Membrane attachment of Slr0006 in *Synechocystis* sp. PCC 6803 is determined by divalent ions

Dalton Carmel, Paula Mulo, Natalia Battchikova and Eva-Mari Aro

Molecular Plant Biology, Department of Biochemistry and Food Chemistry, University of Turku, FIN-20014 Turku, Finland

Corresponding author: Eva-Mari Aro

Address: University of Turku
Department of Biochemistry and Food Chemistry
Molecular Plant Biology
Tykistökatu 6A
Biocity 6th Floor
Turku 20520
Finland

E-mail: evaaro@utu.fi

Tel: +358-2-3338071
Fax: +358-2-3338075

Abstract

Slr0006 is one of the *Synechocystis* sp. PCC 6803 proteins strongly induced under carbon limiting conditions. Slr0006 has no predicted transmembrane helices or signal peptide sequence, yet it was exclusively recovered in the membrane fraction of *Synechocystis*, when the cells were broken in isolation buffers which contain divalent cations and are generally used for photosynthesis studies. Even subsequent washing of the membranes with high salt or various detergents did not release Slr0006, indicating strong binding of the Slr0006 protein to the membranes. Further, DNAse or RNAse treatment did not disturb the tight binding of Slr0006 protein to the membranes. Nevertheless, when the cells were broken in the absence of divalent cations, Slr0006 remained completely soluble. Binding of the Slr0006 to the membrane could not be properly reconstituted if the cations were added after breaking the cells in the absence of divalent ions. This unusual phenomenon has to be considered in identification and localization of other yet uncharacterized cyanobacterial proteins.

Key words: divalent ions; localization; membrane; Slr0006; *Synechocystis*

Introduction

Cyanobacteria are a widespread group of photoautotrophic microorganisms which are commonly used as model organisms in photosynthesis research. They possess internal thylakoid membranes harboring the discrete supra-molecular assemblies of proteins, pigments, and electron carriers to perform complex photochemical redox reactions. In higher plants and algae, thylakoid membranes are structurally differentiated into stacked multilamellar regions called grana, and unstacked stroma thylakoids (Andersson and Anderson 1980; Staehelin 2003). The extent of stacking of the thylakoid membrane depends on various factors like surface interactions between adjacent membranes, lateral movement of proteins in the thylakoid membrane, or spontaneous appression due to Van der Waals attraction. Under *in vitro* conditions, spontaneous stacking of membranes occurs in the presence of cations due to enhanced electrostatic interaction caused by membrane surface charges (Rumak et al. 2010 ; Riviere et al. 1990)**.** In contrast to eukaryotes, cyanobacteria exhibit distinct layers of concentrically organized thylakoid membranes around the periphery of the cell.

The genome of *Synechocystis* sp. PCC 6803 (hereafter called *Synechocystis*) encodes 3672 proteins. Systematic hydropathy analyses showed that 35% of all the predicted gene products encoded within the *Synechocystis* genome are likely to contain transmembrane helixes, and thus to be intrinsic membrane proteins (Paulsen et al. 1998). Additionally, a number of proteins are known to be peripherally bound to the surface of the integral membrane proteins. Although the location of several proteins has been verified experimentally, the majority of the proteins still remain unlocalized. Isolation and separation of membranes and soluble fractions, followed by either 2-D PAGE and mass spectrometric analyses, or 1-D

PAGE and western blotting, are frequently employed methods to identify and localize the proteins. Detailed location of the membrane proteins can be determined by aqueous two phase partitioning (Kwon et al. 2010). However, a number of predicted gene products are lost during the isolation procedure in many proteomics studies. The studies exploiting these techniques have provided massive amount of knowledge about cyanobacterial proteome. We show here that the early steps of the isolation procedure may cause ambiguity of the results. There are various buffers in common use for the isolation procedure of cyanobacterial proteins (Table 1), and in the present study we highlight how the presence of divalent cations during the breakage of *Synechocysits* cells affects the localization of the Slr0006 protein. Presumably, other not yet characterized proteins might show similar behavior.

Materials and methods

Growth conditions

The *Synechocystis* wild type strain was grown in BG-11 medium buffered with 20 mM HEPES-NaOH pH 7.5 at 30°C, under continuous illumination of 50 μmol photons m^{-2} s^{-1} and shaking at 110 rpm (Rippka 1988)**.** The cells grown under 3% CO_2 until A_{750} = 0.8-1.0 were centrifuged, re-suspended in fresh BG-11 medium lacking Na_2CO_3, and the growth was continued under air level CO_2 for 72 hours.

Isolation of total membranes and soluble proteins

Isolation of the membrane and soluble fractions was carried out as follows. *Synechocystis* cells were washed with 50 mM HEPES pH 7.5, and re-suspended in lysis buffer containing 50 mM HEPES pH 7.5, 800 mM sorbitol, and 1 mM ε-amino-*n*-caproic acid. Routinely, the isolation procedure was carried out in the presence of 30 mM $CaCl_2$ (Gombos et al. 1994). In other cases isolation procedure was performed in the presence of different concentrations (0 mM, 1 mM or 10 mM) of $CaCl_2$ or $MgCl_2$ in the lysis buffer. After resuspension, the cells were broken with glass beads (150-212 microns; Sigma) with six repeating cycles of 1 min vortexing and 1 min cooling in ice water bath. The unbroken cells and glass beads were removed (500 g, 5 min, 4ºC), and the cell extract was centrifuged (30000 g, 30 min, 4ºC) to separate the total membrane and soluble fractions. The supernatant and the pellet were stored in -80ºC until use. DNAse and RNAse treatment was carried out by breaking the cells in the presence of 25 mM $CaCl_2$, 5 mM $MgCl_2$ and 25 μg DNAse or RNAse. For $CaCl_2$ reconstitution experiment, cells were broken without divalent cations in the lysis buffer. To one fraction of the total extract, $CaCl_2$ was added to final concentration of 30 mM and incubated on ice for 30 min, whereas the other fraction without $CaCl_2$ was used as control. The reconstituted total extract as well as the control was centrifuged (30000 g, 30 min, 4°C) to separate the membrane and soluble fractions which were analyzed by SDS-PAGE.

Salt wash

100 µg of total membrane proteins were washed with 50 mM Bis-Tris pH 7.0 and suspended in 100 µL 2M NaBr in 50 mM Bis-Tris pH 7.0. The suspension was incubated on ice for 1 hour and then centrifuged at 30000 g for 1 hr at 4^{o}C. The pellet was resuspended in 25 µL 1 x Laemmli buffer. The supernatant was precipitated with 20% TCA (trichloro acetic acid) and centrifuged at 18000 g for 15 min at 4^{o}C. The pellet was washed with ice cold acetone and resuspended in 25 µL of 1 x Laemmli buffer. Equivalent amount of proteins was separated on SDS-PAGE and further analyzed by western blotting.

Detergent treatment

Crude membrane, corresponding to 100 µg of proteins was suspended in 100 µl of 50 mM Bis-Tris pH 7.0. The membrane proteins were solubilized separately with 1.5% of CHAPS {(3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate}, 1.5% DM {n-dodecyl-β-D-maltoside}, 1.5 % SDS {sodium dodecyl sulphate}, 1.5 % OG {n-octyl-β-D-glucopyranoside} and 1.5% Triton X-100 at RT for 1 h with brief mixing every 15 min. After solubilisation, suspensions were centrifuged at 18000 g for 20 min. Supernatants were collected and the pellets were dissolved in 20 µl of 50 mM Bis-Tris pH 7.0 buffer. Equivalent amount of proteins from soluble and insoluble fractions were separated by SDS-PAGE and immuno-detected with Slr0006 antibody.

SDS-PAGE and western blotting

The protein content of the membranes and soluble fractions was determined using Bio-Rad D_c protein assay kit. Mini-PROTEAN II electrophoresis cell (Bio-Rad) was used to separate proteins in 12.5% SDS-PAGE containing 6 M urea (Laemmli 1970). The proteins from the gels were electro-blotted onto PVDF membrane (Immobilon-P; Millipore) using standard procedures. Antibodies against ATP synthase β sununit (Agrisera, Sweden) and custom polyclonal antibodies against Slr0006 (Innovagen AB, Sweden), Flv3 (Antiprot, Germany), NdhD3, D1 and Sll0218 (Eurogentec, Sweden) were used in western blot analysis.

Results and Discussion

Slr0006 is a 23 kDa protein, which is strongly induced in *Synechocystis* under carbon limiting conditions (Battchikova et al. 2010; Carmel et al. 2010). The sequence analysis using PSORTb, Predict protein, SignalP, SOSUI and TMHMM showed neither the presence of transmembrane helices nor a signal peptide (data not shown), which indicates that Slr0006 is likely to be a soluble protein. As a routine localization procedure, we isolated the total membrane and soluble fractions of *Synechocystis* by breaking the cells in lysis buffer containing 30 mM $CaCl_2$ (Gombos et al. 1994). The following analysis of

the fractions by 1-D SDS-PAGE and western blotting revealed that the Slr0006 protein is predominantly located in the membrane fraction (Fig. 1a). Since a wide range of buffers varying especially in the concentration of divalent cations (either $CaCl_2$ or $MgCl_2$) has been used for isolation of various cyanobacterial targets (e.g. membranes and photosystems; see Table I), we tested the effect of divalent ions on the localization of the Slr0006 protein. It was shown that decreasing the concentration of divalent cations in the lysis buffer caused a drastic change in the location of the Slr0006 protein from membrane-bound fraction to the soluble part (Fig. 1b). Occasionally, traces of Slr0006 remained membrane bound even upon breakage of the cells in the absence of divalent cations (Fig. 1b). Location of the Slr0006 was not dependent on the procedure used, as breaking the cells with glass beads (Fig. 1a) or by French press (data not shown) gave identical results.

Notably, Slr0006 behaved similarly both in the presence of $CaCl_2$ and $MgCl_2$ demonstrating that the membrane binding property of Slr0006 is not specific to $CaCl_2$ or $MgCl_2$ but appeared to be a general response to the presence of divalent cations (Fig. 1a). In contrast to Slr0006, the location of the flavoprotein Flv3 in the soluble fraction (Zhang et al. 2009), the binding of the NdhD3 and Sll0218 proteins to the membrane (Zhang et al. 2004), as well as the even distribution of the ATP synthase between the membrane and soluble fractions, was independent of the presence of divalent ions in the isolation buffer (Fig. 1b). Importantly, our recent results show that the membrane binding of other *Synechocystis* proteins, such as Flv2 and Flv4, is also dependent on the level of divalent ions upon breakage of the cells (Zhang et al. unpublished results).

In order to verify whether the *in vitro* membrane attachment of the Slr0006 protein was dependent solely on the presence of divalent ions, the total *Synechocystis* cell extract, isolated in the absence of divalent ions and thus having the Slr0006 protein free in the soluble fraction, was reconstituted with 30 mM $CaCl_2$. Clearly, however, addition of the cations to the cell extract after breakage of the cells had only minor effect on the membrane binding of Slr0006 (Fig. 1c), indicating that the ionic environment during the cell breakage has a major impact on the localization of the Slr0006 protein. Further, the possibility of Slr0006 being trapped in the clustered mass of nucleic acid during the isolation procedure was excluded since breaking the cells in the presence of DNAse or RNAse did not influence the localization of Slr0006 (Fig. 1d).

In membranes, peripheral proteins are temporarily bound either to the lipid polar head groups or to integral proteins by hydrophobic, electrostatic, and other non-covalent interactions. Since these interactions can be displaced *via* treatment with high ionic strength buffer (Speers and Wu 2007), washing of the Slr0006-containing membranes with 2M NaBr was performed to verify the strength of the Slr0006-membrane interaction. Western blot analysis of the total membrane fraction isolated from cells broken in the presence of $CaCl_2$ showed that the high salt wash did not allow release of Slr0006 from the membranes (Fig. 2a). Nevertheless, the salt wash completely released the peripheral β subunit of the ATP synthase from the

membrane (Fig. 2a), indicating that the Slr0006 protein is tightly associated with the membranes by some unknown mechanism(s).

One of the most important steps in the study of a membrane protein is the choice of a detergent that is capable of solubilising the protein of interest without disruption of its natural state. Solubilisation of total membranes with a mild detergent supports the isolation of intact protein complexes which is a valuable tool in studying the membrane proteins. To investigate the possibility that Slr0006 interacts with any of the membrane protein complexes, the total membranes from wild type cells were isolated under standard lysis buffer containing 30 mM $CaCl_2$ and further solubilized with different non ionic, anionic and zwitterionic detergents. Figure 2b demonstrates that the protein was strongly bound to the membrane since 1.5% CHAPS, 1.5% OG and 1.5% TX-100 proved to be ineffective in solubilising Slr0006. SDS, however, being one of the strongest anionic detergents, not only solubilises most of the membranes but also releases Slr0006 to the soluble fraction. Also some solubilization of the Slr0006 protein could be detected by using 1.5% DM (Fig. 2b). This result further emphasizes the tight binding of the Slr0006 protein to the membranes, and hampers the attempts to find the putative binding partners by using blue native gel electrophoresis.

Our results clearly show that the location of the Slr0006 protein, and presumably many others, is dependent on the presence of divalent cations upon isolation of the soluble and membrane fractions. This is an important observation, which may help to avoid the misinterpretation of a soluble to a membrane protein during the proteomic analyses of cyanobacteria. It should be noted, however, that the high concentration of divalent cations frequently used in lysis buffers is out of the range of physiological intracellular concentrations, and that no ultimate conclusions about the *in vivo* location of the Slr0006 can be currently drawn. As the membrane stacking is well known to be dependent on divalent ions (Rumak et al. 2010 ; Riviere et al. 1990), we speculate that the conditions upon cell breakage might result in trapping of the Slr0006 between the membranes, thereby complicating the localization. Nevertheless, the ultimate mechanism behind this phenomenon remains to be resolved.

Acknowledgement

This study was financially supported by the Academy of Finland (Grants number: 118637 and 130075) and the grant from the Maj and Tor Nessling Foundation. MSc. Maija Holmström is thanked for technical assistance, and Drs. Sari Sirpiö and Saijaliisa Kangasjärvi for helpful discussions.

Figure legends

Fig. 1 Effect of divalent cations on localization of Slr0006 and other *Synechocystis* proteins a) Distribution of Slr0006 between the membrane (M) and soluble (S) fractions of *Synechocystis*. The proteins were isolated in the standard lysis buffer containing either 30 mM $CaCl_2$ or 30 mM $MgCl_2$ and 30µg of protein were analyzed by western blotting using Slr0006 antibody b) Distribution of various proteins between the membrane (M) and soluble (S) fraction of *Synechocystis* after lysis of the cells in the presence of 0 mM, 1 mM and 10 mM of $CaCl_2$ or $MgCl_2$. The fractions were analyzed by western blotting using antibodies against Slr0006, NdhD3, Sll0218, ATP synthase β subunit and Flv3 c) Effect of $CaCl_2$-dependent reconstitution on the distribution of Slr0006 between the membrane (M) and soluble (S) fractions of *Synechocystis*. The total cell extract lysed in the absence of divalent cations was supplemented with 30 mM $CaCl_2$ and incubated for 30 min in room temperature and the membrane and soluble fractions were analyzed by western blotting d) Effect of DNAse and RNAse on the localization of Slr0006. Cells were lysed in the presence of 25 mM $CaCl_2$, 5 mM $MgCl_2$, and 25µg of either DNAse or RNAse, as indicated. 30 µg of membrane (M) and soluble (S) protein were separated using PAGE and analyzed by western blotting

Fig. 2 Effect of salt and detergent on the binding of Slr0006 to the membrane a) NaBr treatment. 100 µg of *Synechocystis* total membrane proteins, isolated in the presence of divalent cations, were treated with 2M NaBr for 2-3 min on ice. The insoluble membrane proteins (pellet, P) and soluble peripheral protein (supernatant, S) were analyzed by western blotting b) Treatment of membranes with various detergents. Total membranes were isolated from *Synechocystis* cells in standard buffer containing 30 mM $CaCl_2$ and 100 µg membrane proteins were solubilized with following detergents: 1.5% CHAPS; 1.5% DM; 1.5% SDS; 1.5%OG; 1.5% TX-100 at room temperature for 30 min. The insoluble (pellet, P) and soluble fractions (supernatant, S) were analyzed by western blotting

References

Andersson B, Anderson JM (1980) Lateral heterogeneity in the distribution of chlorophyll-protein complexes of the thylakoid membranes of spinach chloroplasts Biochim Biophy Acta 593: 427-440.

Battchikova N, Vainonen JP, Vorontsova N, Keranen M, Carmel D, Aro EM (2010) Dynamic changes in the proteome of *Synechocystis* 6803 in response to CO_2 limitation revealed by quantitative proteomics J Prot Res 9: 5896-5912.

Carmel D, Battchikova N, Holmström M, Mulo P, Aro EM (2010) Knock-out of low CO_2-induced *slr0006* gene in *Synechocystis* sp. PCC 6803: Consequences on growth and proteome Proceedings of the 15th International Congress on Photosynthesis, Beijing, China (in press).

Dühring U, Irrgang KD, Lunser K, Kehr J, Wilde A (2006) Analysis of photosynthetic complexes from a cyanobacterial *ycf37* mutant Biochim Biophys Acta 1757: 3-11.

Gombos Z, Wada H, Murata N (1994) The recovery of photosynthesis from low-temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance PNAS 91: 8787-8791.

Hung CH, Huang JY, Chiu YF, Chu HA (2007) Site-directed mutagenesis on the heme axial-ligands of cytochrome b_{559} in photosystem II by using cyanobacteria *Synechocystis* PCC 6803 Biochim Biophys Acta 1767: 686-693.

Klinkert B, Ossenbühl F, Sikorski M, Berry S, Eichacker L, Nickelsen J (2004) PratA, a periplasmic tetratricopeptide repeat protein involved in biogenesis of photosystem II in *Synechocystis* sp. PCC 6803 J Biol Chem 279: 44639-44644.

Komenda J, Tichy M, Prasil O, Knoppova J, Kuvikova S, de Vries R, Nixon PJ (2007) The exposed N-terminal tail of the D1 subunit is required for rapid D1 degradation during photosystem II repair in *Synechocystis* sp PCC 6803 The Plant Cell 19: 2839-2854.

Kubota H, Sakurai I, Katayama K, Mizusawa N, Ohashi S, Kobayashi M, Zhang P, Aro EM, Wada H (2010) Purification and characterization of photosystem I complex from *Synechocystis* sp. PCC 6803 by expressing histidine-tagged subunits Biochim Biophys Acta 1797: 98-105.

Kwon J, Ohb J, Parka C, Chob K, Kimb SI, Kimb S, Leed S, Bhakd J, Norling B, Choib JS (2010) Systematic cyanobacterial membrane proteome analysis by combining acid hydrolysis and digestive enzymes with nano-liquid chromatography–Fourier transform mass spectrometry J Chrom 1217: 285–293.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. T4 Nature 227: 680-685.

Ma W, Mi H (2008) Effect of exogenous glucose on the expression and activity of NADPH dehydrogenase complexes in the cyanobacterium *Synechocystis* sp. strain PCC 6803 Plant Physiol Biochem PPB / Societe francaise de physiologie vegetale 46: 775-779.

Mann NH, Novac N, Mullineaux CW, Newman J, Bailey S, Robinson C (2000) Involvement of an FtsH homologue in the assembly of functional photosystem I in the cyanobacterium *Synechocystis* sp. PCC 6803 FEBS Lett 479: 72-77.

Norling B (2000) Purification of Cyanobacterial Thylakoid, Plasma, and Outer Membranes by Two-Phase Partitioning In: Hatti-Kaul R (ed) Aqueous Two-Phase Systems. Methods in Biotechnology Vol. 11 Humana Press Inc., Totowa, NJ pp. 185-192.

Omata T, Murata N (1984) Isolation and characterisation of three types of membranes from cyanobacterium (blue green algae) *Synechocystis* PCC 6714 Arch Microbiol 139: 113-116.

Paulsen IT, Sliwinski MK, Saier MH (1998) Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities J Mol Biol 277: 573-592.

Rippka R (1988) Isolation and purification of cyanobacteria Meth Enzymol 167: 3-27.

Riviere ME, Arrio B, Steffan I, Molitor V, Kuntner O, Peschek GA (1990). Changes of some physical properties of isolated and purified plasma and thylakoid membrane vesicles from the freshwater cyanobacterium *Synechococcus* 6301 (*Anacystis nidulans*) during adaptation to salinity Arch Biochem Biophys 280: 159-166.

Rumak I, Gieczewska K, Kierdaszuk B, Gruszecki WI, Mostowska A, Mazur R, Garstka M (2010) 3-D modelling of chloroplast structure under (Mg(2+)) magnesium ion treatment. Relationship between thylakoid membrane arrangement and stacking Biochim Biophys Acta 1797: 1736-1748.

Speers AE, Wu CC (2007) Proteomics of integral membrane proteins-theory and application Chem Rev 107: 3687-3714.

Staehelin LA (2003) Chloroplast structure: from chlorophyll granules to supra-molecular architecture of thylakoid membranes Photosynth Res 76: 185-196.

van Thor JJ, Jeanjean R, Havaux M, Sjollema KA, Joset F, Hellingwerf KJ, Matthijs HC (2000) Salt shock-inducible photosystem I cyclic electron transfer in *Synechocystis* PCC6803 relies on binding of ferredoxin:$NADP^+$ reductase to the thylakoid membranes via its CpcD phycobilisome-linker homologous N-terminal domain Biochim Biophys Acta 1457: 129-144.

Zhang P, Allahverdiyeva Y, Eisenhut M, Aro EM (2009) Flavodiiron proteins in oxygenic photosynthetic organisms: Photoprotection of Photosystem II by Flv2 and Flv4 in *Synechocystis* sp. PCC 6803 PLoS ONE 4: e5331.

Zhang P, Battchikova N, Jansen T, Appel J, Ogawa T, Aro EM (2004) Expression and functional roles of the two distinct NDH-1 complexes and the carbon acquisition complex NdhD3/NdhF3/CupA/Sll1735 in *Synechocystis* sp PCC 6803 Plant Cell 16: 3326-40.

Abstract

Slr0006 is one of the *Synechocystis* sp. PCC 6803 proteins strongly induced under carbon limiting conditions. Slr0006 has no predicted transmembrane helices or signal peptide sequence, yet it was exclusively recovered in the membrane fraction of *Synechocystis*, when the cells were broken in isolation buffers which contain divalent cations and are generally used for photosynthesis studies. Even subsequent washing of the membranes with high salt or various detergents did not release Slr0006, indicating strong binding of the Slr0006 protein to the membranes. Further, DNAse or RNAse treatment did not disturb the tight binding of Slr0006 protein to the membranes. Nevertheless, when the cells were broken in the absence of divalent cations, Slr0006 remained completely soluble. Binding of the Slr0006 to the membrane could not be properly reconstituted if the cations were added after breaking the cells in the absence of divalent ions. This unusual phenomenon has to be considered in identification and localization of other yet uncharacterized cyanobacterial proteins.

Table 1 Concentrations of divalent ions in the lysis buffer used in various studies of cyanobacteria

Concentration of divalent ions in lysis buffer	**Target studies**	**References**
50 mM $CaCl_2$	Photosystem II	(Mann et al. 2000)
30 mM $CaCl_2$	Membranes	(Gombos et al. 1994)
25 mM $CaCl_2$, 5 mM $MgCl_2$	Membranes	(Duhring et al. 2006)
10 mM $CaCl_2$, 10 mM $MgCl_2$	Photosystem II	(Komenda et al. 2007)
5 mM $CaCl_2$, 10 mM $MgCl_2$	Photosystem I	(Kubota et al. 2010)
5 mM $CaCl_2$, 5 mM $MgCl_2$	Cytochrome b_{559}	(Hung et al. 2007)
10 mM $MgCl_2$	Membranes	(Klinkert et al. 2004)
10 mM $MgCl_2$	NDH-I complex	(Ma and Mi 2008)
5 mM $MgCl_2$	Membranes	(van Thor et al. 2000)
No divalent ions	Membranes	(Norling 2000)
No divalent ions	Membranes	(Omata and Murata 1984)

Line figure

Click here to download high resolution image

Figure 1

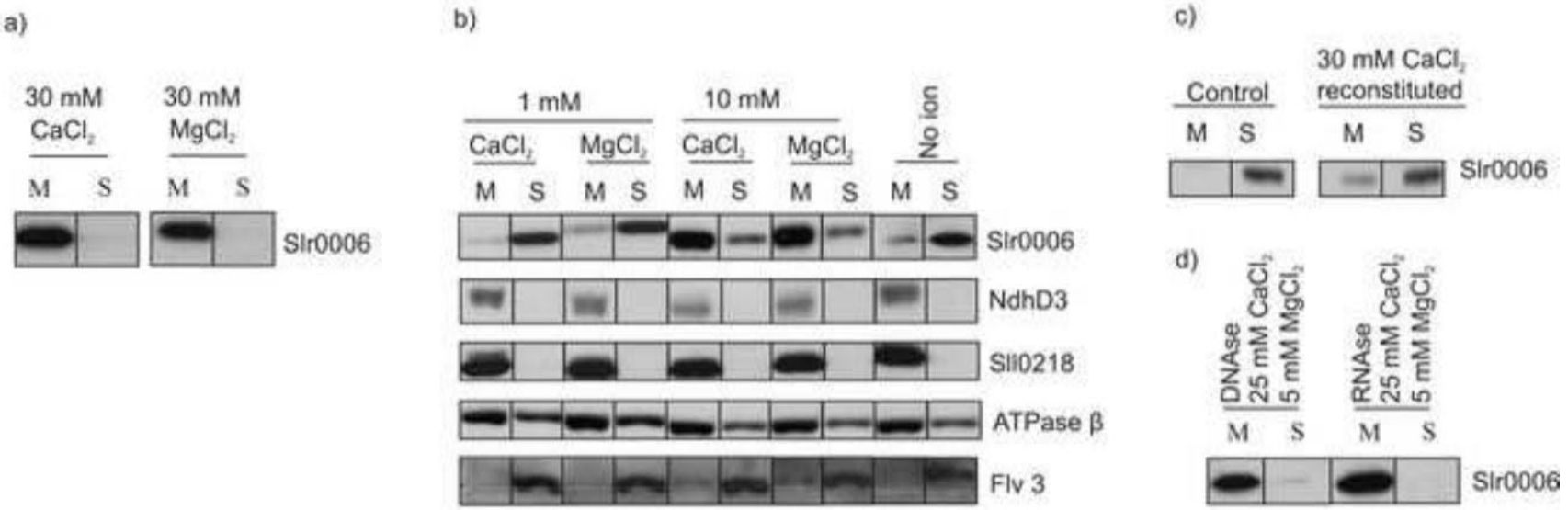

Figure 2