**Probing high and low affinity $Mg^{2+}$ binding at recombinant functional sites of plasma membrane $H^{+}$-ATPase from yeast with trinitrophenyl nucleotide analogues**

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Trinitrophenyl (TNP)-nucleotides are an useful tool for the characterization of ATP binding sites in P-type ATPase family, since these nucleotide derivatives increase their fluorescence drastically upon high affinity binding to a more hydrophobic environment. We used TNP-AXP (TNP-ATP or TNP-AMP) to test both nucleotide and $Mg^{2+}$ binding sites of the recombinant nucleotide-binding (N) or nucleotide-binding and phosphorylation (NP) domains of yeast PMA1. The N and NP domains showed nucleotide binding sites, with higher affinities (1 to 3 µM) than those previously described for the recombinant sites of SERCA. As observed for this later, the presence of $Mg^{2+}$ in the medium quenches the florescence of bound nucleotides, without significantly changing the nucleotide affinity. In addition, we observed that fluorescence quench is greater for the NP domain at low concentrations of $Mg^{2+}$ (< 5 mM), while only importantly affected above this concentration for the N domain. Our results suggest the existence of high and low affinity $Mg^{2+}$ sites at the NP domains, but only low affinity $Mg^{2+}$ site at the N domain. In addition, these results pinpoint the important role of conserved residues at the P and hinge domains for $Mg^{2+}$ binding in the active site of P-ATPases.

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