Studies on Cyclophilin-D and NAADP on Ca^{2+}-mediated events

PhD thesis

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Introduction:

Calcium is one of the most important and versatile signalling agent that determines a vide variety of intra- and intercellular processes. The patterns of changes in the concentration of calcium are tightly regulated by several extracellular factors, as well as various intracellular second messenger pathways. Compartmentalization and signal-induced release of calcium from intracellular organelles (e.g. endoplasmic/sarcoplasmic reticulum, lysosomes, mitochondria, Golgi membrane, nucleus) or the extracellular space forms the basis of calcium signal transduction. Nicotinic acid-adenine dinucleotide phosphate (NAADP) is rapidly emerging as an intracellular second messenger mediating calcium release from mainly the acidic calcium stores (e.g. lysosomes, and derivatives: microsomes). The NAADP-induced Ca^{2+}-mobilization is usually limited in space and time, acting as a trigger that may be propagated and generalized by Ca^{2+}-induced Ca^{2+}-release (CICR) via ryanodine receptors and inositol-1,4,5-trisphosphate ($InsP_3$) receptors (e.g. oocyte-fertilization) or serves as a local effector of e.g. insulin-secretion from pancreatic β-cells, or neurite outgrowth and neurotransmitter secretion.

Mitochondria play a more complex role in intracellular calcium homeostasis, acting as a ‘calcium sink’ and vice versa, changes of matrix calcium concentration determines several mitochondrial functions, expanding from regulation of key steps of the Szentgyörgyi-Krebs cycle to mitochondrial permeability transition (mPT). Reversible or irreversible permeability transition occurs in response to different intra- and extracellular signal transduction pathways or metabolic changes converging to enhanced ROS-production or highly elevated matrix calcium concentration or high free phosphate (P_i) concentration. Cyclophilin-D (CypD) is an important, calcium-sensitive regulator of the permeability transition pore’s opening, promoting permeability transition

Objectives:

#1 The primary aim of the present thesis was to clarify the modulatory role of cyclophilin-D in the Ca^{2+}-induced permeability transition and it's relation to the bioenergetic state of mitochondria. We first verified that substrate-starved mitochondria are indeed more susceptible to Ca^{2+}-induced permeability transition and showed that genetic deletion of CypD provides similar protection to that of cyclosporin-A in wild-type, substrate-starved mitochondria. Also, we performed parallel recordings of swelling and Ca^{2+} uptake into mitochondria in the presence of Ru360 or an uncoupler. These results led to the question whether high Ca^{2+} acts on the surface of mitochondria or is entering mitochondria by a RuRed/Ru360-independent pathway. To shed light among these possibilities, we challenged *in situ* mitochondria with Ru360 or Ruthenium Red, and recorded Ca^{2+} uptake capabilities. Next, we clarified the contribution of the different complexes of the electron transport chain to Ca^{2+}-induced swelling by using inhibitors of Complex I, III and IV. Finally, we demonstrated the diverse effect of the ablation of cyclophilin-D on the swelling of *in situ* mitochondria of neurons and astrocytes. Moreover, we showed that the deletion of cyclophilin-D not only delayed initial swelling of neuronal mitochondria induced by excitotoxicity (glutamate-glycine), but it also renders neurons less susceptible to delayed calcium deregulation.

#2 On the other hand, in regard to cytosolic Ca^{2+}-signalization, the main objective of our work was to establish NAADP as a distinct pathway for Ca^{2+} release in hepatocyte microsomes. Firstly, we defined the actively loaded microsomal Ca^{2+}store to be thapsigargin-, but not bafilomycin A1-sensitive. In the next step, we verified that NAADP was indeed active in mobilizing Ca^{2+} from rat liver microsomes and we compared the Ca^{2+} releasing potential of NAADP to those of $InsP_3$ and cADPR. Also, we demonstrated the lack of cross-desensitization between NAADP, cADPR and $InsP_3$ as an evidence for separate receptors for each Ca^{2+}-mobilizing second messengers. Focusing on the properties of the NAADP-dependent Ca^{2+} release, we provided an NAADP

dose-dependence curve, as well as pH- and pCa-dependence curves for the NAADP-induced Ca^{2+} efflux from liver microsomes. We verified for the first time in vertebrate tissues the unique inactivation pattern of the NAADP-mediated Ca^{2+} release, where 'per se' non-Ca^{2+}-mobilizing doses of NAADP are able to abolish the effect of a saturating quantum of NAADP. Furthermore, we incubated pre-loaded microsomes with thapsigargin and bafilomycin A1 to support the notion that the one-pool model is applicable for the Ca^{2+} release from the microsomal Ca^{2+} stores. Finally, by demonstrating the pharmacological properties of the NAADP-induced Ca^{2+} efflux, we provided strong evidence for NAADP being an independent mediator from $InsP_3$ and cADPR in liver microsomes.

Methods:

Isolation of brain mitochondria from WT and cypD KO mice - C57Bl/6

WT and KO for cyclophilin-D littermate mice were a kind gift from Drs. Nika Danial and Anna Schinzel, from Howard Hughes Medical Institute and Dana-Farber Cancer Institute, Harvard Medical School. Mice were cross-bread for 8 generations prior to harvesting brain tissues from WT and KO age-matched animals for the purpose of mitochondrial isolation and culturing of neurons and astrocytes. Non-synaptic brain mitochondria from adult male WT and KO for CypD mice (aged 87-115 days) were isolated on a Percoll gradient as described previously (Sims et al., 1990) with minor modifications detailed in (Chinopoulos C. et al., 2003). All animal procedures were carried out according to the local animal care and use committee (Egyetemi Állatkíserleti Bizottság) guidelines.

Preparation of rat liver microsomes

Liver microsomes were prepared as described previously (Fleishner and Kraus-Friedmann, 1986). Briefly, Sprague-Dawley rat liver was homogenised in an ice-cold medium of 0.32 M sucrose, 20 mM MOPS-buffer (pH 7.2), 0.5 mM EGTA also containing 1 mM dithiothreitole (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitors and centrifuged at 2000x*g* for 15 minutes at 4 °C. The supernatant was centrifuged at

15,000x*g* for 45 minutes, and the resulting supernatant was collected and further centrifuged at 100,000x*g* for 90 minutes. Finally the pellet was resuspended in a solution containing 0.32 M sucrose, 20 mM MOPS (pH=7.2), 1 mM DTT and 0.2 mM PMSF. Protein concentration was set for ~20 mg/ml which was measured by the Lowry assay using bovine serum albumine as standard. The samples were frozen in liquid nitrogen and stored at -80 °C until required.

Mitochondrial membrane potential ($\Delta\Psi_m$) determination

$\Delta\Psi_m$ was estimated using fluorescence quenching of the cationic dye safranine O, because of its accumulation inside energized mitochondria (Akerman et al., 1976). Mitochondria (1 mg) were added to 2 ml of an incubation medium containing 120 mM KCl, 20 mM Hepes (acid), 10 mM potassium phosphate, 1 mM $MgCl_2$, 0.005 mM EGTA, 5 mM potassium glutamate, 5 mM potassium malate, 0.001 mM cyclosporine A, 0.05 mM AP_5A, 0.5 mg/ml BSA and 5 µM safranine O (pH 6.8 or pH 7.8). Fluorescence was recorded in a Hitachi F-4500 spectrofluorimeter (Hitachi High Technologies, Maidenhead, UK) at a 5 Hz acquisition rate, using 495 and 585 nm excitation and emission wavelengths, respectively. Experiments were performed at 37 °C. To convert safranine O fluorescence into millivolts (mV), a voltage–fluorescence calibration curve was constructed. To this end, safranine O fluorescence was recorded in the presence of 2 nM valinomycin and stepwise increasing $[K^+]$ (in the 0.2–120 mM range), which allowed calculation of $\Delta\Psi_m$ by the Nernst equation, assuming a matrix $[K^+]$ of 120 mM (Akerman et al., 1976).

Ca^{2+} uptake of isolated mitochondria

Mitochondrial-dependent removal of medium Ca^{2+} was followed using the impermeant hexapotassium salt of the fluorescent dye Calcium Green 5N (CaGreen) (Molecular Probes, Portland, OR, USA). CaGr (500 nM) was added to a 2 ml medium containing mitochondria (0.125 mg/ml) and 120 mM KCl, 10 mM Tris, 5 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.6. Substrates were added where indicated. All experiments were performed at 37 °C. Fluorescence

intensity was measured in a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) using 517 nm excitation and 535 nm emission wavelengths.

Measurement of mitochondrial swelling

Swelling of isolated mitochondria was assessed by measuring light scatter at 520 nm in a GBC UV/VIS 920 spectrophotometer. Mitochondria were added at a final concentration of 0.125 mg/ml to 2 ml of medium containing 120 mM KCl, 10 mM Tris, 5 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.6. Substrates were added where indicated. At the end of each experiment, the non-selective pore-forming peptide alamethicin (40 µg) was added as a calibration standard to cause maximal swelling. All experiments were performed at 37 °C.

Active loading of microsomes with Ca^{2+} and Ca^{2+} release assay

Ca^{2+} uptake and release were measured using $^{45}Ca^{2+}$ isotope to detect Ca^{2+} movements. The microsomes were diluted in a solution of 150 mM KCl, 20 mM MOPS (pH 7.2), 0.5 mM $MgCl_2$, 10 µM Ca^{2+}. In each experiment, 20-40 nCi $^{45}CaCl_2$ was used per assay point. The Ca^{2+} uptake was started by injecting 1 mM of ATP in the solution at room temperature. Ca^{2+} release was preformed by adding 100 µM EGTA in the presence or absence of the Ca^{2+} releasing agents (10 µM $InsP_3$, 10 µM cADPR and 10 µM NAADP). The $^{45}Ca^{2+}$ remaining in the vesicles was determined by filtration of 0.5 ml microsomes through a nitrocellulose Millipore filter (HAWP, 0.45 µm pore size) under vacuum. The filters were washed with 5 ml of quench solution (150 mM KCl, 20 mM MOPS (pH 7.2), 10 mM $MgCl_2$ and 1 mM $LaCl_3$) to lower the rate of unspecifically bound radioactivity. The radioactivity retained on the filter was measured by standard scintillation counting.

Passive loading of microsomes and Ca^{2+} release

Liver microsomes were passively loaded with 5mM $^{45}CaCl_2$ (20-40 nCi per assay point) by incubation for at least 5 hours in an ice-cold medium containing 150 mM KCl, 20 mM MOPS (pH 7.2), $^{45}Ca^{2+}$ and 5 mM Ca^{2+}. Passive loaded vesicles were diluted 10-fold into a Ca^{2+} releasing medium containing 150 mM KCl, 20 mM MOPS (pH 7.2) and 500 µM of EGTA to

adjust pCa to 6 at room temperature and Ca^{2+} releasing agonists. The Ca^{2+} release was stopped by 5-fold dilution with the same quench solution described above, then the samples were filtrated through Millipore filters and washed by 5 ml of quench solution. The retained radioactivity was measured by standard scintillation counting.

Results and discusion:

#1 *Complex contribution of Cyclophilin-D in brain-specific mitochondrial permeability transition induced by* Ca^{2+}

Firstly, we challenged isolated brain mitochondria by $CaCl_2$ in the presence and absence of glutamate and malate, and recorded light scattering spectrophotometrically at 520 nm. We used a three-pulse $CaCl_2$ protocol for this, and all subsequent similar experiments: 20 µM $CaCl_2$ was given at 100 sec, followed by 200 µM $CaCl_2$ at 300 sec and again at 500 sec. The addition of 20 µM $CaCl_2$ to substrate-supplemented or substrate-starved brain mitochondria of wild type mice did not cause a decrease in light scatter; instead, a cessation in the baseline decrease in light scatter was observed. However, the subsequent 200 µM $CaCl_2$ pulse induced a large decrease in light scatter in substrate-starved, but not substrate-supplemented mitochondria. The next 200 µM $CaCl_2$ pulse given at 500 sec did not induce any further changes in light scatter for the substrate-starved mitochondria, but it caused a minor change in substrate-supplemented mitochondria. The effect of the first 200 µM $CaCl_2$ pulse was cyclosporin A-sensitive, however, the second addition of 200 µM $CaCl_2$ overrode the protective effect of Cys A. Subsequent experiments benefited from the availability of cyclophilin-D knock-out mice. Our results obtained from substrate-starved mitochondria from CypD-KO mice were strikingly similar to those obtained from CysA-treated WT mice. The presence of substrates, however, did not provide additional protection in the CypD-KO mitochondria. These results show that high-Ca^{2+} loads can induce PTP in the absence of substrates. In our hands, absence of substrates prevented isolated mitochondria from building a

membrane potential of higher than -10 mV, a condition where mitochondrial Ca^{2+} uptake is unfavorable. Indeed, recordings of extramitochondrial Ca^{2+} by Calcium Geeen 5N revealed that in the absence of substrates, mitochondria were unable to perform Ca^{2+} sequestration, yet exhibited large changes in light scatter. Electron microscopy imaging of mitochondria that exhibited large changes in light scatter confirmed that this was due to swelling.

To address the site of action of Ca^{2+} on the light scatter, we pre-treated mitochondria with the Ca^{2+} uniporter inhibitor, Ru360 at a concentration that was found to prevent the uptake of extramitochondrial Ca^{2+} and showed that WT mitochondria still exhibited high-Ca^{2+}-induced changes in light scatter in the presence of Ru360. The lack of effect of Ru360 was also observed in the presence of Cys A or when the effect of Ca^{2+} was compared in WT versus CypD-KO mitochondria. The presence of the uncoupler completely depolarizing mitochondria failed to afford extra protection against high-Ca^{2+} induced swelling. Furthermore, the presence of the uncoupler negated the protective effects of substrates in WT mitochondria. The failure of Ru360 to protect against the Ca^{2+}-induced large changes in light scatter could be explained by assuming that Ca^{2+} acted on the extramitochondrial side. Surprisingly, isolated mitochondria loaded with Fura 2 and imaged under wide-field epifluorescence microscopy showed robust increases in matrix-entrapped Fura 2 fluorescence when perfused with a buffer containing 0.1 mM $CaCl_2$. This effect exhibited only a partial sensitivity to Ru360 (10 µM) and ruthenium red (10 µM), arguing against the assumption that Ca^{2+} was acting exclusively on an extramitochondrial site when inducing changes in light scatter.

Next, we pretreated mitochondria with complex I (rotenone or piericidin A), complex III (myxothiazol or stigmatellin), and complex IV (KCN) inhibitors to address the contribution of respiratory chain components to the protective effect of substrates on Ca^{2+}-induced changes in light scatter. The emerging picture based on our results is that rotenone or piericidin A afforded protection from Ca^{2+}-induced changes in light scatter, irrespective of the presence or abscence of substrates, while myxothiazol, stigmatellin and KCN not only falied to confer protection, they also negated the protective effect of substrates. High concentrations of myxothiazol and stigmatellin (10

μM and 2 μM, respectively) that also block complex I binding to a different site, failed to afford protection, as opposed to rotenone and piericidin A.

To address the role of CypD in the opening of brain-specific PTP *in situ*, swelling of CypD-deficient versus wild type mitochondria within neurons or astrocytes of the same culture were compared during Ca^{2+} overload, induced by addition of calcimycin (1 μM 4Br-A23187) and visualized by wide-field epifluorescence imaging of mitochondrially targeted DsRed2. Neurons and astrocytes were distinguished by their different mitochondrial morphology and mitochondrial swelling was monitored by calculation of changes in mean mitochondrial diameters using the thinness ratio technique. The onset of swelling was defined by the sudden decrease in the forementioned thinness ratio. Both WT and cypD-KO mitochondria within neurons swelled and fragmented in response to Ca^{2+} overload induced by the addition of calcimycin within 600-800 sec. When NaCN was co-applied with calcimycin in a glucose-free media in the presence of 2 mM 2-deoxyglucose, swelling of mitochondria was almost immediate in both wild type and CypD-KO neurons. However, Ca^{2+} overload of uncoupled mitochondria (by co-application of 1 μM SF 6847) triggered swelling at a significantly earlier time in CypD-KO than in wild type neurons. In contrast to the neurons, Ca^{2+} overload of uncoupled mitochondria triggered swelling at a significantly earlier time in wild type than in CypD-KO astrocytes.

Finally, in order to expose neuronal in situ mitochondria to high-Ca^{2+} challenge by an alternative mechanism, we exposed neurons to excitotoxic levels of glutamate and glycine, in the absence of Mg^{2+}. Glutamate exposure triggered a biphasic mitochondrial swelling response comprised of an initial, 1st and a well separated, delayed 2nd drop. The 1st drop of thinness ratio invariably coincided with the initial $[Ca^{2+}]_i$-response to glutamate and the 2nd drop to the secondary, irreversible rise of $[Ca^{2+}]_i$, termed delayed calcium deregulation, DCD. In cultures prepared from CypD-KO mice the 1st phase of mitochondrial swelling was detected only in 60% of the neurons and only 40 % of CypD-KO neurons exhibited the secondary swelling of mitochondria during DCD. In addition, the initial swelling of mitochondria was significantly delayed in CypD-KO neurons compared to wild type, while the

time of onset of the secondary mitochondrial swelling was not statistically different.

#2 *Ca^{2+} release triggered by NAADP in hepatocyte microsomes*

Hepatic microsomal vesicles rapidly sequestered $^{45}Ca^{2+}$ in the presence of ATP, with an uptake of 4.0 ± 0.2 nmol/mg protein (n=13). The maximum of Ca^{2+} uptake was found within 5-10 and about 90% of the specifically retained microsomal Ca^{2+} was rapidly released by ionomycin (5 µM). The Ca^{2+} accumulation of microsomes was nearly abolished by 1 µM thapsigargin (a selective inhibitor of SERCA), while 1 µM bafilomycin A1 (an established blocker of the V-type ATP-ase) does not affect substantially the Ca^{2+} uptake mechanisms of liver microsomes. In the light of these results, it is the SERCA that represents the main mechanism responsible for the active loading of liver microsomes.

NAADP (10 µM), $InsP_3$ (10 µM) and cADPR (10 µM) induced a fast Ca^{2+} efflux, which differed significantly from control microsomes (CICR). After 5 seconds of Ca^{2+} release, the total amount of Ca^{2+} efflux elicited by CICR was 0.165 ± 0.06 nmol/mg protein, $InsP_3$ released 0.7 ± 0.09 nmol Ca^{2+}/mg protein, while cADPR elicited 0.821 ± 0.1 nmol Ca^{2+}/mg protein. Under the same conditions, NAADP released 0.42 ± 0.08 nmol Ca^{2+}/mg protein. Thus NAADP is a potent, but somewhat less effective Ca^{2+} releasing messenger than cADPR and $InsP_3$ in liver hepatocyte microsomes. NAADP induced Ca^{2+} release in rat liver microsomes in a dose-dependent manner, with a half-maximal concentration (EC_{50}) of 0.93 ± 0.1 µM. Next, we tested subsequent Ca^{2+} release from actively loaded liver microsomes by cADPR, $InsP_3$ and NAADP in the presence of an ATP-regenerating system. NAADP managed to elicit maximal Ca^{2+} efflux when applied after cADPR and $InsP_3$ have already been probed. Thus cross-desensitization to $InsP_3$ and cADPR by NAADP did not occur. This result further supports the view that NAADP acts upon a Ca^{2+} release mechanism distinct from that of $InsP_3$ and cADPR from rat liver microsomes. When tested for self-desensitization, first injection of a subthreshold concentration of NAADP (0.1 µM) to microsomes at the third

minute during active loading did not result in substantial Ca^{2+} release by itself, however after 2 minutes of incubation, 10 µM NAADP released only 0.14 ± 0.04 nmol Ca^{2+}/mg protein compared to 0.39 ± 0.04 nmol/mg protein Ca^{2+} released from non pre-incubated microsomes. Thus, NAADP may function as its own specific antagonist with an IC_{50} of 30 nM. The curves of dose dependency and residual Ca^{2+} efflux after pre-incubation with varying concentrations of NAADP form a U-shape as NAADP desensitize its receptors with an IC_{50} that is one order of magnitude lower than the EC_{50}. When actively loaded microsomes were incubated for at least 5 minutes with bafilomycin A1 (1 µM), we found that both NAADP (10 µM) and cADPR (10 µM) elicited an entirely normal Ca^{2+} release response. On the other hand, pre-treatment with thapsigargin (1 µM) reduced the amount of Ca^{2+} efflux elicited by NAADP, similarly to that of cADPR. Based on our results the Ca^{2+} release from microsomes can be described with a one-pool model, being a mixture of Ca^{2+} stores deriving from both lysosomes and the ER, filled mainly by SERCA and containing receptors for $InsP_3$, cADPR and NAADP.

We show that the pCa response curves of the $InsP_3$ and cADPR appeared to be bell-shaped with an optimal pCa at 7 and 6 respectively, however, the NAADP-induced Ca^{2+} release was fairly independent of the extravesicular Ca^{2+} concentration. Also, we found that the NAADP-induced Ca^{2+} release in hepatocyte microsomes was not affected by changing the pH of the incubation buffer from 6.4 to 7.8, however, the response to cADPR was mostly dependent on pH, showing an optimal pH of 7.2. NAADP- and cADPR-triggered Ca^{2+} release from liver microsomes was differentially affected by pH, providing further evidence that these agonists signal through functionally distinct pathways. Finally, heparin (100 µg/ml, a well-established inhibitor of the $InsP_3$R-s) inhibited the Ca^{2+} release elicited by $InsP_3$ and did not alter the effect of cADPR and NAADP. The RyR antagonists (ryanodine [5 µM] and ruthenium red [5 µM]), blocked the cADPR-induced Ca^{2+} efflux, leaving that of $InsP_3$ and NAADP unaltered. The L-type Ca^{2+} receptor blockers, verapamil (100 µM) and diltiazem (100 µM) abolished specifically, but only partially the Ca^{2+} releasing effect of NAADP having minimal effect on the Ca^{2+} release by $InsP_3$ and cADPR. To sum up, neither heparin, nor ryanodine and ruthenium red were able to block substantially the

NAADP-induced Ca^{2+} release, while verapamil and diltiazem were effective inhibitors of NAADP receptors strengthening the notion that the NAADP mediated Ca^{2+} release in hepatocyte microsomes is indeed distinct and independent from that of $InsP_3$ and cADPR.

List of publications:

Related to the present thesis

Mándi M., Tóth B., Timár Gy. and Bak J. (2006): Ca^{2+} release triggered by NAADP in hepatocyte microsomes.
Biochem. J. **395**: 233-238.
[IF: 4.100 (2006)]

Mándi M. & Bak J. (2008): Nicotinic acid adenine nucleotide dipohosphate (NAADP) and Ca^{2+} mobilization.
J. Recept. Signal. Transduct. Res. **23 (3)**, 163-184.
[IF: 1.540 (2008)]

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Biophys.J. **96**, 2490-2504.
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[IF: 3.139 (2009)]

Chinopoulos, C., Gerencsér, AA., Mándi, M., Máthé, K., Törőcsik, B., Dóczi, J., Turiák, L., Kiss, G., Konrád, Cs., Vajda, Sz., Vereczki, V., Oh, RJ. and Ádám-Vizi, V. (2010): Forward operation of adenine nucleotide translocase during F0F1-ATPase reversal: critical role of matrix substrate-level phosphorylation. FASEB J. **24 (7)**, 2405-2416.
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Dóczi, J., Turiák, L., Vajda Sz., Mándi, M., Törőcsik, B., Gerencsér, A.A., Kiss, G., Konrád, Cs., Ádám-Vizi, V., and Chinopoulos, C. (2011): Complex contribution of Cyclophilin-D to Ca^{2+}-induced permeability transition in brain mitochondria, with relation to the bioenergetic state.
J. Biol. Chem. **286 (8)**, 6345-6353.
[IF: 5.52 (2009)]

Not related to the present thesis

Konrád, Cs.; Kiss, G.; Törőcsik, B.; Lábár, J.; Gerencsér, A.A.; Mándi, M.; Ádám-Vizi, V., and Chinopoulos, C. (2011): A distinct sequence in the adenine nucleotide translocase from Artemia franciscana embryos is associated with insensitivity to bongkrekate and atypical effects of adenine nucleotides on Ca^{2+} uptake and sequestration. FEBS J. **278 (5)**, 822-836.
[IF: 3.139 (2009)]