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Thapsigargin affinity purification of intracellular P_{2A}-type Ca^{2+} ATPases ☆

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ABSTRACT

The ubiquitous sarco(endo)plasmic reticulum (SR/ER) Ca^{2+} ATPase (SERCA2b) and secretory-pathway Ca^{2+} ATPase (SPCA1a) belong both to the P_{2A}-type ATPase subgroup of Ca^{2+} transporters and play a crucial role in the Ca^{2+} homeostasis of respectively the ER and Golgi apparatus. They are ubiquitously expressed, but their low abundance precludes purification for crystallization. We have developed a new strategy for purification of recombinant hSERCA2b and hSPCA1a that is based on overexpression in yeast followed by a two-step affinity chromatography method biasing towards properly folded protein. In a first step, these proteins were purified with the aid of an analogue of the SERCA inhibitor thapsigargin (Tg) coupled to a matrix. Wild-type (WT) hSERCA2b bound efficiently to the gel, but its elution was hampered by the high affinity of SERCA2b for Tg. Therefore, a mutant was generated carrying minor modifications in the Tg-binding site showing a lower affinity for Tg. In a second step, reactive dye chromatography was performed to further purify and concentrate the properly folded pumps and to exchange the detergent to one more suitable for crystallization. A similar strategy was successfully applied to purify WT SPCA1a. This study shows that it is possible to purify functionally active intracellular Ca^{2+} ATPases using successive thapsigargin and reactive dye affinity chromatography for future structural studies. This article is part of a Special Issue entitled: 11th European Symposium on Calcium.

1. Introduction

Members of the superfamily of P-type ATPase actively transport specific ions across various biological membranes with the use of energy derived from ATP hydrolysis. Archetypical members of this superfamily are the (α1 subunit of the) Na^+/K^+-ATPase and the sarco (endo)plasmic reticulum (SR/ER) Ca^{2+} ATPase SERCA1a, i.e., two highly specialized ion transporters that, because of their unusually high abundance, were the first to be purified for crystallization [1,2]. In the last decade, more than 20 different crystal structures representing nine different conformational states have been reported for SERCA1a that together cover nearly the entire reaction cycle providing detailed insights in the Ca^{2+} pumping mechanism [3–5]. However, because SERCA1a is present only in the highly specialized SR of fast-twitch skeletal muscle, this isoform can hardly be considered as the typical intracellular Ca^{2+} pump.

The structure–function of the housekeeping Ca^{2+} pumps found in the intracellular compartments, i.e., the paraloguous SERCA2b and the secretory-pathway Ca^{2+} ATPase SPCA1a are only poorly understood. So far, due to their low tissue expression, these proteins evaded purification, which is required for more extensive functional characterization and crystallization. Both pumps belong to the P_{2A}-type ATPase subgroup and transport Ca^{2+} ions back into the major intracellular Ca^{2+} stores, respectively, the ER and the Golgi apparatus [4]. These pumps help to maintain a low cytosolic Ca^{2+} concentration in most cell types, but they also maintain the high luminal Ca^{2+} concentration required for vital cell functions, such as protein folding, cell division and signaling [6].

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Among the Ca^{2+}-transport ATPases, only the SERCA-type pumps possess two high-affinity Ca^{2+}-transport sites (site I and site II). Consequently, they can transport two Ca^{2+} ions per ATP hydrolyzed, whereas the SPCAs transport only one Ca^{2+} ion (binding to a site corresponding to site II of SERCA). Despite this important functional difference between SERCAs and SPCAs, their overall sequence similarity clearly demonstrates a close relation (29% sequence identity). Both types of Ca^{2+} pumps cycle between two major conformations: E1, with the high-affinity Ca^{2+}-binding site(s) facing the cytoplasm, and E2, in which the same Ca^{2+}-binding site (s) lower their affinity for the ion and reorient to the organellar lumen [7,8].

The housekeeping SERCA2b pump markedly differs from other SERCA isoforms by an extra 11th transmembrane (TM) region and luminal C-terminus. Based on extensive mutagenesis, a structural model of SERCA2b was recently developed, predicting intramolecular interactions of the 2b tail with upstream regions of the pump (i.e., within the membrane and in the ER lumen). This interaction would stabilize the pump in the E1 conformation, which at least partially explains its higher apparent affinity for Ca^{2+} [9,10]. Solving the crystal structure of SERCA2b might provide additional support for these structural and functional predictions. Thereto, the protein needs to be purified from an overexpression system such as *Saccharomyces cerevisiae*. Related P-type ATPases like SERCA1a, H^+-ATPase and PfATP6 were already successfully overexpressed in yeast for purification [11–15]. In a previously described method, a biotin acceptor domain (BAD)-tag attached at the C-terminus of the protein was used to purify recombinant SERCA1a from yeast by means of streptavidin affinity chromatography [16]. The presence of a tag at the C-terminus may ensure that only full-length expressed proteins are purified. However, based on the SERCA2b structural predictions, a C-terminal tag would interfere with proper docking of the 2b tail to the luminal side of the pump [10]. Therefore, the BAD system or related approaches using other affinity tags could only be used when attaching the tag to the N-terminus, where it not necessarily allows the selection of correctly translated and folded proteins.

In this study, we describe a new affinity purification strategy based on thapsigargin (Tg), which avoids the use of affinity tags and guarantees the purification of properly folded ATPases. Tg is a sesquiterpene lactone derived from the plant *Thapsia garganica* [17], which inhibits SERCA in the sub-nanomolar range such that it fully blocks SERCA activity in almost stoichiometric amounts [18]. It binds to SERCA in a hydrophobic, funnel-like cavity open to the cytosol formed by TM3, TM5 and TM7 and prevents the movement of the helices relative to each other (Fig. 1). This explains the inhibitory effect on the pump [19]. Because amino acids lining the binding pocket for Tg in SERCA1a are fully conserved in SERCA2b, the crystal structure of SERCA1a is highly informative regarding the Tg-binding site in SERCA2b. Note that SPCA1 also shows some sensitivity toward Tg inhibition, but in a Tg concentration range of at least 1000-fold higher (still micromolar) compared to SERCA [20].

Here, we report a proof of principle of a novel technique for affinity purification of properly folded SERCA2 and SPCA1 from a yeast overexpression system using Tg and ATP analogues (respectively Tg_a, i.e., 2–12 ADT, and reactive dye). This method will enable future crystallization attempts of these housekeeping pumps.

2. Materials and methods

2.1. Heterologous expression of human SERCA2b and SPCA1a in yeast cells

The human (h) SERCA2b and SPCA1a cDNAs were amplified by PCR and cloned by Gateway (Invitrogen) recombination in a custom-made

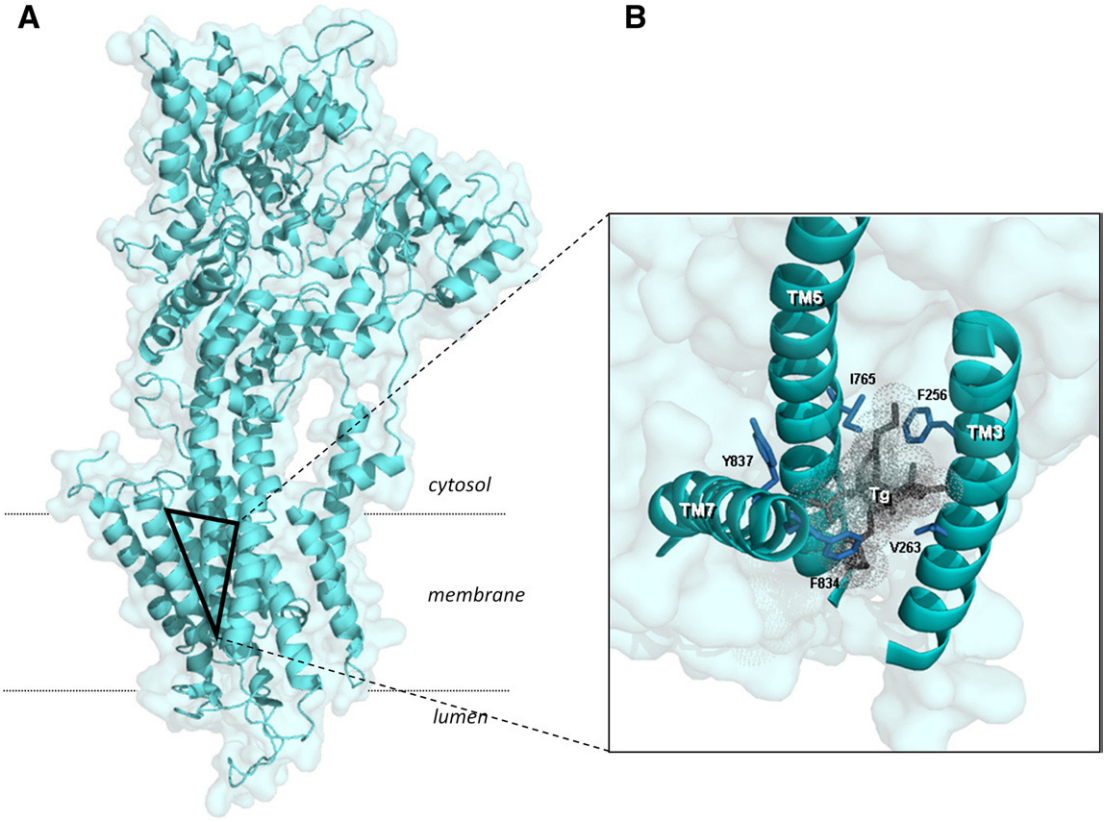

Fig. 1. 3-Dimensional view of the SERCA1a and its thapsigargin-binding site. The 3-dimensional structure of SERCA1a (2AGV [46]) is shown in the E2 conformation stabilized by the SERCA inhibitor thapsigargin (Tg). The Tg-binding site is indicated by the triangle (A). Tilted and enlarged view of the Tg-binding site, flanked by TM3, TM5 and TM7 (B). Important residues contributing to the binding site are depicted in stick representation.