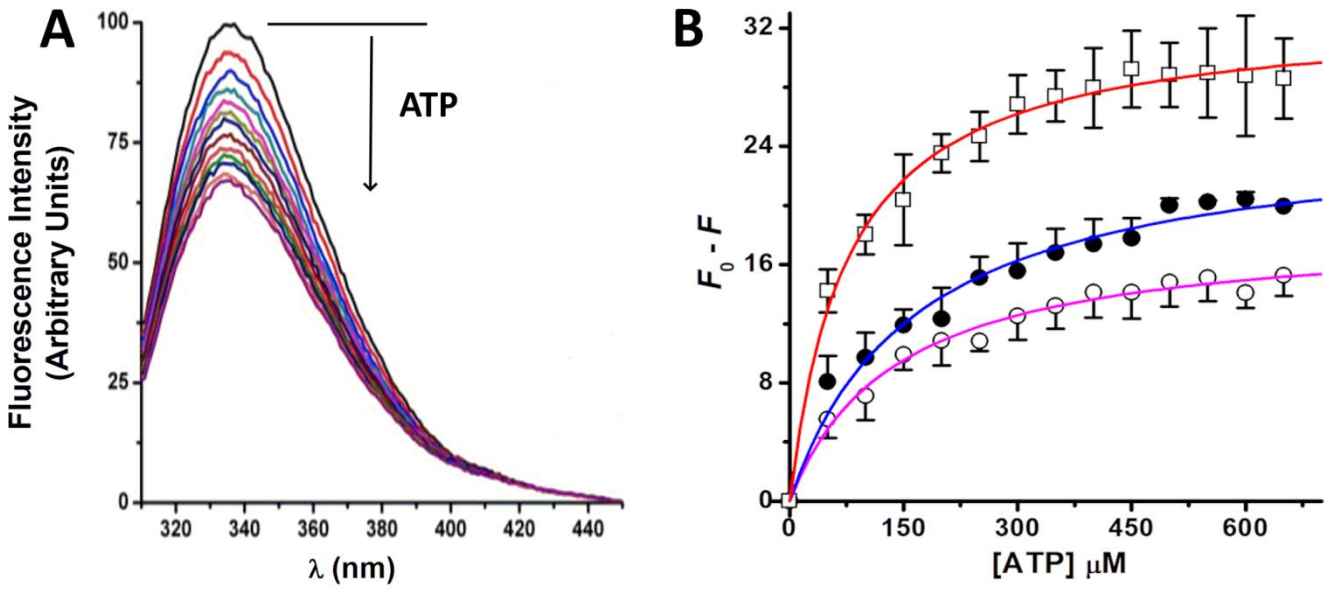



**Figure 4.** Effect of pH on ATP binding to the H⁺-ATPase N-domain. **A)** ATP binding mediates intrinsic fluorescence quenching of the recombinant N-domain at pH 7. The N-domain was suspended in 10 mM phosphate pH 7.0 and fluorescence spectra were obtained upon excitation of the N-domain at λ = 295 nm and in the absence and presence of different concentrations of ATP (0 to 600 μM) at 25 °C. **B)** ATP binding to the recombinant N-domain at different pH. N-domain intrinsic fluorescence quenching was performed as described in panel A at different pH : ○ 6.0, ● 7.0, and □ 8.0. Fluorescence intensities at wavelength (λ) of 338 nm were plotted *versus* ATP concentration, and data fitted to Equation 1 (Eq. 1) by nonlinear regression. Calculated nucleotide binding parameters are shown in Table 1.

A recombinant H⁺-ATPase N-domain was used to study *in vitro* the binding of calcium (Figure 3). An expression plasmid containing the nucleotide sequence coding for the H⁺-ATPase N-domain was generated, and the protein was expressed and isolated to high purity (Figure 3), as described in methods. The N-domain folded properly as determined by circular dichroism spectroscopy (Shown below) and intrinsic fluorescence (Figure 4A); maximum fluorescence intensity at λ of 336-338 nm. Importantly, a sole Trp524 residue is found in the H⁺-ATPase N-domain, that is useful for structural studies and ligand binding.[45,46]

The effect of pH on binding of nucleotides was determined by intrinsic fluorescence changes of the N-domain (Figure 4). N-domain intrinsic fluorescence was quenched by both ATP and ADP (not shown) at all pH tested (6.0, 7.0, and 8.0); Figure 4A shows fluorescence quenching at pH 7.0. Quenching data (fluorescence intensity at λ= 338 nm versus nucleotide concentration) displayed a hyperbolic pattern (Figure 4B), that was analyzed by fitting the data by non-linear regression to Equation 1 (Eq. 1):[47]

$$F_0 - F = (V_{max} \cdot [nucleotide])/(K_d + [nucleotide]) \quad (1)$$

where $F_0$ is the fluorescence in the absence of nucleotide, $F$ is the fluorescence at a given nucleotide concentration, $\Delta F_{max}$ is the maximum change in fluorescence, and $K_d$ is the dissociation constant of nucleotide (ATP or ADP) from binding site.

ATP affinity showed a significant variation on pH of the medium (Figure 4B, Table 1). Notably, the highest affinity for ATP was observed at alkaline pH (8.0); hence, the change of the protonation state of at least one amino acid residue close to binding site has a role in increasing ATP binding to the N-domain;[48,49] the γ-phosphate of ATP do appears to be directly involved in the interaction, similarly as reported in the $Ca^{2+}$-ATPase.[47]

**Table 1.** Effect of pH on the nucleotide binding parameters in the recombinant H⁺-ATPase N-domain.[a]

| Nucleotide | pH | $K_d$ (μM) | $\Delta F_{max}$ (%) |
|---|---|---|---|
| ADP | 6 | - | - |
| | 7 | 173 ± 10 | 20.4 ± 0.4 |
| | 8 | 70 ± 9 | 16.9 ± 0.4 |
| ATP | 6 | 140 ± 15 | 18.4 ± 0.6 |
| | 7 | 165 ± 24 | 25.2 ± 1.2 |
| | 8 | 77 ± 6 | 33.0 ± 0.6 |

[a] N-domain intrinsic fluorescence quenching data were used to calculate parameter values using Equation 1 (Eq. 1) by non-linear regression.

The effect of calcium-binding in the structure of the H⁺-ATPase N-domain, was also studied by changes in intrinsic fluorescence of the protein.[45–47] Phosphate ion is one of the main components of the cell cytoplasm;[50] however, it forms

complexes with calcium ions (at a $Ca^{2+}$/phosphate molar ratio of 1-1.3) leading to its precipitation.[51,52] In *S. cerevissiae*, total phosphorus concentration was determined to be ~300 mM, while free phosphate concentration appears wide and ranges between 10 to 75 mM.[53] In order to have biological significance, ligand binding assays in enzymes have to be performed under closely realistic conditions.[53] Hence, it was decided to use 10 mM phosphate buffer solution for the calcium binding assays; in this regard, calcium addition (0-500 μM) to phosphate buffer did not increase or decrease the fluorescence baseline (SI 3A). The addition of calcium to the suspended recombinant N-domain resulted in ~20% intrinsic fluorescence intensity quenching (Figure 5A) with a saturation pattern (Figure 5B). Intrinsic fluorescence quenching data at λ of 338 nm was fitted to equation 2 (Eq. 2) by non-linear regression:

$$F_0 - F = (\Delta F_{max} \cdot [Ca^{2+}])/(K_d + [Ca^{2+}]) \quad (2)$$

where $F_0$ and $F$ are the N-domain intrinsic fluorescence intensity in the absence and presence of calcium, $\Delta F_{max}$ is the maximum change in N-domain intrinsic fluorescence, and $K_d$ is the $Ca^{2+}$ apparent dissociation constant.[47] The calculated binding parameters for $Ca^{2+}$ were: $\Delta F_{max}$= 20.09±0.53%, and $K_d$= 119.43±10.28 μM (Figure 5B). In this regard, cytoplasmic calcium concentration is low (0.05 - 0.5 μM),[53] calcium affinity probably is higher in the whole $H^+$-ATPase than in the isolated N-domain, and might be modulated by local cytoplasmic conditions near the plasma membrane, e.g., by pH and the presence of other ions.

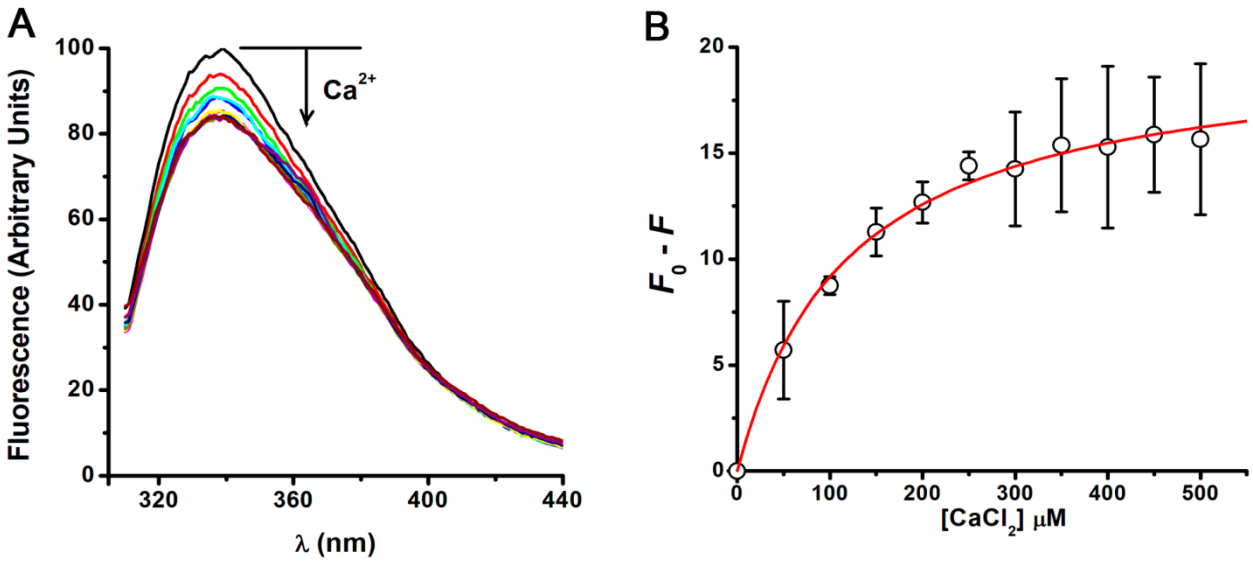



**Figure 5.** Calcium binding to the recombinant $H^+$-ATPase N-domain. **A)** Calcium-binding quenched (~20%) the intrinsic fluorescence intensity of the recombinant N-domain. Fluorescence spectra were obtained upon excitation of the N-domain at λ = 295 nm in the absence and presence of different concentrations of calcium (0 to 500 μM) at 25 °C. **B)** Plot of N-domain fluorescence intensity change ($F_0$ – $F$) at λ = 338 nm versus calcium concentration. Fluorescence intensity data were fitted to equation 2 (Eq. 2) by non-linear regression, and binding parameters were calculated: $\Delta F_{max}$=20.09 ±0.53%, and $S_{0.5}$= 119.43±10.28 μM.

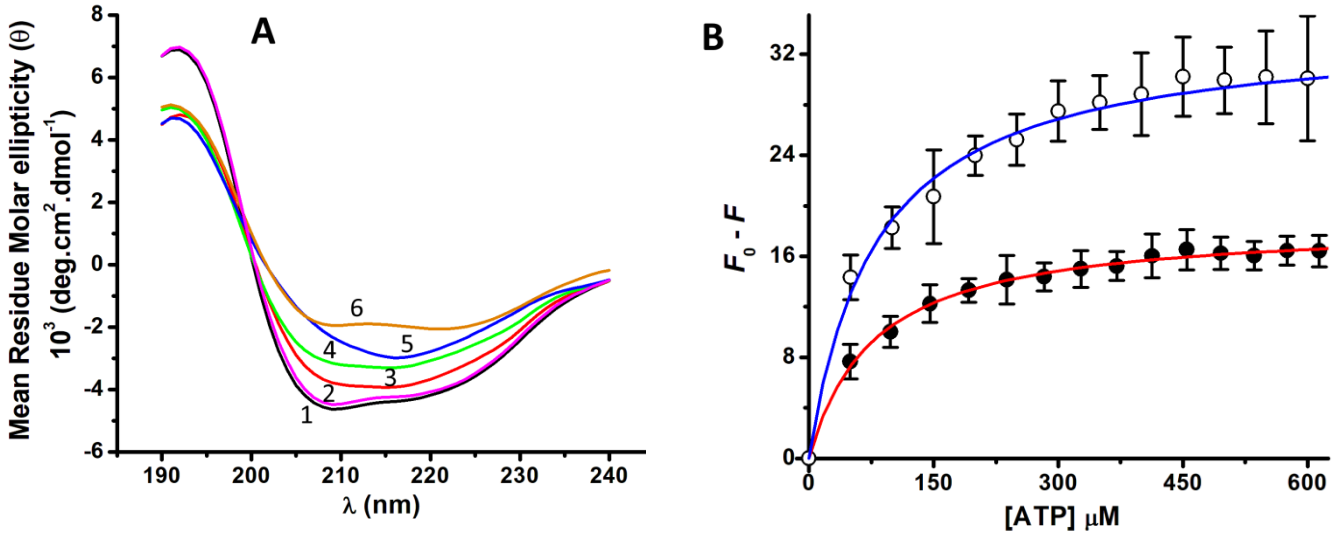



**Figure 6. A)** Circular dichroism spectra of the recombinant $H^+$-ATPase N-domain. CD spectra lines correspond to 1) the purified N-domain (10 μM) and in the presence of 2) 200 μM $Ca^{2+}$, 3) 200 μM $Ca^{2+}$ and 100 μM ATP, 4) 200 μM $Ca^{2+}$ and 200 μM ATP, 5) 200 μM $Ca^{2+}$ and 300 μM ATP, and 6) 300 μM ATP. **B)** Effect of calcium on ATP binding to the recombinant N-domain at pH 8.0. Intrinsic fluorescence quenching of N-domain by ATP addition was performed in the presence of 200 μM calcium (●); N-domain fluorescence quenching data in the absence of calcium (O) at pH 8.0 is replotted as reference. N-domain was suspended in 10 mM phosphate buffer (pH 8.0), containing 200 μM calcium. Protein excitation was at λ = 295 nm at 25 °C. Fluorescence spectra was recorded after ATP addition and data analyzed as described in Figure 5. Binding parameters for ATP in the presence of calcium were calculated: $K_d$= 77.38 ± 4.03 μM and $\Delta F_{max}$ = 18.66 ± 0.21.

**Table 2.** Effect of calcium and ATP on the secondary structure of the recombinant $H^+$-ATPase N-domain.[a]

| | | Fractional secondary structure content | | | | | |
|---|---|---|---|---|---|---|---|
| $Ca^{2+}$ (μM) | ATP (μM) | Hr | Hd | Sr | Sd | turn | unrd |
| 0 | 0 | 0.06 | 0.08 | 0.21 | 0.11 | 0.22 | 0.31 |
| 200 | 0 | 0.07 | 0.08 | 0.21 | 0.11 | 0.22 | 0.31 |
| 200 | 100 | 0.03 | 0.06 | 0.25 | 0.12 | 0.22 | 0.32 |
| 200 | 200 | 0.02 | 0.05 | 0.27 | 0.13 | 0.22 | 0.31 |
| 200 | 300 | 0.01 | 0.05 | 0.27 | 0.14 | 0.22 | 0.31 |
| 0 | 300 | 0.04 | 0.02 | 0.27 | 0.14 | 0.21 | 0.31 |

[a]Abreviations: Hr, regular α-helix; Hd, distorted α-helix; Sr, regular β-sheet; Sd, distorted β-sheet; unrd, disordered structure. Fractional content of secondary structure in the N-domain was calculated using the CONTIN method and SP43 protein reference set.[54,55]

N-domain intrinsic fluorescence quenching upon calcium-binding suggested a slight loosening (increased disorder) of the N-domain structure (Figure 5),[56] that is, the internal structure becomes more solvent accessible. Notably, the binding of calcium did not affect significantly the secondary structure content of the N-domain (α-helix, β-sheet, turns, and coiled-coil), as no significant changes were observed in the circular dichroism spectrum upon calcium addition (Figure 6A, Table 2). In contrast, ATP binding decreased the amount of α-helix, but increased that of β-sheet (Figure 6A, Table 2). In this regard, the presence of $Ca^{2+}$ seems to stabilize part of the α-helix structure (Figure 6A, Table 2), probably as a result of binding to the predicted binding sites A and B, which locate at α-helix structures (Figure 2). The presence of calcium (200 μM), however, decreased the affinity for ATP in the N-domain at pH 8.0 notably (Figure 6B); this seem to be due to the effect on N-domain structure (Table 2) rather than to calcium interaction with Asp420 that locates at the nucleotide binding site; the distance between Phe451 and Asp420 residues in the nucleotide binding site increases from ~4.8 to ~10.4 Å as showed by MDS in the presence of calcium (SI 6).

In this regard, the calculated calcium affinity appears too low to bind calcium in the yeast cytoplasm.[1,57] Nonetheless, the influx of calcium through the VGCC may probably lead to a transient localized high calcium concentration close to the $H^+$-ATPase,[2,26] e.g., in cardiac cells, calcium concentration reaches ~73 μM in sarcoplasmic reticulum calcium release sites.[58,59] High calcium concentration increases the probability of binding to the $H^+$-ATPase N-domain.[20,60] If that occurs, therefore it would be quick and transitory.[20,60] *In vitro* experiments showed that calcium concentrations higher than 100 μM completely inhibit ATPase activity, probably in part by decreasing ATP affinity, as shown here in Figure 6B, while *in vivo* calcium concentrations between 10 and 50 μM are required to stimulate (indirectly) proton pump activity.[61]

Determining the existence of calcium-binding sites in proteins is difficult.[30,33,35,39] This, is mostly because of the lack of a spectroscopic signal for $Ca^{2+}$ (SI 2A).[30] Nonetheless, energy

transfer to $Tb^{3+}$ (a lanthanide ion) has been the method of choice to determine calcium-binding sites in proteins.[28] $Tb^{3+}$ displays a similar coordination preference as $Ca^{2+}$;[62] both cations are oxophilic and have a similar ionic radii.[62] Thus, $Tb^{3+}$ has become amply used as a probe for calcium-binding sites in proteins.[28,62,63] In this regard, aromatic (Trp and Tyr) amino acid residues present in proteins and located close to calcium-binding sites are usually good energy donors to sensitize $Tb^{3+}$ luminescence.[62,64–67] Interestingly, in the $H^+$-ATPase N-domain, Tyr443 and Tyr498 residues are located close to Asp402 and Asp403, and to Glu496 and Glu499 in the *in silico* identified calcium binding sites A and B, respectively (Figure 7).[30,68,69] The $R_0$ distance (50% efficiency in Föster energy transfer) between the aromatic amino acid residue and $Tb^{3+}$ has been stated to be between 5 - 10 Å.[70,71] In this regard, the calculated molecular distances between the bound $Ca^{2+}$ and Tyr443/Tyr498 residues were around 10 Å (Figure 7), close enough for energy transfer.[56,69–71]

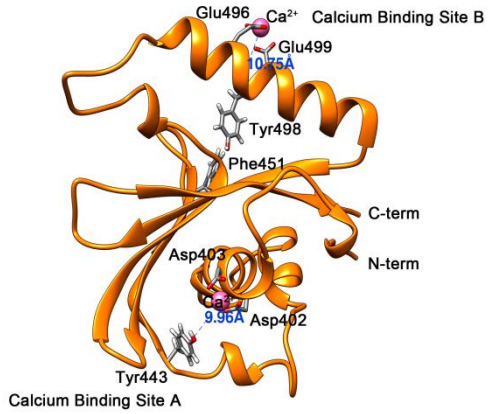



**Figure 7.** Molecular distance between calcium and the closest aromatic residues in calcium binding sites A and B. Measurements of molecular distances were performed using the software UCSF Chimera (https://www.rbvi.ucsf.edu/chimera).[72] The calculated molecular distance between calcium in binding site A with Tyr443 was 9.96 Å, while, in binding site B, the distance between calcium and Tyr498 was 10.75 Å. The Phe451 residue, that interacts with the adenine group of ATP during binding is shown as a reference.

Similarly as calcium, terbium forms non-soluble complexes with phosphate ion notably.[73] In this regard, $Tb^{3+}$ showed a slight effect on fluorescence baseline (~5%) of the medium, probably because of its known spectroscopic properties. This effect was observed between wavelenghts (λ) 310 - 400 nm (SI 3B), where proteins emit fluorescence, while, above λ of 400 nm no $Tb^{3+}$ luminescence signal was observed (SI 3B). Purified recombinant $H^+$-ATPase N-domain was titrated with $Tb^{3+}$, and binding was monitored by $Tb^{3+}$ luminescence emission after excitation at λ= 295 nm (Figure 8). $Tb^{3+}$ luminescence emission spectra showed maximum intensity peaks at λ of 490 ($^5D_4 \rightarrow {}^7F_6$) and 545 nm ($^5D_4 \rightarrow {}^7F_5$) (Figure 8),[74,75] in agreement with published results.[62,69,71,76]

$Tb^{3+}$ cooperative binding has been reported in prothrombin and calf brain S-100 protein.[28] However, caution must be taken when using $Tb^{3+}$ as a non-native ion to study the effect of calcium binding in proteins, as suggested recently.[77] Further, because of its high affinity for phosphate ions,[73] $Ca^{2+}$ and $Tb^{3+}$ have been used to precipitate phosphoproteins.[78–80] Glu residues at protein surface nonetheless appears to interact with metal mono-phosphate,[81,82] thus, it should not rule out any of the phosphate species as that which interacts with the predicted binding sites.[73,82] Larger calcium (hydroxyapatite) and terbium phosphate structures are ruled out to interact as they require either chemically modified Glu residues or long polyGlu peptide sequences.[81] Nonetheless, Figure 8 experiment was repeated in a cationic environment (SI 4), namely, in Tris buffer (pH 8.0). Similar results were observed upon titration of the $H^+$-ATPase N-domain with $Tb^{3+}$ (SI 4); however, relatively higher fluorescence changes were observed at low concentrations of $Tb^{3+}$, which indicates a higher affinity of the binding site. The characteristic greenish luminescence of terbium was observed with the naked eye when the sample in the quartz fluorescence cuvette was irradiated with UV light (inset in SI 4). It was concluded, therefore, that terbium binds to the N-domain in either anionic or cationic media.

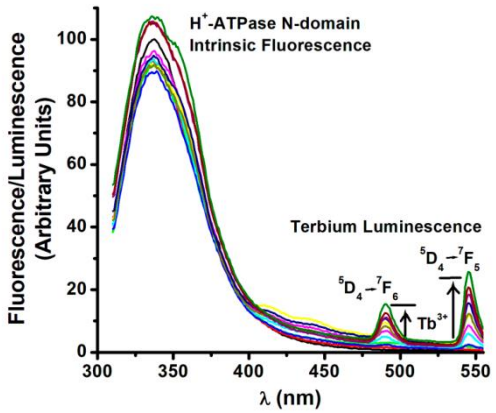



**Figure 8.** $Tb^{3+}$ luminescence emission when in the presence of the recombinant $H^+$-ATPase N-domain. $Tb^{3+}$ (0-920 μM) was added stepwise to the N-domain and fluorescence spectra were obtained upon excitation at λ= 295 nm.

Several controls of fluorescence titration with $Ca^{2+}$ and $Tb^{3+}$ were performed under identical experimental conditions using subtilisin C (a well known calcium binding protein), hen egg lysozyme (that does not bind calcium as the equine milk lysozyme), and alcohol dehydrogenase (ADH) from yeast (proposed to bind calcium at monomer interface) (SI 5).[42,83–85] In this regard, in all these proteins $Ca^{2+}$ and $Tb^{3+}$ addition did result in a decrease of the protein intrinsic fluorescence, while showing a slight (non-significant) luminescence intensity at λ of 490 and 545 nm in the presence of $Tb^{3+}$ (SI 5, $Ca^{2+}$ results not shown). Nonetheless, only subtilisin C showed a similar pattern with N-domain in fluorescence variation upon $Tb^{3+}$ addition; a decrease in intrinsic fluorescence intensity at initial additions (up to 100 μM), that increased at subsequent additions, the above probably was due to macromolecular complexes formation as the increase in fluorescence signal was avoided when applying moderate constant stirring. Interestingly, similarly to the N-

domain subtilisin C contains a sole Trp residue and numerous (15) Tyr residues that locate at the protein surface, but away enough from calcium binding sites for energy transfer, therefore, no significant $Tb^{3+}$ luminescence signal was observed notably (SI 5A). Additonally, the existence of unspecific interaction of $Tb^{3+}$ with aromatic amino acids at the protein surface was also discarded.

Tyr443 and Tyr498 capability for energy transfer sensitizing the bound $Tb^{3+}$ was tested. The molecular distance between the calcium binding site A/B and the Trp524 residue after 50 ns MDS was 17.7 and 23.4 Å (SI 7), respectively; thus contribution of Trp524 to $Tb^{3+}$ luminescence seems minimal. Nonetheless, to discard this contribution, the Trp524 residue was chemically modified in the purified recombinant $H^+$-ATPase N-domain, using *N*-bromosuccinimide (NBS) (SI 8).[45,86] NBS modifies the indole group of tryptophan to oxindole, a non-fluorescent group.[87–89] Thus, part (~55%) of the $H^+$-ATPase N-domain intrinsic fluorescence was quenched by NBS treatment (SI 8).[46,47] In this regard, chemical modification with NBS does not affect overall protein folding as the maximum peak of fluorescence emission did not show a bathochromic shift to λ of 352 nm (Figure 9 and SI 8), as expected for an unfolded protein. Further, NBS-modified proteins are still able to bind ligands, as observed in the N-domain $Ca^{2+}$-ATPase.[86] In the chemically modified N-domain, both $Tb^{3+}$ luminescence peaks ($^5D_4 \rightarrow {}^7F_6$ and $^5D_4 \rightarrow {}^7F_5$) did increase (Figure 9). Therefore, energy transfer to bound $Tb^{3+}$ seems to occur from Tyr443/Tyr498 residues (Figure 7, 8, and 9) with high efficiency, similarly as reported by others.[28,69–71,90] In this regard, N-domain excitation at λ of 280 nm did generate a more consistent and less noisy fluorescence spectra when in presence of $Tb^{3+}$ notably (SI 9); further, the increase in fluorescence peak at λ of 358 nm did not occur.

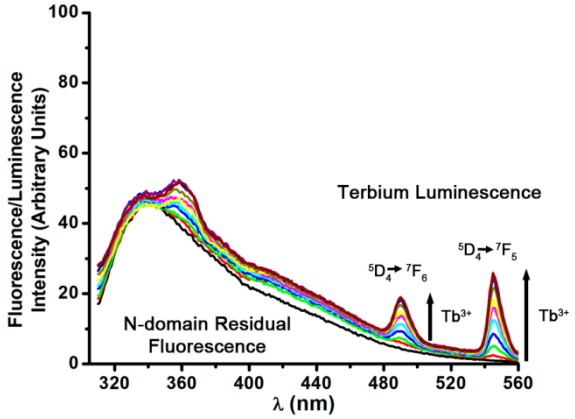



**Figure 9.** $Tb^{3+}$ luminescence emission in the presence of a NBS-modified N-domain. $Tb^{3+}$ (0-400 μM) was added stepwise to the chemically modified N-domain and fluorescence spectra was obtained upon excitation at λ= 295 nm.

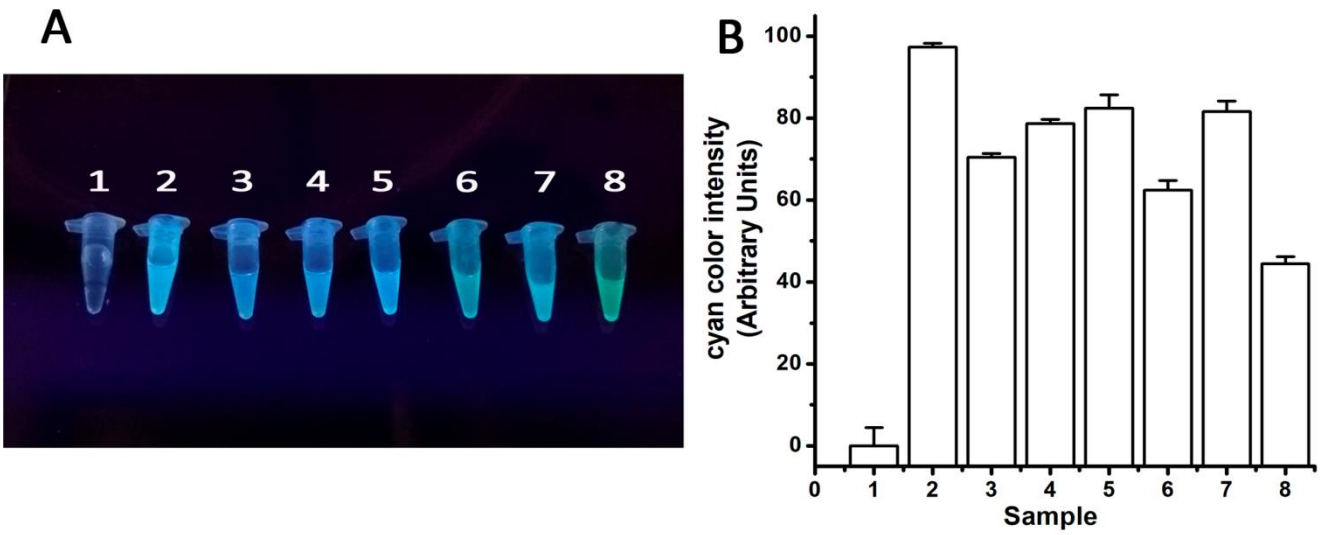



**Figure 10. A)** Effect of different chemical compounds on $Tb^{3+}$ luminescence when in the presence of the recombinant $H^+$-ATPase N-domain. $Tb^{3+}$ (0.5 mM) in solution (1) alone and in the presence (2) of $H^+$-ATPase N-domain (20 μM), plus: (3) $Ca^{2+}$ (500 μM); (4) NBS (1 mM); (5) SDS (20%); (6) EDTA (0.5 mM); (7) guanidinium hydrochloride (1 M); (8) acrylamide (5 mM). Solutions were irradiated with UV light using a transilluminator. **B)** Plot of cyan color intensity from A. Color intensity was determined using the software ImajeJ (https://imagej.nih.gov/ij/index.html).

The $Tb^{3+}$ luminescence peaks $^5D_4 \rightarrow {}^7F_6$ and $^5D_4 \rightarrow {}^7F_5$ transitions locate in the blue and green regions of the visible electromagnetic spectrum, respectively,[75] i.e., visible with the unaided eye. A solution made of $Tb^{3+}$ and the recombinant $H^+$-ATPase N-domain did show light emission of cyan color upon UV light irradiation (Figure 10A); observation of $Tb^{3+}$ luminescence with the bared eyes supports the prediction that $H^+$-ATPase N-domain structure contains calcium-binding sites. The effect of different chemicals/compounds on $Tb^{3+}$ luminescence intensity emission was tested (Figure 10A and 10B), as follows (tubes 1 to 8): $Tb^{3+}$ alone did not display luminescence, but it did in the presence of the purified

recombinant $H^+$-ATPase N-domain; calcium did compete with $Tb^{3+}$ for binding sites (higher affinity for $Tb^{3+}$, apparently); Tyr443/Tyr498 did energy-transfer to sensitize $Tb^{3+}$ in an NBS-modified N-domain (most of the luminescence is retained);[86,87] the anionic detergent SDS unfolded the N-domain, but this did not significantly affect $Tb^{3+}$ binding notably, the above probably because of the continuous location of Asp and Glu residues at polypeptide chain, i.e., overall folding seems not to be crucial for calcium binding (Figure 2);[91] EDTA did chelate $Tb^{3+}$ decreasing its availability for binding;[92] guanidinium hydrochloride (GndHCl) unfolded the N-domain,[93] and the effect on $Tb^{3+}$ binding was similar to that of SDS thus supporting the above suggestion. Acrylamide presence quenched the fluorescence of aromatic amino acid residues,[94] thus resulting in less energy transfer to $Tb^{3+}$. Notably, the typical green luminescence of $Tb^{3+}$ ($^5D_4 \rightarrow {}^7F_5$ transition) was mainly observed when in the presence of the fluorescence quencher acrylamide. Future experiments will clarify some apparent inconsistencies, like the lack of effect of SDS and GndHCl in $Tb^{3+}$ binding.

$Tb^{3+}$ has been used to study the nucleotide-binding site of the plasma membrane $H^+$-ATPase from *Schizosaccharomyces pombe*.[95] $Tb^{3+}$ complexes with ATP to form Tb-ATP which is hydrolyzed by the $H^+$-ATPase; notably, Tb-ATP competes with Mg-ATP for the binding site.[95] In this regard, Ekberg *et al* (2010) identified the $Tb^{3+}$ binding site in the plasma membrane $H^+$-ATPase from the plant *Arabidopsis thaliana* by X-ray crystallography,[96] the $Tb^{3+}$ binding site locates in the N-domain close to the ATP binding site,[96] Asp487 appears as the sole amino acid residue interacting with $Tb^{3+}$. Thus, $Tb^{3+}$ has been also useful to identify $Mg^{2+}$ binding sites (where it binds) when in complex with nucleotides.[95,96]

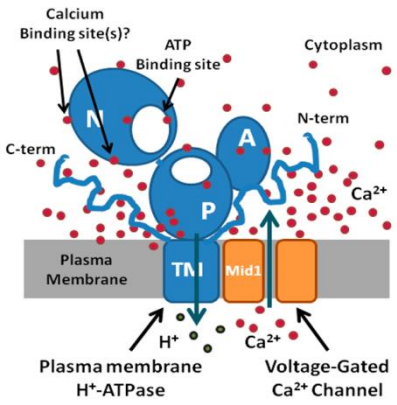



**Figure 11.** Cartoon of the interaction of the plasma membrane $H^+$-ATPase with Mid1, a subunit of the voltage-gated $Ca^{2+}$ channel (VGCC). After activation, $Ca^{2+}$ influx to yeast cytoplasm encounters immediately with the $H^+$-ATPase cytoplasmic domains: N-domain (N); P-domain (P); A-domain (A); C- and N-terminal (C- and N-term) unstructured segments. The transmembrane domain (TM) interacts with Mid1 a subunit of VGCC.[26] Putative calcium-binding sites locate at the N-domain (this work); calcium binding to N-domain generate modulatory effects on the structure/affinity of the $H^+$-ATPase.

## Conclusion

In yeast, calcium signaling mediates plasma membrane $H^+$-ATPase activation by glucose.[10,14] Phosphorylation of the C-terminal domain of $H^+$-ATPase by protein kinase (Ptk2) has been the most studied activation mechanism.[11,14,15,97] Nonetheless, the existence of other activity modulating mechanisms in the $H^+$-ATPase has been suggested,[2,7,14] e.g., high proton concentration (low pH) may induce the release of the C-terminal domain from interaction with cytoplasmic domains,[5] thus leading to $H^+$-ATPase activation. In this regard, protons compete with calcium in target enzymes/proteins,[98,99] e.g., in $Ca^{2+}$-ATPase, mutation of Asp residues located in the L6-7 loop decreases enzyme turnover and ATP affinity in the N-domain.[100] In yeast, it has been reported that $H^+$-ATPase interacts physically with Mid1 (a subunit of the voltage-gated $Ca^{2+}$ channel).[26] Under this arrangement, calcium influx meets with the $H^+$-ATPase cytoplasmic structure, where potential interactions of calcium ions may occur with the enzyme domains (Figure 7). Thus, whether calcium exerts directly some type of modulation of the $H^+$-ATPase N-domain structure and activity (nucleotide affinity) seems probable (Figure 6 and Table 2);[61] others $H^+$-ATPase domains seem to contain calcium-binding sites. The close dependence between proton pumping and calcium influx to the cytoplasm supports this suggestion.[7,8,26,60] *In silico* amino acid sequence analysis and MDS in the presence of $Ca^{2+}$[27,101] seem to be a good initial strategy to predict the existence of calcium-binding sites in proteins.[28–30,39,102] MDS is particularly helpful in identifing $Ca^{2+}$ binding sites on protein surfaces where two carboxylates are close together. In the $H^+$-ATPase N-domain, the binding sites I, II, and V described in SI Table 1 together with that determined by MDS appear to be the most likely for calcium binding. The binding site formed by Glu496 and Glu499 located at the $H^+$-ATPase N-domain appeared consistently in the *in silico* experiments. Notably, both Glu residues are non-conserved, while the phenotype of the *mid-1* knockout mutant is different between fungus and yeast species;[103,104] likely as an indicative of the ability/inability to grow in the diverse calcium-containing media. Nonetheless, the results of *in vitro* experiments (protein intrinsic fluorescence and terbium luminescence) suggest the presence of calcium-binding site(s) in the $H^+$-ATPase N-domain from *S. cerevisiae*. Further research is required to confirm the predicted Asp/Glu residues as those forming the calcium-binding site(s).

## Experimental Section

The N-domain amino acid sequence was analyzed for the presence of calcium-binding sites by submission to the online software MIB2 (Metal ion binding site prediction and modeling server, http://combio.life.nctu.edu.tw/MIB2/). The N-domain 3D structure was extracted from the hexamer $H^+$-ATPase crystal structure (PDB ID 7VH5 and 7VH6, inhibited and active states, respectively) and used for MDS.[5] MDS of the $H^+$-ATPase N-domain in the presence of 30 calcium ions were performed using the software GROMACS 5.1.2 software (https://www.gromacs.org/)[101] and a standard protocol as described by Lindahl,[44] and validated using troponin C (a calcium-binding protein) (PDB ID 1TN4) and horse milk lysozyme (PDB ID 2EQL), briefly: the canonical NVT ensemble and isobaric-isothermal ensemble (NPT), 50,000 steps each, were used to perform the system equilibration. Then, 50 ns (extensive to 200 ns when mentioned) MD simulations were performed. The generated results were visualized using the software VMD (http://www.ks.uiuc.edu/Research/vmd/) and UCSF chimera (https://www.cgl.ucsf.edu/chimera/).[72,105]

Plasmid (for protein expression) construction harboring the nucleotide sequence coding for the $H^+$-ATPase N-domain (comprising 387-534 amino acid residues plus a His6-tag for protein affinity purification) from *S. cerevisiae* was performed, briefly: the nucleotide sequence segment 1159-1602 (encoding the N-domain) of the gen PMA1/YGL008C was synthesized by GenScript™ and cloned in-frame in the expression plasmid pET28a (kanamycin resistance and a T7lac promoter), using the enzymes *Eco*R I and *Bam*H I; thus resulting in the plasmid pET28aNDHATPase. Competent *E. coli* BL21 DE3 cells were transformed with plasmid pET28aNDHATPase and transformants cells selected for kanamycin resistance in LB solid medium. Protein expression was performed by an overnight preculture at 37 °C of the *E. coli* cells in 50 mL LB liquid medium supplemented with kanamycin (50 μg/mL), then the 50 mL *E. coli* preculture was added to 450 ml fresh LB liquid medium, and culture was continued by incubation at 37 °C and constant stirring. Protein expression ($H^+$-ATPase N-domain $His_6$-tag) was initiated by IPTG addition (0.5 mM final concentration) when optical density (OD) (λ=660 nm) of the culture reached a value of 0.7 AU. Cell culture was continued for 4 h at 37 °C and with constant stirring. Cells were harvested by centrifugation (3,500*g*), for 10 minutes at 4°C. The cell pellet was resuspended in lysis buffer and subjected to sonication for 2.5 min, with 15 s ultrasonic pulses. The cell homogenate was centrifuged (17,000*g*) for 40 min at 4 °C, and the non-soluble fraction was discarded. The expressed N-domain $His_6$ tag was purified from the supernatant by immobilized metal ion affinity chromatography (IMAC) using a Ni-Sepharose chromatography column His TRAP HP™ (GE Healthcare™). The bound protein was eluted using a step-increasing concentration gradient of imidazole (10, 50, 250, and 500 mM) in 50 mM Tris buffer (pH 8.0) and 300 mM NaCl. PD-10 (desalting column, GE Healthcare™) was used to remove imidazole and change the suspending medium to 10 mM phosphate buffer (pH 8.0); or 10 mM Tris-HCl (pH 8.0) when indicated. N-domain $His_6$–tag molecular weight and purity were determined by SDS-PAGE. Protein concentration was determined using the Lowry assay[106] and human serum albumin as standard and corroborated spectrophotometrically by absorbance (λ=280 nm) using an extinction coefficient (ε) of 11,460 $M^{-1}{\cdot}cm^{-1}$. Protein folding was determined by intrinsic fluorescence spectrum emission after excitation at the wavelength (λ) of 295 nm; the folded N-domain shows a center of spectral mass at λ of 338 nm. The purified protein was stored at 4 °C until use.

Calcium and $Tb^{3+}$ binding to the recombinant $H^+$-ATPase N-domain (and control proteins: subtilisin C, hen egg lysozyme, and yeast alcohol dehydrogenase) was determined by intrinsic fluorescence changes at 25 °C; the N-domain contains a sole Trp524 residue that displays fluorescence intensity changes upon ligand binding.[45,46] The N-domain (5 μM) was suspended in 1 mL of 10 mM phosphate buffer, pH 8.0 (or 10 mM Tris-HCl, pH 8.0 when indicated). Nucleotides (ADP and ATP), and metal ions ($CaCl_2$ and $TbCl_3$) were added stepwise until no further change in N-domain intrinsic fluorescence/$Tb^{3+}$ luminescence was observed, accordingly. After each addition (2 min), fluorescence/luminescence was recorded in a spectrofluorometer Shimadzu RF5301PC after excitation at λ=295 nm; experiments were performed three times. N-domain was chemically modified by NBS addition (stepwise, 0 – 22 μM) and titrated with $Tb^{3+}$ as described above. Experimental data were analyzed by nonlinear regression to equations 1 (Eq. 1) and 2 (Eq. 2) using the software Microcal™ Origin® 6.0 (Northampton, MA, USA).

## Acknowledgments

CLG-F is a fellow of Conacyt-México (466820). This work received no external funding.

Competing Interests: The authors declare no competing interests.

**Keywords:** calcium-binding site • plasma membrane $H^+$-ATPase • molecular dynamics simulation • terbium luminescence • terbium binding • energy transfer

## References

[1] M. Lange, E. Peiter, *Frontiers in microbiology*, 2019, 10, 3100.
[2] S. Groppi, F. Belotti, R. L. Brandão, E. Martegani, R. Tisi, *Cell Calcium*, 2011, 49, 376–386.
[3] J. Cui, J. A. Kaandorp, P. M. A. Sloot, C. M. Lloyd, M. V. Filatov, *FEMS Yeast Research*, 2009, 9, 1137–1147.
[4] Y. Ruiz-Granados, V. De La Cruz-Torres, J. Sampedro, *Molecules*, 2019, 24, 958.
[5] P. Zhao, C. Zhao, D. Chen, C. Yun, H. Li, L. Bai, *Nature Communications*, 2021, 12, 6439.
[6] R. Serrano, *FEBS Letters*, 1983, 156, 11–14.
[7] J. Li, W. Huang, X. Wang, T. Tang, Z. Hua, G. Yan, *World Journal of Microbiology and Biotechnology*, 2010, 26, 1181–1186.
[8] M. J. M. Trópia, A. S. Cardoso, R. Tisi, L. G. Fietto, J. L. R. Fietto, E. Martegani, I. M. Castro, R. L. Brandão, *Biochemical and Biophysical Research Communications*, 2006, 343, 1234–1243.
[9] I. Romero, A. M. Maldonado, P. Eraso, *Biochemical Journal*, 1997, 322, 823–828.
[10] L. E. M. Bouillet, A. S. Cardoso, E. Perovano, R. R. Pereira, E. M. C. Ribeiro, M. J. M. Trópia, L. G. Fietto, R. Tisi, E. Martegani, I. M. Castro, R. L. Brandão, *Cell Calcium*, 2012, 51, 72–81.
[11] P. Eraso, M. J. Mazón, F. Portillo, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 2006, 1758, 164–170.
[12] F. Portillo, P. Eraso, R. Serrano, *FEBS Letters*, 1991, 287, 71–74.
[13] M. A. A. Souza, M. J. Trópia, R. L. Brandão, *Microbiology*, 2001, 147, 2849–2855.
[14] D. D. Castanheira, E. P. Santana, F. Godoy-Santos, R. H. S. Diniz, F. Faria-Oliveira, R. R. Pereira, M. J. M. Trópia, I. M. Castro, R. L. Brandão, *FEMS Yeast Research*, , DOI:10.1093/femsyr/fox088.
[15] R. R. Pereira, D. Castanheira, J. A. Teixeira, L. E. M. Bouillet, E. M. C. Ribeiro, M. M. J. Trópia, F. Alvarez, L. F. M. Correa, B. E. F. Mota, L. E. F. R. Conceição, I. M. Castro, R. L. Brandão, *FEMS Yeast Research*, 2015, 15, fov003.
[16] E. Estrada, P. Agostinis, J. R. Vandenheede, J. Goris, W. Merlevede, J. François, A. Goffeau, M. Ghislain, J. Francois, A. Goffeau, M. Ghislain, *Journal of Biological Chemistry*, 1996, 271, 32064–32072.
[17] M. J. Mazón, P. Eraso, F. Portillo, *FEMS Yeast Research*, 2015, 15, fov030.
[18] B. C. Monk, M. Niimi, M. G. Shepherd, *Journal of Bacteriology*, 1993, 175, 5566–5574.
[19] L. Merlini, O. Dudin, S. G. Martin, *Open Biology*, 2013, 3, 130008.
[20] N. Carbó, N. Tarkowski, E. P. Ipiña, S. P. Dawson, P. S. Aguilar, *Molecular Biology of the Cell*, 2017, 28, 501–510.
[21] M. Bagnat, K. Simons, *Proceedings of the National Academy of Sciences of the United States of America*, 2002, 99, 14183–14188.
[22] S. M. Kurian, A. Lichius, N. D. Read, *Journal of Fungi*, 2022, 8, 90.
[23] J. L. Galzi, S. Bertrand, P. J. Corringer, J. P. Changeux, D. Bertrand, *EMBO Journal*, 1996, 15, 5824–5832.
[24] J. J. Jayasundar, J. Xing, J. M. Robinson, H. C. Cheung, W.-J. Dong, *PLoS ONE*, 2014, 9, e87135.
[25] S. Tobwala, D. K. Srivastava, *Advances in Enzyme Research*, 2013, 1, 17–29.
[26] T. Cho, A. Ishii-Kato, Y. Fukata, Y. Nakayama, K. Iida, M. Fukata, H.

Iida, *Genes to Cells*, 2017, 22, 94–104.
[27] Y. F. Lin, C. W. Cheng, C. S. Shih, J. K. Hwang, C. S. Yu, C. H. Lu, *Journal of Chemical Information and Modeling*, 2016, 56, 2287–2291.
[28] H. G. Brittain, F. S. Richardson, R. B. Martin, *Journal of the American Chemical Society*, 1976, 98, 8255–8260.
[29] R. C. K. Cheng, B. S. Zhorov, *European Biophysics Journal*, 2010, 39, 825–838.
[30] C. Yanyi, X. Shenghui, Z. Yubin, Y. J. Jie, *Science China. Chemistry*, 2010, 53, 52–60.
[31] M. S. Cates, M. L. Teodora, G. N. Phillips, *Biophysical Journal*, 2002, 82, 1133–1146.
[32] R. W. Wallace, E. A. Tallant, M. E. Dockter, W. Y. Cheung, *Journal of Biological Chemistry*, 1982, 257, 1845–1854.
[33] H. Deng, G. Chen, W. Yang, J. J. Yang, *Proteins: Structure, Function, and Bioinformatics*, 2006, 64, 34–42.
[34] M. Kohagen, M. Lepšík, P. Jungwirth, *Journal of Physical Chemistry Letters*, 2014, 5, 3964–3969.
[35] X. Hu, Q. Dong, J. Yang, Y. Zhang, *Bioinformatics*, 2016, 32, 3260–3269.
[36] X. Cao, X. Hu, X. Zhang, S. Gao, C. Ding, Y. Feng, W. Bao, *PLoS ONE*, 2017, 12, e0183756.
[37] C. H. Lu, C. C. Chen, C. S. Yu, Y. Y. Liu, J. J. Liu, S. T. Wei, Y. F. Lin, *Bioinformatics*, 2022, 38, 4428–4429.
[38] Z. Dolatkhah, S. Javanshir, A. S. Sadr, J. Hosseini, S. Sardari, *Journal of Chemical Information and Modeling*, 2017, 57, 1246–1257.
[39] A. Garg, D. Pal, *Proteins: Structure, Function and Bioinformatics*, 2021, 89, 1125–1133.
[40] M. M. Harding, *Acta Crystallographica Section D: Biological Crystallography*, 2004, 60, 849–859.
[41] L. J. McGuffin, J. D. Atkins, B. R. Salehe, A. N. Shuid, D. B. Roche, *Nucleic Acids Research*, 2015, 43, W169–W173.
[42] J. Zeng, K. R. Rao, K. Brew, R. Fenna, *Journal of Biological Chemistry*, 1990, 265, 14886–14887.
[43] A. Nurisso, A. Daina, R. C. Walker, in *Homology Modeling: Methods and Protocols*, eds. A. J. W. Orry and R. Abagyan, Springer Science+Business Media, 2012, vol. 857, pp. 137–173.
[44] E. R. Lindahl, in *Molecular Modeling of Proteins. Methods in Molecular Biology*, ed. A. Kukol, Humana Press, Totowa, NJ, 2008, vol. 443, pp. 3–23.
[45] J. G. Sampedro, Y. G. Ruiz-Granados, H. Nájera, A. Téllez-Valencia, S. Uribe, *Biochemistry*, 2007, 46, 5616–22.
[46] J. G. Sampedro, H. Nájera, S. Uribe-Carvajal, Y. G. Ruiz-Granados, *Journal of Fluorescence*, 2014, 24, 1849–1859.
[47] E. D. Páez-Pérez, V. De La Cruz-Torres, J. G. Sampedro, *Biochemistry*, 2016, 55, 6751–6765.
[48] R. A. Alberty, *Journal of Physical Chemistry B*, 2000, 104, 9929–9934.
[49] A. V. Onufriev, E. Alexov, *Quarterly Reviews of Biophysics*, 2013, 46, 181–209.
[50] Y. Lei, B. Song, R. D. van der Weijden, M. Saakes, C. J. N. Buisman, *Environmental Science & Technology*, 2017, 51, 11156–11164.
[51] H. Carlsson, H. Aspegren, N. Lee, A. Hilmer, *Water Research*, 1997, 31, 1047–1055.
[52] D. Watrobska-Swietlikowska, *Scientific Reports*, 2019, 9, 10525.
[53] K. van Eunen, J. Bouwman, P. Daran- Lapujade, J. Postmus, A. B. Canelas, F. I. C. Mensonides, R. Orij, I. Tuzun, J. van den Brink, G. J. Smits, W. M. van Gulik, S. Brul, J. J. Heijnen, J. H. de Winde, M. J. Teixeira de Mattos, C. Kettner, J. Nielsen, H. V Westerhoff, B. M. Bakker, *The FEBS Journal*, 2010, 277, 749–760.
[54] N. Sreerama, R. W. Woody, *Analytical Biochemistry*, 2000, 287, 252–260.
[55] N. Sreerama, R. W. Woody, *Analytical Biochemistry*, 1993, 209, 32–44.
[56] E. T. Bell, J. D. B. Featherstone, J. E. Bell, *Archives of Biochemistry and Biophysics*, 1989, 271, 359–365.
[57] R. Kellermayer, R. Szigeti, M. Kellermayer, A. Miseta, *FEBS Letters*, 2004, 571, 55–60.
[58] J. J. Saucerman, D. M. Bers, *Biophysical Journal*, 2008, 95, 4597–4612.
[59] C. Soeller, M. B. Cannell, *Biophysical Journal*, 1997, 73, 97–111.
[60] T.-Y. Ma, M.-A. Deprez, G. Callewaert, J. Winderickx, *Cell Calcium*, 2021, 100, 102479.
[61] R. R. Lew, *Plant Physiology*, 1989, 91, 213–216.
[62] L. J. Martin, B. Imperiali, in *Methods in Molecular Biology*, ed. R. Derda, Springer Science+Business Media, New York, 1st edn., 2015, vol. 1248, pp. 201–220.
[63] L. J. Martin, M. J. Hähnke, M. Nitz, J. Wöhnert, N. R. Silvaggi, K. N. Allen, H. Schwalbe, B. Imperiali, *Journal of the American Chemical Society*, 2007, 129, 7106–7113.
[64] C. S. Bonnet, M. Devocelle, T. Gunnlaugsson, *Organic and Biomolecular Chemistry*, 2012, 10, 126–133.
[65] L. Calisti, M. C. Trabuco, A. Boffi, C. Testi, L. C. Montemiglio, A. des Georges, I. Benni, A. Ilari, B. Taciak, M. Białasek, T. Rygiel, M. Król, P. Baiocco, A. Bonamore, *PLOS ONE*, 2018, 13, e0201859.
[66] A. S. Goryashchenko, M. G. Khrenova, A. A. Bochkova, T. V. Ivashina, L. M. Vinokurov, A. P. Savitsky, *International Journal of Molecular Sciences*, 2015, 16, 16642–16654.
[67] A. K. Di Gennaro, L. Gurevich, E. Skovsen, M. T. Overgaard, P. Fojan, *Physical Chemistry Chemical Physics*, 2013, 15, 8838–8844.
[68] C. W. V. Hogue, J. P. MacManus, D. Banville, A. G. Szabo, *Journal of Biological Chemistry*, 1992, 267, 13340–13347.
[69] J. E. Allen, G. L. McLendon, *Biochemical and Biophysical Research Communications*, 2006, 349, 1264–1268.
[70] C. W. V. Hogue, J. P. MacManus, D. Banville, A. G. Szabo, *Journal of Biological Chemistry*, 1992, 267, 13340–13347.
[71] J. P. MacManus, C. W. Hogue, B. J. Marsden, M. Sikorska, A. G. Szabo, *Journal of Biological Chemistry*, 1990, 265, 10358–10366.
[72] E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, *Journal of Computational Chemistry*, 2004, 25, 1605–1612.
[73] F. H. Firsching, *Journal of Chemical and Engineering Data*, 1992, 37, 497–499.
[74] C. M. Spangler, C. Spangler, M. Schäerling, *Annals of the New York Academy of Sciences*, 2008, 1130, 138–148.
[75] F. S. Richardson, *Chemical Reviews*, 1982, 82, 541–552.
[76] J. L. Vázquez-Ibar, A. B. Weinglass, H. R. Kaback, *Proceedings of the National Academy of Sciences of the United States of America*, 2002, 99, 3487–92.
[77] S. C. Edington, A. Gonzalez, T. R. Middendorf, D. B. Halling, R. W. Aldrich, C. R. Baiz, *Proceedings of the National Academy of Sciences of the United States of America*, 2018, 115, E3126–E3134.
[78] Y. Güzel, M. Rainer, M. R. Mirza, G. K. Bonn, *Analytical and Bioanalytical Chemistry*, 2012, 403, 1323–1331.
[79] X. Zhang, J. Ye, O. N. Jensen, P. Roepstorff, *Molecular and Cellular Proteomics*, 2007, 6, 2032–2042.

[80] M. Pink, N. Verma, F. Polato, G. K. Bonn, H. A. Baba, A. W. Rettenmeier, S. Schmitz-Spanke, *Journal of Proteomics*, 2011, 75, 375–383.

[81] R. Fujisawa, Y. Wada, Y. Nodasaka, Y. Kuboki, *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*, 1996, 1292, 53–60.

[82] M. Tavafoghi, M. Cerruti, *Journal of the Royal Society, Interface*, 2016, 13, 20160462.

[83] X. De Bolle, C. Vinals, J. Fastrez, E. Feytmans, *Biochemical Journal*, 1997, 323, 409–413.

[84] M. Mizuguchi, M. Nara, Y. Ke, K. Kawano, T. Hiraoki, K. Nitta, *European Journal of Biochemistry*, 1997, 250, 72–76.

[85] N. A. M. Azrin, M. S. M. Ali, R. N. Z. R. A. Rahman, S. N. Oslan, N. D. M. Noor, *Biotechnology and Applied Biochemistry*, , DOI:10.1002/bab.2309.

[86] V. De la Cruz-Torres, Y. Cataño, M. Olivo-Rodríguez, J. G. Sampedro, *Journal of Fluorescence*, 2020, 30, 483–496.

[87] T. F. Spande, B. Witkop, in *Methods in Enzymology*, Academic Press, 1967, vol. 11, pp. 498–506.

[88] B. F. Peterman, K. J. Laidler, *Archives of Biochemistry and Biophysics*, 1980, 199, 158–164.

[89] T. F. Spande, N. M. Green, B. Witkop, *Biochemistry*, 1966, 5, 1926–1933.

[90] J. Wang, L.-X. Zhang, Z.-L. Liu, H.-G. Liang, L. Yang, X.-Y. Hu, *Photosynthesis Research*, 1995, 44, 297–302.

[91] M. S. Nouraei, H. Faghihi, A. R. Najafabadi, A. H. M. Zadeh, M. Darabi, A. Vatanara, *AAPS PharmSciTech*, 2019, 20, 327.

[92] W. G. Gonzalez, V. Ramos, M. Diaz, A. Garabedian, J. C. Molano-Arevalo, F. Fernandez-Lima, J. Miksovska, *Biochemistry*, 2016, 55, 1873–1886.

[93] J. M. Scholtz, G. R. Grimsley, C. N. Pace, in *Methods in enzymology*, 2009, vol. 466, pp. 549–565.

[94] M. R. Eftink, C. A. Ghiron, *Analytical Biochemistry*, 1981, 114, 199–227.

[95] M. Ronjat, J. J. Lacapere, J. P. Dufour, Y. Dupont, *Journal of Biological Chemistry*, 1987, 262, 3146–3153.

[96] K. Ekberg, B. P. Pedersen, D. M. Sørensen, A. K. Nielsen, B. Veierskov, P. Nissen, M. G. Palmgren, M. J. Buch-Pedersen, *Proceedings of the National Academy of Sciences of the United States of America*, 2010, 107, 21400–21405.

[97] A. Goossens, N. de la Fuente, J. Forment, R. Serrano, F. Portillo, *Molecular and Cellular Biology*, 2000, 20, 7654–7661.

[98] E. M. Blanchard, R. J. Solaro, *Circulation Research*, 1984, 55, 382–391.

[99] G. L. Smith, D. A. Eisner, *Circulation*, 2019, 139, 2358–2371.

[100] G. Lenoir, M. Picard, J. V Møller, M. le Maire, P. Champeil, P. Falson, *Journal of Biological Chemistry*, 2004, 279, 32125–32133.

[101] D. Van Der Spoel, E. Lindahl, B. Hess, G. Groenhof, A. E. Mark, H. J. C. Berendsen, *Journal of Computational Chemistry*, 2005, 26, 1701–1718.

[102] J. H. Kaplan, *Annual Review of Biochemistry*, 2002, 71, 511–535.

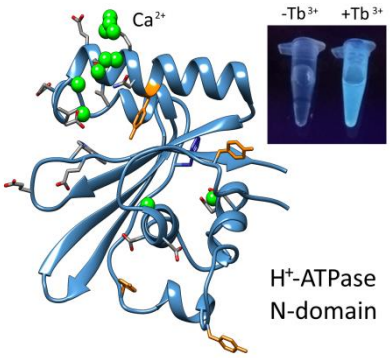
[103] R. R. Lew, Z. Abbas, M. I. Anderca, S. J. Free, *Eukaryotic Cell*, 2008, 7, 647–655.

[104] H. Iida, H. Nakamura, T. Ono, M. S. Okumura, Y. Anraku, *Molecular and Cellular Biology*, 1994, 14, 8259–8271.

[105] W. Humphrey, A. Dalke, K. Schulten, *Journal of Molecular Graphics*, 1996, 14, 33–38.

[106] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randal, *Journal of Biological Chemistry*, 1951, 193, 265–75.

## Entry for the Table of Contents




The nucleotide-binding domain (N-domain) of the plasma membrane $H^+$-ATPase appears to contain five calcium-binding sites as identified by amino acid sequence analysis and molecular dynamics simulations. Calcium binding was tested in a recombinant N-domain by intrinsic fluorescence quenching and energy transfer sensitized $Tb^{3+}$ luminescence. Calcium binding probably modulates $H^+$-ATPase structure and nucleotide affinity.

A non-higlighted version of the revised manuscript

# Identification of Calcium-Binding Sites in the Nucleotide Binding Domain of the Plasma Membrane $H^+$-ATPase from *Saccharomyces cerevisiae*

Claudia L. Gómez-Flores,[b] and José G. Sampedro*[a]

[a] Ph.D. C. L. Gómez-Flores
Instituto de Física, Universidad Autónoma de San Luis Potosí.
Avenida Chapultepec 1570, Privadas del Pedregal. San Luis Potosí. C.P. 78295, SLP, México
[b] Prof. J. G. Sampedro (*Corresponding author)
Instituto de Física, Universidad Autónoma de San Luis Potosí.
Avenida Chapultepec 1570, Privadas del Pedregal. San Luis Potosí. C.P. 78295, SLP, México
E-mail: sampedro@dec1.ifisica.uaslp.mx

**RESPONSE TO REVIEWERS**

**Reviewer 2:** This manuscript is certainly improved, but there are still important issues that need to be addressed.

**1.** Primarily, the authors did not address my previous comment about phosphate. While I appreciate that there is phosphate in cells and it is useful to mimic physiological conditions, including phosphate is simply not appropriate in experiments involving divalent and trivalent metal ions, because those ions' complexes with phosphate are highly insoluble. The results of every titration experiment require knowledge of the soluble metal concentration at every point, but this is not possible when some (unknown) amount of the added metal is insoluble. Therefore, the $K_d$ values as currently reported cannot be correct - notably, they are overestimated. It's pretty easy to repeat the experiments in a different buffer like HEPES or MOPS or Tris (ok for Ca or Tb), and such experiments would yield lower Kd values, which in turn should make the authors' case stronger - a $K_d$ of, say, 5 µM for Ca binding would certainly be more plausibly relevant in vivo than a value of 120 µM. (The argument on p. 6 about how a Kd of 120 uM could be relevant in vivo isn't very convincing.) Inside cells, the problem of phosphate precipitating metals is probably mostly avoided because there are many other ligands that keep the metal ions soluble - but of course it's not reasonable to include all of these ligands in an in vitro experiment. If the purpose of the experiment is to report a reproducible Kd value, however, then it must be done using a buffer that does not precipitate the metal ions used in the titration. Remember that if the Kd in a direct titration ends up being similar to the protein concentration, a titration using Ca-chelator complexes to control the free $Ca^{2+}$ concentration needs to be done (method of Pozzan and Tsien).

**Re: Given that several equilibriums are involved, including that of the N-domain protein, it is difficult to determine the actual concentration of free calcium. Therefore, we agree that the calculated Kd value for calcium is incorrect. We repeated the experiment using Tris buffer, as suggested by the reviewer. Interestingly, we observed terbium luminescence (see SI 4 plot), similar to that observed in the original experiment using phosphate buffer. It is important to note that Tris at pH 8.0 is mostly cationic. Thus, calcium seems to be bound irrespective of the ionic composition of the medium. Higher changes in fluorescence at low terbium concentrations (see SI 4) suggest higher binding site affinity, as discussed above by the**

**reviewer. Detailed experiments on this issue are underway, and the results will be published. We acknowledge the reviewer's recommendation and comments.**

**2.** The reason I suggested controls and experiments in the absence of phosphate was not because I thought that precipitated Ca phosphate or Tb phosphate might have much fluorescence but rather because if there is precipitate in the sample there will be light scattering that could end up non-specifically quenching fluorescence. Ca addition is observed to quench the protein fluorescence (Figure 5), so this mechanism must be considered as possible under the authors' conditions. This is another reason why it is imperative that the experiments not be performed in a phosphate buffer.

**Re: As requested by the reviewer, experiments in the absence of phosphate were performed (see SI 4). Using a Tris buffer solution (10 mM), the plot in SI 4 was generated, and it clearly shows the same result, probably of better quality than with phosphate buffer, with only minor differences. An additional experiment using Bis-Tris yielded similar results but was not included in the article because the pH was different. Under these conditions, the sole calcium complexes formed are those with the N-domain.**

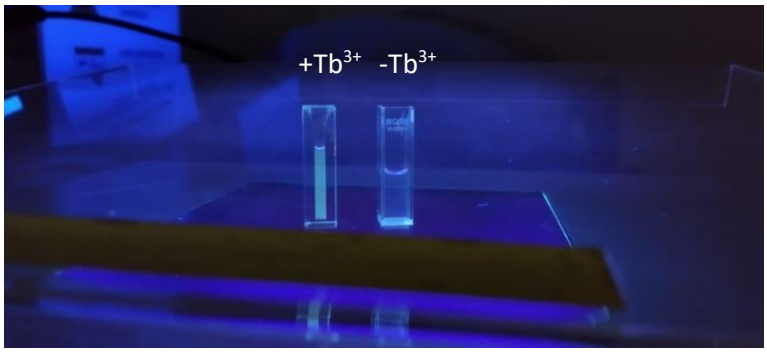
**3.** Figure 4: the authors should check whether some of the Trp fluorescence quenching in their ATP titrations is due to inner filter effect from the strong absorption from ATP (ATP will absorb some of the incident light, resulting in a decrease in Trp fluorescence. I would guess that could still be a problem even at 295 nm excitation. There are standard ways to correct for this: see Analytical Biochemistry 96, 448, 1979. Inner filter effect might also account for the different degrees of Trp quenching at different pHs. If this is contributing, the authors may need to reevaluate some of their conclusions on p. 4 and elsewhere.

**Re: The inner filter effect has been corrected as requested by the reviewer. Unlike in previous versions, the hyperbolicity of the curves in Figures 4B and 6B now clearly reach their asymptotes. Thanks to the reviewer's observations, these figures have been significantly improved. It should also be noted that the ATP concentrations tested were not particularly high. The maximum concentration tested was 0.6 mM. The parameters in Table 1 have been updated accordingly.**

**4.** Without any evidence from mutagenesis experiments for any specific sites, the authors have to be careful about their conclusions about where Ca is binding. All they can say is that Tb is binding reasonably close to a Trp or Tyr (probably Tyr because I agree with the authors that the data suggest energy transfer is not occurring from the Trp), and Ca seems to weakly compete for the Tb sites (Figure 10).

**Re: The experiment was repeated (see SI 9), but this time the sample was excited at a wavelength (λ) of 280 nm instead of 295 nm, which is more appropriate for tyrosine (Tyr) excitation. The resulting spectra were less noisy, suggesting a more "effective" energy transfer system. Nevertheless, we agree with the reviewer´s point about the absence of mutagenesis experiments. Therefore, in the concluding paragraph (page 9, right column, lines 27-32), we state that several amino acid residues are probably involved in calcium binding. Considering the above, we expressed a small fragment of the N-domain containing binding site V (see SI Table 1) including a Tyr residue. As shown in the included image, which is for reviewing**

**purpose only, we obtained results similar to those in Figure 8 in Tris buffer (10 mM, pH 8.0). The experimental details will be published.**




**5.** Figure 6B: Was magnesium present in the ATP titrations? It should be shown that the effect of calcium on this same protein cannot be replicated by 0.5-1 mM magnesium (the estimates for intracellular $Mg^{2+}$ concentration in the literature).

**Re: As described in the methods section, magnesium was not present in the shown ATP titrations. In fact, the experiment including $Mg^{2+}$ in ATP titrations was performed: the $Mg^{2+}$ presence decreases the affinity of the N-domain for ATP (not shown), and MDS showed no binding to $Mg^{2+}$ (see page 2, right column, lines 58-60).**

**Minor comments:**

**1.** p. 1, column 2, line 31: change "indicated" to "suggested." Simulations suggest possible sites; they do not indicate that those sites exist.

**Re: The change was performed as requested. Now it is read in p. 1, column 2, line 30-32 the following:**

> **molecular dynamics simulations (MDS) results suggested the presence of two calcium-binding sites.**

**2.** p. 1, column 2, line 49: both mono- and bidentate coordination are involved in Ca2+ binding by carboxylates

**Re: The correction in line 49 (p. 1, column 2) was performed as requested by reviewer. Now it is read:**

> **the coordination of the calcium ions mainly occurs with the mono- and bidentate chemical structures of these amino acids**

**3.** p. 2, column 1, line 44-47: the discussion of the number of oxygen atoms needed for $Ca^{2+}$ coordination is a bit confusing. In saying that 2, 3, or 4 oxygen atoms is a possible binding site, I assume the authors are referring to purely the ligands derived from the protein, not the total number of ligands (including solvent) - I'm not aware of any real Ca binding sites that have fewer than 6 ligands. Anyway, please be more specific about this point.

**Re: Lines 44-47 (p. 2, column 1, now lines 52-55) were rewritten to clarify the coordination of $Ca^{2+}$ by oxygen atoms from amino acid residues in proteins. The text now reads as follows:**

> **The number of oxygen atoms from amino acid residues in proteins required for calcium binding is four to eight, [33,40] although calcium coordination by three or two oxygen atoms from amino acids does occur, albeit less commonly.**

**4.** p. 2, column 2, line 44: I did not understand what the authors were saying here: "to note is that pH of the medium is important in both states, more acidic for the active state notably"

**Re: The line was deleted in order to avoid confusing the reader. The pH of the medium is always important for protein structure.**

**5.** p. 2, column 2: there shouldn't be data "not shown." Showing the data for the first "not shown" is particularly important, in my opinion

**Re: The data that is not shown refers to the initial MDS that was performed for the initial submission. In other words, this data was originally included in the manuscript. The initial work was done with an online-generated model. The results differ slightly from those obtained with the crystal (already included as SI 1A and 1B, and SI Table 1) of the "recently" published oligomeric structures of the $H^+$-ATPase. This is why it is not longer shown.**

**6.** Top p. 3: While I'm glad that the authors showed simulation data for troponin C and lysozyme, the troponin C results are not very convincing. They do suggest a few of the ligands, but the "site" that it proposes is not a reasonable site, probably because the MD does not do a good job of simulating the conformational change of the protein upon Ca binding. I don't agree with the authors that these are "slight differences" most likely due to the MD simulating a solution condition and the structure being from a crystal – while the latter may contribute somewhat, I think the large discrepancy between the real Ca binding sites and the simulated ones is because of the conformational change. Anyway, the authors should be more transparent about this. The conclusion at the end on p. 9 that MD simulations "seem to be a good strategy to predict the existence of calcium-binding sites in proteins" is not strong - the MD seems to only have found places where two carboxylates are close together.

**Re: Using MDS to study the interaction between calcium and biomolecules is not a new concept. It has previously been performed with phospholipid bilayers, as described by Melcrova et al. (2016).**

> **Melcrová, A., Pokorna, S., Pullanchery, S. et al. The complex nature of calcium cation interactions with phospholipid bilayers. Sci Rep 6, 38035 (2016). https://doi.org/10.1038/srep38035**

**Nonetheless, we agree with the reviewer – the MDS appears useful to find places on the protein surface where two carboxylates are close together -, that is calcium coordination by two bidentate (four oxygen atoms) amino acid residues. Hence, we modified the sentence (p. 9, right column, lines 25-29) as follows:**

> ***In silico*** **amino acid sequence analysis and MDS in the presence of $Ca^{2+}$[27,101] seem to be a good initial strategy to predict the existence of calcium-binding sites in proteins.[28–30,39,102] MDS is particularly helpful in identifing $Ca^{2+}$ binding sites on protein surfaces where two carboxylates are close together.**

**7.** Table 1 and elsewhere: too many significant figures. Please report values with a reasonable number of significant figures (most likely no more than 3).

**Re: Significant figure in Table 1 and Table 2 were adjusted to a minimum.**

**8.** Figure 6A: check the units of molar ellipticity. The stated values seem about 3 orders of magnitude too low.

**Re: The units of molar ellipticity were checked and corrected in Figure 6A. We appreciate the reviewer request. Thank you.**