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Biochemical characterization of two thylakoid lumenal cyclophilins – TLP20 and TLP40

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Introduction

With the advancement in our understanding of the regulation and stress protection of the photosynthetic process, the thylakoid lumen has become increasingly more interesting. Recent studies demonstrated that the lumen contains a large number of proteins, which may be involved in the regulation of photosynthesis (Bricker et al., 2001; Kieselbach et al., 1998; Peltier et al., 2000). One of the proteins present in the thylakoid lumen is the cyclophilin-like protein TLP40 (Fulgosi et al., 1998) that was the first protein with PPIase activity identified in the thylakoid lumen and the first complex immunophilin detected in plants. PPIases are of high importance in all living cells since they catalyse the isomerisation of prolyl bonds during protein folding. Apart from their role in protein folding some PPIases are involved in a multitude of regulatory cellular processes including cell signalling, biogenesis and activities of several receptors (Schiene-Fischer and Yu, 2001). PPIases are divided into Cyclophilins or FK506/rapamycin binding proteins (FKBP) due to their sensitivity to the inhibitors Cyclosporin A (CsA) or FK506/rapamycin, respectively. In this study we have focused on proteins with PPIase activity to find out more about their function in the thylakoid lumen.

Materials and methods

Protein purification. Lumenal proteins were purified according to Kieselbach et al. (1998) with the following exception. The buffer during Yeda-press contained 50 mM MES, pH 6.0, 50 mM NaCl, 100 mM sucrose. TLP20 and TLP40 were separated on a Sepharose Q column (Amersham Pharmacia Biotech) equilibrated with 50 mM MES, pH 6.0, 50 mM NaCl. TLP40 was retained on the column while TLP20 was collected in the unbound material. The Sepharose Q column with the bound TLP40 was re-equilibrated with 20 mM Tricine, pH 7.8, (buffer A) and TLP40 was eluted with buffer A + 350 mM NaCl. This fraction was then diluted 3 times in buffer A and loaded onto a Resource Q column (Amersham Pharmacia Biotech). TLP40 was eluted with a 0-300 mM NaCl gradient in buffer A and further purified

on a Superose 75 column (Amersham Pharmacia Biotech). The unbound fraction with TLP20 was applied onto a Poros HS column (Applied Biosystems) equilibrated with 50 mM MES, pH 6.0, and eluted with a 0-1 M NaCl gradient.

Preparation of randomised inside-out vesicles (R-IO-vesicles). Inside-out particles with the same overall composition of thylakoid membrane components were made essentially according to Andersson (1986). To remove extrinsic proteins bound to the lumenal side, the vesicles were washed with 0.5 M NaCl in a 10 mM Na-phosphate buffer pH 6.5, 100 mM sucrose before they were stored at -80°C in aliquots of 10 µg chlorophyll in 10 mM Na-phosphate buffer, pH 5.5, 5mM NaCl, 100 mM sucrose.

Binding and activity assay. R-IO-vesicles containing 10 µg of chlorophyll were incubated with 0.5µg of TLP40 in 10 mM Na-phosphate buffer, 5 mM NaCl, 100 mM sucrose in the final volume of 75 µl. The pH during standard conditions was 5.5. Na-phosphate buffers with pH 5.0-8.0 were used to study pH-dependence. After 10 minutes incubation at 22°C the suspension was centrifuged at 100 000xg for 25 minutes. The pellet and supernatant were separated and the latter was dried in a SpeedVac Concentrator SVC100H (Savant). The pellet was washed once by resuspension in the same buffer and centrifuged as above. The dried supernatant and pellet were subjected to SDS-PAGE according to Laemmli (1970) and Western blotting with antibodies raised against TLP40. The bands were visualized and quantified using a LAS1000 CCD-camera (Fujifilm) with the Image Gauge V3.46 software.

The PPIase activity was measured essentially according to Fulgosi et al. (1998) using a coupled reaction involving chymotrypsin.

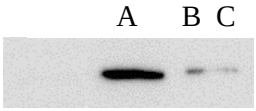

Fig. 1. Re-binding of TLP40 to R-IO-vesicles identified by immunoblotting. A) RIO-vesicles, B) the bound and C) the unbound fraction of TLP40 after addition to R-IO-vesicles.

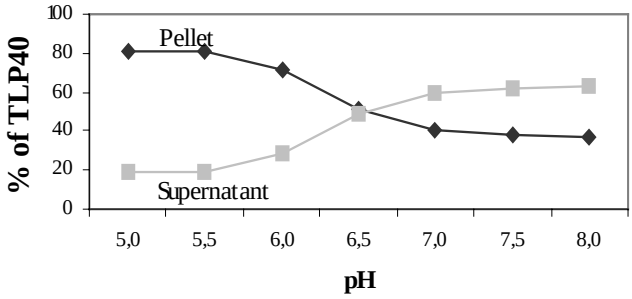

Fig. 2. pH-dependence of TLP40 binding to R-IO-vesicles. The amount of TLP40 present in pellet and supernatant, respectively, at different pH. The amount of bound TLP40 is decreased at higher pH values.

Results and discussion

To obtain more insights into the interactions between TLP40 and the thylakoid membrane we have developed a new assay, which allowed us to study the reversible binding of TLP40 with inside-out vesicles from thylakoid membranes under various conditions. We have used inside-out vesicles (IO) (Andersson, 1986), which have been made homogeneous with regard to the distribution of the membrane components (R-IO-vesicles). By using these vesicles and adding purified TLP40, for the first time, we have been able to demonstrate the rebinding of TLP40 to thylakoid membranes (Fig. 1). Under our conditions a majority of the added TLP40 binds to the membrane (~80%) and less than 5% is removed during the following wash of the R-IO-vesicles (data not shown). To investigate the influence of pH on the binding we preformed reassociation experiments at different pH values ranging from 5.0-8.0. Our experiments show the highest interaction at low pH (5.0-5.5) (Fig. 2) while the extent of binding is decreased at higher pH. These results together with the fact that TLP40 is released from R-IO-vesicles during high salt wash reveal an ionic type of interaction with the thylakoid membrane.

Recently, Rokka *et al*. (2000) have reported increased dissociation of TLP40 from the thylakoid membrane into the lumen during moderate heat treatment. Thus, we used the same temperatures, 22°C, 27°C, 35°C and 42°C, during incubation in our assay to perform the reconstitution studies. Our results show a decreased rebinding at higher temperatures (Fig. 3) suggesting a decreased affinity to the membrane at elevated temperatures. TLP40 is known to interact with a PP2A-type phosphatase (Fulgosi et al., 1998; Rokka et al., 2000; Vener et al., 1999) but it has also been reported to interact with the cytochrome b_6/f complex (Andersson et al., 1998). The fact that some TLP40 is still bound at high pH may suggest the presence of multiple binding sites, but more experiments have to be performed to elucidate the detailed properties and sites for these interactions.

During purification of TLP40 from spinach thylakoids we have identified an additional PPIase, which was separated from TLP40 during the first chromatographic step on a Sepharose Q column. Subsequent purification on a Poros HS column resulted in a highly enriched fraction containing four major proteins. In contrast to TLP40 the additional PPIase is of conventional size, ~20 kDa (hence named TLP20). The PPIase activity in the highly enriched fraction of TLP20 is higher than the activity of TLP40 (data not shown). The PPIase activity has also been tested in the presence of the immunosuppressive drugs Cyclosporin A (CsA) and FK506. It is inhibited at very low concentrations (nM) of CsA but no effect on the activity could be detected even at µM concentrations of FK605 (Fig. 4). These results demonstrate that TLP20 belongs to the cyclophilin family of PPIases and is substantially more susceptible to inhibition by CsA than TLP40 (Vener et al., 1999).

The N-terminal peptide of TLP20 sequenced by mass spectrometry has been used to search the *Arabidopsis thaliana* database and a homologous protein was found. This protein contains an N-terminal signal peptide, targeted to the chloroplast, followed by a cyclophilin-like PPIase-domain.

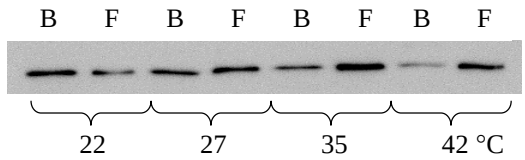

Fig. 3. Temperature dependence of TLP40 binding to R-IO-vesicles. Western blot shows bound (B) and free (F) TLP40 during elevated temperatures.

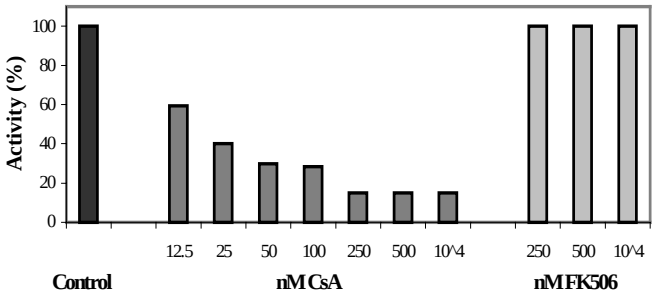

Fig. 4. Inhibition of TLP20 PPIase activity with Cyclosporin A (CsA) and FK506. The activity is inhibited at nM concentrations of CsA but is not affected with FK506.

The discovery of two cyclophilins, in addition to other lumenal PPIases predicted in the databases, emphases the importance of the thylakoid lumen as a photosynthetic regulatory compartment. The involvement of highly active conventional small PPIases in general catalysis of protein folding implies specific regulatory functions of complex immunophilins, such as TLP40. In the latter case the distinct protein-binding domains may direct the PPIase activity towards designated processes controlling maintenance and regulation of the photosynthetic process. In the case of TLP40 this is suggested to specifically relate to reversible thylakoid protein phosphorylation.

Acknowledgements

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