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REVIEW

Carbonic anhydrase subunits of the mitochondrial NADH dehydrogenase complex (complex I) in plants

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The mitochondrial nicotinamide adenine dinucleotide, reduced (NADH) dehydrogenase complex (complex I) of plants has a molecular mass of about 1000 kDa and is composed of more than 40 distinct protein subunits. About three quarter of these subunits are homologous to complex I subunits of heterotrophic eukaryotes, whereas the remaining subunits are unique to plants. Among them are three to five structurally related proteins that resemble an archaebacterial γ-type carbonic anhydrase (γCA). The γCA subunits are attached to the membrane arm of complex I on the matrix-exposed side and form an extra spherical domain. At the same time, they span the inner mitochondrial membrane and are essential for assembly of the protein complex. Expression of the genes encoding γCA subunits is reduced if plants are cultivated in the presence of elevated CO_2 concentration. The functional role of these subunits within plant mitochondria is currently unknown but might be related to photorespiration. We propose that the complex I–integrated γCAs are involved in mitochondrial HCO_3^- formation to allow efficient recycling of inorganic carbon for CO_2 fixation in chloroplasts under high light conditions.

The respiratory chain of plant mitochondria

Mitochondrial respiration is based on oxidoreductases that transfer electrons from reducing equivalents (NADH and flavin adenine dinucleotide, reduced ($FADH_2$) to molecular oxygen. In most eukaryotes, four multisubunit complexes are involved in respiratory electron transport, the NADH dehydrogenase complex (complex I), succinate dehydrogenase (complex II), cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV). Additional so-called 'alternative' oxidoreductases occur in some groups of organisms, especially in plants (Møller 2002, Rasmusson et al. 2004, Siedow and Umbach 1995). As a consequence, respiratory electron transport is branched. However, also the 'classical' oxidoreductase complexes of the respiratory chain are quite special in plants because they include several plant-specific subunits. Some of these proteins introduce side activities into the protein complexes of the respiratory chain. For example, the two subunits of the mitochondrial processing peptidase form an integral part of complex III in plants (Braun et al. 1992, 1995, Eriksson et al. 1994). Complex I of plants contains about 10 plant-specific subunits (Cardol et al. 2004, Heazlewood et al. 2003). In fact, the overall molecular mass of plant complex I is clearly larger than the one of mammalian complex I as revealed by direct comparison of these complexes by

Abbreviations – BCTs, bicarbonate transporters; CAL, carbonic anhydrase like; CAM, γCA of *Methanosarcina thermophila*; ccm, CO_2 concentration mechanisms; C_i, inorganic carbon; γCA, γ-type carbonic anhydrase; EC, Enzyme Commission; EM, electron microscope; $FADH_2$, flavin adenine dinucleotide, reduced; LβH, left-handed-β-parallel; NADH, nicotinamide adenine dinucleotide, reduced; NDH-1, NAD(P)H dehydrogenase type 1; PAGE, polyacrylamide gel electrophoresis; RubisCO, D ribulose 1,5 diphosphate carboxylase-oxygenase; SDS, sodium dodecye sulphate; TCA, tricarboxylic acid; T-DNA, DNA fragment derived.

one-dimensional Blue-native polyacrylamide gel electrophoresis (Jänsch et al. 1995). Three to five of the plant-specific complex I subunits have molecular masses of about 30 kDa and resemble γ-type carbonic anhydrases (γCAs) (Perales et al. 2004). A 68-kDa subunit represents l-galactono-1,4-lactone dehydrogenase, which catalyses the terminal step of mitochondrial ascorbic acid biosynthesis (Millar et al. 2003). However, this protein only forms part of a smaller version of complex I of unknown function (H.-P. Braun, unpublished results). Also respiratory complexes II and IV include some plant-specific subunits of unknown function, which most likely introduce side activities into these oxidoreductases (Eubel et al. 2003, Millar et al. 2004). This review summarizes recent results on the CA subunits of complex I from plants. A hypothesis on the function of these subunits is presented.

Carbonic anhydrases

CAs (Enzyme Commission (EC) 4.2.1.1) are zinc-containing metalloenzymes that catalyse the interconversion of CO_2 and HCO_3^-. The first enzyme was discovered in human erythrocytes (Meldrum and Roughton 1933) but meanwhile corresponding activities have been described in many organisms, including animals, plants, eubacteria and archaebacteria (Hewett-Emmett and Tashian 1996). CAs play important roles in many physiological processes linked with decarboxylation or carboxylation reactions, e.g. during photosynthesis and respiration. They also participate in transport of inorganic carbon (C_i) to actively photosynthesising cells or away from actively respiring cells. CAs probably evolved as enzymes facilitating *trans*-membrane CO_2 transport and took on a secondary metabolic role later in metazoan evolution (Henry 1996). CAs are encoded by at least five distinct, evolutionarily unrelated gene families. Correspondingly, these enzymes are assigned to four classes designated α, β (two different subclasses), γ and δ (Sawaya et al. 2006, So et al. 2004). For this review we mainly focus on γCA proteins.

So far, only one representative of the γCA family has been physiologically and biochemically characterized, the γCA of the archaeon *Methanosarcina thermophila* ('CAM'; Alber and Ferry 1994). CAM is a homotrimer composed of proteins in left-handed-β-helical-fold conformation. Although it is assumed that CAM binds zinc like all other classes of CAs, iron-substituted forms of the enzyme exhibit the highest CO_2 hydration rates. It thus is possible that CAM binds a different cofactor instead of zinc (Tripp et al. 2004). High-resolution crystal structures with bicarbonate bound to the active site of CAM have allowed to predict the residues directly involved in catalysis (Iverson et al. 2000), which meanwhile were confirmed by site-directed mutagenesis (Tripp and Ferry 2000, Tripp et al. 2002). Three His residues (His 81, 117 and 122) are essential to coordinate the metal ion. CO_2 binds adjacent to the zinc-bound hydroxyl group at a position, which still is not precisely clear. Solvent-accessible Gln-75, which orients the zinc-bound hydroxide for attack on CO_2, is important for CO_2 hydration activity. Also Glu-62 is important for the CO_2 hydration step, although the specific function is unknown. Glu-84 functions as a proton shuttle residue. Arg-59 is important for the assembly of monomers into the native trimer. It also is essential for the CO_2 hydration step and is postulated to bind bicarbonate. Arg 59 is indirectly hydrogen bonded to the active site zinc through a network that includes Asp 61, Asp76, His 81 and His 117 (Iverson et al. 2000, Tripp et al. 2002).

There are many open-reading frames in archaea, bacteria and cyanobacteria whose sequences are significantly similar to that of CAM. Most residues important for catalysis are well conserved in these homologues (the three His coordinating a metal ion, Arg 59, Asp 76 and Gln 75). However, in some cases the active site residues Glu 62 and Glu 84 are not conserved (Iverson et al. 2000, Tripp et al. 2002). To date, none of these homologous proteins has been shown to actually exhibit CA activity.

Gamma CA subunits of complex I in plants

Discovery of the γCA subunits

Complex I has been characterized for several plants by chromatographic or electrophoretic procedures (Combettes and Grienenberger 1999, Herz et al. 1994, Jänsch et al. 1996, Leterme and Boutry 1993, Rasmusson et al. 1994, Trost et al. 1995). The subunit composition of the purified complex was investigated by sodium dodecye sulphate (SDS)-PAGE in combination with direct protein sequencing by cyclic Edman degradation. Some of the obtained N-terminal sequences showed significant similarities to complex I subunits of *Neurospora* or beef but several others could not be assigned (Herz et al. 1994, Leterme and Boutry 1993). One example is the 29-kDa subunit of complex I from potato and a corresponding 30-kDa subunit of complex I from bean.

Recently, systematic proteome analyses uncovered several previously unknown mitochondrial proteins in *Arabidopsis* (Kruft et al. 2001, Millar et al. 2001). Among them, two proteins are homologous to the 29-/30-kDa subunit of complex I from bean and potato (termed 'similar to unknown protein from *Rickettsia prowazekii* [gene RP516]' in Kruft et al. 2001). Later, proteome analyses based on Blue-native/SDS-PAGE revealed that these proteins form part of complex I in *Arabidopsis*, rice and *Chlamydomonas* (Cardol et al. 2004, Heazlewood

et al. 2003, Perales et al. 2004, Sunderhaus et al. 2006). *Arabidopsis* complex I was shown to include five structurally related subunits of this type, complex I of rice at least two and complex I of *Chlamydomonas* three. Thus, small protein families occur for this subunit in all plants investigated. All proteins include hexapeptide repeat (PaaY) motifs and originally have been annotated as 'ferripyochelin binding protein-like' (Cardol et al. 2004, Heazlewood et al. 2003) on the basis of sequence similarity with a corresponding protein of *Pseudomonas aeruginosa* (P. Sokol et al., University of Calgary, Canada, unpublished results). However, annotation of this prokaryotic protein was recently corrected. It now is annotated as an unknown PaaY-containing protein similar to CAs/acetyltransferases of the 'isoleucine patch superfamily' (accession number AAG07140).

Assignment of the plant-specific 29-/30-kDa subunits of complex I representing CAs was first suggested by Parisi et al. (2004). Structural modelling of these proteins revealed a left-handed-β-parallel (LβH) conformation. Sequence comparisons including more than a hundred homologous sequences of plants showed highest conservation of these proteins to CAM of *M. thermophila*, which also belongs to the 'isoleucine patch superfamily'. The functionally important amino acids His 81, His 117, His 122 (zinc coordination), as well as Arg 59, Asp 61, Gln 75 and Asp 76 are conserved between CAM and most of the complex I subunits of plants. Two other functionally important residues (Glu 62 and Glu 84 of CAM) are missing, but alternative amino acids were identified that may substitute their roles (Parisi et al. 2004). Accordingly, it was proposed that the novel complex I subunits represent a PaaY-containing family showing characteristics of γCAs. Representatives of the novel family are present in plant mitochondria and bacteria but absent in mammals and fungi. Nowadays, this family is designated gamma CA–like family (accession number 51174). Compared with bacteria, γCAs from plants carry N-terminal extensions, which exhibit the typical properties of mitochondrial targeting sequences.

The *Arabidopsis* γCA protein family is represented by five members. Three of them contain nearly all functionally important amino acids: γCA1 (At1g19580), γCA2 (At1g47260) and γCA3 (At5g66510). The two other members are more divergent proteins: γCAL1 (CAL, carbonic anhydrase like; At5g63510) and γCAL2 (At3g48680). All photosynthetic eukaryotes examined so far contain at least one γCA and one γCAL (Perales et al. 2004).

Localization of the γCA subunits

All five *Arabidopsis* γCA/γCAL subunits were found to be associated with mitochondrial complex I (Heazlewood et al. 2003, Sunderhaus et al. 2006). Mitochondrial localization of the proteins was also shown by in vitro import experiments. Presequences of 3–5 kDa are proteolytically removed after transport in mitochondria is completed (Parisi et al. 2004, Perales et al. 2004). The suborganellar localization of γCA and γCAL proteins was studied by two-dimensional Blue-native/SDS-PAGE in combination with immunoblotting using a polyclonal antibody directed against γCA2, which recognizes all γCA and γCAL proteins (Perales et al. 2004). Immune signals were nearly exclusively found in the 30 kDa range of the vertical rows representing complex I and the $I + III_2$ and $I_2 + III_4$ supercomplexes on the two-dimensional gels. However, some minor immune signals were also visible in the 30-kDa region of smaller protein complexes, which might represent assembly intermediates of complex I or other unknown structures. Further investigations were carried out for the γCA2 protein using an antibody monospecific for this protein (Sunderhaus et al. 2006). γCA2 is exclusively present in the membrane fraction of complex I. Carbonate treatment of isolated mitochondrial membranes did not allow extraction of the protein, indicating a direct anchoring of γCA2 within the inner mitochondrial membrane.

Direct interaction of CA and CAL proteins was shown by the yeast two-hybrid system (Perales et al. 2004). γCA proteins are able to form homodimers, but interaction between γCAL and γCA proteins is stronger than between γCA proteins themselves. In contrast, direct interaction between γCAL proteins could not be monitored using this experimental system. The PaaY domain proved to be absolutely required for stable interaction. Surprisingly, two-hybrid screens using γCA2 allowed to identify only γCAL proteins but neither complex I subunits nor other γCAs. This could be interpreted in favour of a γCA/γCAL complex, which has to be assembled before its association with complex I (Perales et al. 2004).

Localization of γCA proteins within complex I was addressed by Blue native/Blue-native PAGE in combination with mass spectrometry (Sunderhaus et al. 2006). Using this gel system, complex I (1000 kDa) becomes divided into two subcomplexes of 600 and 400 kDa, which represent the membrane and the matrix arm of this complex. Spots representing these subcomplexes were directly cut out of the gel, trypsinated and analysed by mass spectrometry. Four of the five γCA subunits were identified in the 600-kDa membrane arm. None of them were found in the 400-kDa matrix arm (Sunderhaus et al. 2006).

The topological localization of γCA2 within the membrane arm of complex I was investigated by protease protection experiments using isolated mitoplasts (mitochondria lacking the outer mitochondrial membrane).

Comparative immunoblotting analyses of treated and untreated fractions revealed that γCA2 is protease protected to a large extend (Sunderhaus et al. 2006). However, presence of a 28-kDa degradation product of low abundance indicates that a small part of γCA2 might be exposed to the mitochondrial intermembrane space. It thus was concluded that the γCAs from *Arabidopsis* form part of the membrane arm of complex I and that their LβH domains point towards the mitochondrial matrix most likely interacting with each other. The precise subunit arrangement of this so-called CA domain is presently unknown but deserves further investigations. Interestingly, analysis of *Arabidopsis* complex I by single particle electron microscopy revealed a spherical extra domain, which is attached to the central part of the membrane arm on its matrix side (Fig. 1, Dudkina et al. 2005, Sunderhaus et al. 2006). This domain is absent in complex I particles of all other investigated organisms, except for the alga *Polytomella*, which is closely related to *Chlamydomonas*. Thus, presence of CA subunits within complex I correlates with the occurrence of this extra matrix domain, which most likely represents these proteins. The size of this domain would nicely correspond to a γCA/CAL trimer like reported for CAM.

Characterization of *Arabidopsis* γCA knock out mutants

Homozygous *Arabidopsis* knockout mutants carrying a DNA fragment derived from insertion in genes encoding γCA subunits were generated to investigate their physiological role. Separation of mitochondrial protein complexes by Blue-native PAGE or sucrose gradient ultracentrifugation revealed drastically reduced complex I levels in a *γca2* mutant and to a lesser extent in *γca3* mutant (Perales et al. 2005). Furthermore, the mitochondrial I + III_2 supercomplex was very much reduced in *γca2* mutant plants. Remaining complex I had normal molecular mass and also included the spherical extra domain attached to its membrane arm as described above, suggesting substitution of the γCA2 subunit by one of the structurally related subunits of γCA family (Perales et al. 2005, Sunderhaus et al. 2006).

Surprisingly, development of *Arabidopsis γca* mutants was normal under standard growth conditions (Perales et al. 2005). However, a suspension cell culture generated from γca2 mutant plants exhibited clearly reduced growth rates and respiration. Amounts of singular complex I subunits were reduced, suggesting specific protein degradation or downregulation of the corresponding nuclear and mitochondrial genes. Abundances of all other protein complexes and alternative oxidoreductases were largely unchanged between mutant and wild-type cells, except for the formate dehydrogenase complex, which was slightly induced, and adrenodoxin (mitochondrial ferredoxin), which was reduced in protein fractions of mutant cells. In summary, comparative characterization of mitochondrial proteins from wild-type and *γca* cells revealed an important role of γCA2 for complex I assembly.

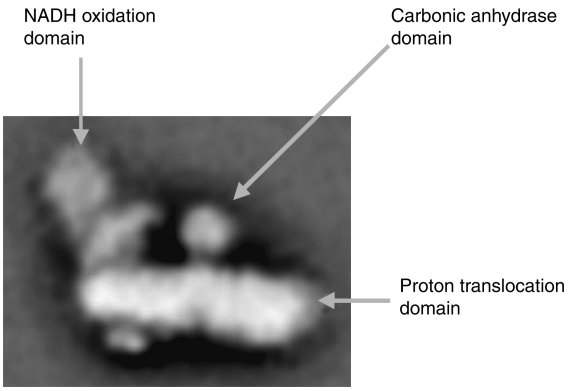

Fig. 1. Structure of complex I from *Arabidopsis* (reproduced from Sunderhaus et al. 2006, with permission). The carbonic anhydrase subunits form a characteristic extra domain, which protrudes into the mitochondrial matrix.

Activity of γCA subunits

Until present, there is no direct physiological evidence of CA activity of complex I in plants. Several efforts to determine this activity have been performed using different biochemical fractions, including purified *Arabidopsis* complex I and γCA proteins overexpressed in *Escherichia coli* (E. Zabaleta and H.-P. Braun, unpublished results). Also, in gel CA activity assays have been carried out using Blue-native gels. Possible reasons for these negative results were discussed previously (Perales et al. 2005). However, CA activity of this group of plant-specific complex I subunits is strongly supported by computer modelling. As summarized above, most of the residues important for catalysis are conserved between the prototype CAM and plant γCAs (Parisi et al. 2004, Perales et al. 2004). Also, antibodies directed against CAM specifically recognize the mitochondrial γCAs (Parisi et al. 2004). On the other side, it currently cannot be ruled out that the γCA/γCAL subunits of complex I are inactive with respect to CA activity and only bind CO_2 and/or bicarbonate in the context of a different physiological process.

Recently, the crystal structure of a protein of the archaea *Pirococcus horikoshii*, which is closely related to CAM, was deposited at the Macromolecular Structure Database of the European Bioinformatic Institute

(Jeyakanthan, J. and Tahirov, T.H., http://www.ebi.ac.uk/msd-srv/msdlite/atlas/summary/1v67.html). The structure includes bound Zn and HCO_3^-. Because all residues of this protein involved in Zn- and HCO_3^- binding are completely conserved in the γCA/γCAL subunits of *Arabidopsis* complex I, they definitely should be able to bind these two ligands.

Electron microscope (EM) analysis of complex I revealed a cavity within the membrane arm of complex I on the intermembrane-exposed side directly in opposite to the location of attachment of the extra spherical domain on the matrix side of the membrane arm (Fig. 1). It therefore was speculated that the complex I–integrated γCAs might not only be involved in CO_2–HCO_3^- interconversion but at the same time catalyse bicarbonate transport across the inner mitochondrial membrane (Sunderhaus et al. 2006). This transport could be driven by the proton gradient across the inner membrane. If complex I indeed would represent a proton-driven bicarbonate translocase, inactivity of the γCA subunits under in vitro conditions could be because of the absence of the proton gradient necessary for catalysis.

Involvement of complex I–integrated γCA/γCAL subunits in CO_2 metabolisms is also supported by transcriptome analyses for *Arabidopsis*. Currently, more than 500 *Arabidopsis* microarray experiments are publicly available at Stanford-Microarray Database (http://genome-www5.stanford.edu/cgi-bin/scriptIndex.pl). Expression of the genes encoding γCA1 or γCA2 is very constant under all physiological conditions tested. However, both genes are clearly repressed (>80%) if *Arabidopsis* was cultivated in the presence of an elevated CO_2 concentration (700 ppm) (Perales et al. 2005). This means that the γCA subunits of complex I could be especially important if the CO_2 concentration is low. Indirect evidence for involvement of γCA2 in mitochondrial one-carbon metabolism also comes from the observation that formate dehydrogenase is upregulated in the *γca2* mutant line. The fact that upregulation of this protein has not been observed in mutants lacking other complex I subunits (Pineau et al. 2005, Sabar et al. 2000) indicates a specific relationship between the plant γCA proteins and one-carbon metabolism.

Possible functional roles of γCAs in plant mitochondria

What could be the physiological role of CAs in mitochondria? Animal cells are known to have several α-type CAs, two of which are localized in mitochondria (CA VA and VB, reviewed in Nishimori et al. 2005). These αCAs are of low abundance. VA was first discovered and only is present in hepatocytes (Dodgson et al. 1984). It is speculated to be involved in maintenance of bicarbonate production for carboxylation reactions of several important biosynthetic pathways, such as lipogenesis, gluconeogenesis and ureagenesis, among others (Dodgson and Forster 1986). VB has a much wider tissue distribution (Shah et al. 2000), suggesting a different physiological role. However, bicarbonate production in mitochondria for the above mentioned biosynthetic pathways most likely is not important in plants, because the pathways do not occur in plant mitochondria. Therefore, the complex I–integrated γCAs of plants must have a different role.

Also in *Chlamydomonas*, two mitochondrial CAs were described (Eriksson et al. 1996). They belong to the βCA family and are distinct from the much later discovered γCAs present within complex I of this organism (Cardol et al. 2004). Both βCAs are encoded by two almost identical nuclear genes, which are expressed in the light at low external CO_2 concentrations (Eriksson et al. 1998, Villand et al. 1997). Like in the case of the complex I–integrated γCAs, activity of the βCAs, which most likely are localized within the mitochondrial matrix, so far could not be monitored. Different hypothesis were suggested concerning their function: (1) CO_2–HCO_3^- interconversion by the βCAs might be important for buffering matrix H^+ concentration upon initiation of photorespiration when cells are transferred from high to low CO_2 conditions (Eriksson et al. 1996). However, it was shown that the βCA genes are also expressed at high CO_2 concentrations [0.2% (v/v) in air], if sufficient NH_4^+ (1–10 m*M*) is available (Giordano et al. 2003). (2) HCO_3^- formation by the mitochondrial βCAs might be important for anaplerotic reactions, which require C_i to build up C4 compounds for the tricarboxylic acid cycle via β-carboxylations (Giordano et al. 2003). The provision of C_i for these reactions could be crucial to sustain amino acid and protein synthesis. (3) HCO_3^- formation possibly is important for limiting loss of CO_2 caused by photorespiration (Raven 2001). Prerequisite for this hypothesis is the presence of a bicarbonate transporter within the mitochondrial membranes, which so far has not been described. In contrast to CO_2, HCO_3^- could be transported actively from mitochondria to the cytoplasm and afterwards into plastids, allowing recycling of excess of mitochondrial CO_2 for carbon fixation by D ribulose 1,5 diphosphate carboxylase-oxygenase. Alternatively it was suggested that the exported HCO_3^- might be used for NH_4^+ fixation (Raven 2001). Indeed, expression of the βCAs from *Chlamydomonas* was shown to be modulated by NH_4^+ supply as well as CO_2 supply (Giordano et al. 2003).

The *Arabidopsis* genome includes several genes encoding proteins of the α- and βCA families (Moroney et al. 2001). Targeting prediction analyses using the

deduced protein sequences does not indicate presence of αCAs within mitochondria. In contrast, one of the six βCAs encoded by the *Arabidopsis* mitochondrial genome (At1g58180) is predicted to have mitochondrial localization using several different targeting prediction software tools. However, this protein never was detected in mitochondrial fractions by proteome analyses and therefore either has a different subcellular location or is of very low abundance. In consequence, the five γCA/γCAL proteins of complex I seem to represent the only mitochondrial CAs in higher plants.

Interesting insights into the functional role of CAs come from investigations with cyanobacteria, which might be helpful to better understand the role of the complex I–integrated CAs in plants. Also in cyanobacteria, involvement of a complex I–like enzyme [termed NAD(P)H dehydrogenase type 1 (NDH-1) complex in cyanobacteria and plastids of higher plants] in carbon dioxide metabolism was reported, which is important in the context of a carbon dioxide concentration mechanism (reviewed by Badger and Price 2003). NDH-1 complexes are known to have multiple functions in both cyanobacteria and higher plants. Common for both groups of organisms is a function of the NDH-1 complexes in respiration (chlororespiration in chloroplasts) and in cyclic electron transport around photosystem I (Munekage et al. 2004). A number of distinct types of NDH-1 complexes were described for β-cyanobacteria, which were found to have different physiological roles: a conventional respiratory NDH-1 complex (NDH-$I_{1/2}$, or NDH-1L and NDH-1M, Battchikova et al. 2005) and two specialized NAD-1 complexes termed NDH-1_3 (also designated NDH-1S) and NDH-1_4 complexes involved in a CO_2 uptake mechanism. The latter two complexes include so-called ChpY/Cup A and ChpX/Cup B polypeptides. The Chp proteins are integral membrane proteins directly catalysing conversion of CO_2 into HCO_3^-, which is linked to electron transfer and proton translocation within the NDH-$1_{3/4}$ complexes (Herranen et al. 2004, Maeda et al. 2002, Zhang et al. 2005). Although Chp proteins do not exhibit sequence homologies to any member of the known CA protein families, two conserved histidine residues and one conserved cysteine residue are believed to allow coordination of Zn (Battchikova et al. 2005). The Chp proteins possibly should be defined as an additional independent class of CAs.

By analogy, the complex I–integrated γCAs of plant mitochondria might also catalyse a proton driven interconversion of CO_2 and HCO_3^- as discussed above. However, in contrast to cyanobacteria, which use these mechanisms to actively increase their internal C_i concentration, plant mitochondria rather have a problem with excess of CO_2 because of catabolic reactions within the mitochondrial matrix, especially during photorespiration. Interestingly, subunits of an NDH-1_3 like complex were recently also described for thylakoid membranes of higher plants, whose abundance increases under conditions of C_i limitation (Herranen et al. 2004, Zhang et al. 2005, reviewed in Badger et al. 2006). However, involvement of these proteins in CO_2–HCO_3^- interconversion has not been shown.

In general, research with cyanobacteria points to a linkage of CO_2 hydration at the plasma membrane and proton translocation across this membrane (Badger and Price 2003, Badger et al. 2006). It was suggested that electron transport–dependent proton translocation across membranes causes formation of local alkaline pockets (Kaplan and Reinbold 1999). Because the equilibrium of CO_2–HCO_3^- interconversion shifts very much to the HCO_3^- side at pH $>$ 8, the alkaline pockets are an ideal microlocation for CAs involved in HCO_3^- generation. This might be the reason for the presence of complex I–integrated CA activities in cyanobacteria and plant mitochondria, which most likely arose by convergent evolution.

Cyanobacteria also contain a protein homologous to γCAs. This protein forms part of the carboxysome and is termed CcmM (product of the *ccmM* gene; ccm, CO_2 concentration mechanisms). It so far was not shown to have CA activity. The protein comprises 539 amino acids and has a bipartite structure, which resembles CAM within its N-terminal and the small subunit of RubisCO within its C-terminal half. Its role in CO_2 metabolism is so far unclear. Disruption of the *ccmM* gene in the cyanobacterium *Synechocystis* sp. PCC 6803 causes carboxysome deficiency and impaired growth at ambient CO_2 conditions (Berry et al. 2005, Ludwig et al. 2000). Physiological characterization of this protein might give new insights into the functional role of the mitochondrial γCA protein family.

In summary, although direct evidence for CA activity of the γCA/γCAL subunits of mitochondrial complex I in plants is still lacking, data from a large number of sources discussed above indicate that these proteins are involved in CO_2 metabolism, most likely represent CAs and possibly also are involved in proton-driven bicarbonate transfer across the inner mitochondrial membrane. Downregulation of the γCA genes upon cultivation of *Arabidopsis* in the presence of elevated CO_2 concentration, which is known to repress photorespiration, points to a role of the γCA subunits in the context of this metabolic pathway. We therefore suggest involvement of the γCA subunits in HCO_3^- formation to allow efficient recycling of mitochondrial C_i for CO_2 fixation in chloroplasts (Fig. 2) in accordance with the hypothesis of Raven (2001).

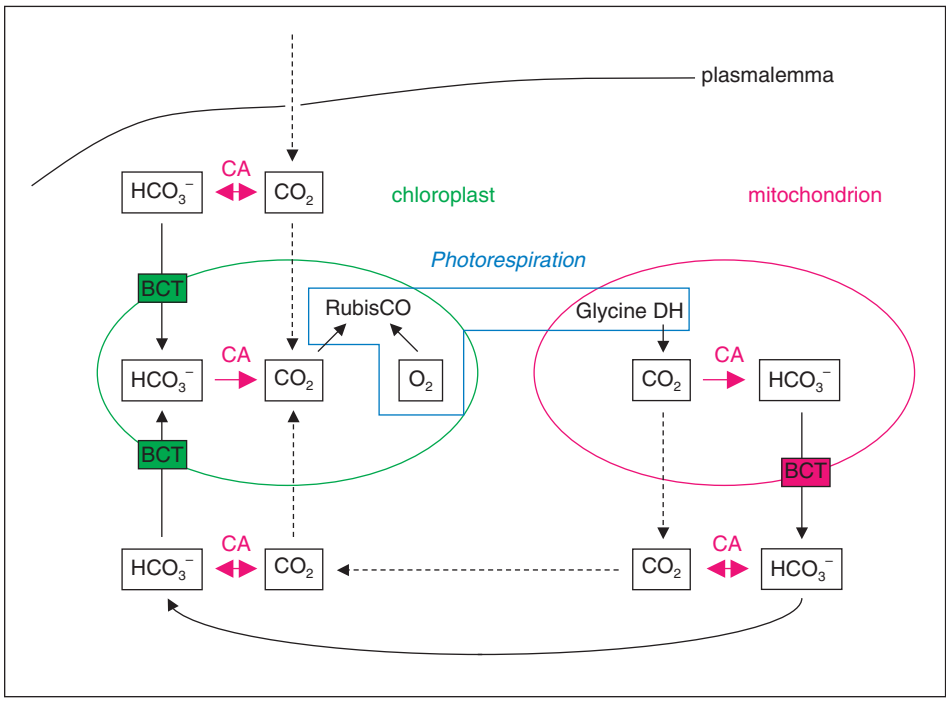

Fig. 2. Model for the function of the complex I–integrated carbonic anhydrases (CAs) in plants. Under high light conditions, CO_2 concentration decreases in chloroplasts because of RubisCO activity. At the same time, CO_2 concentration increases in mitochondria because of the oxygenation side activity of RubisCO in combination with the decarboxylation activity of the mitochondrial glycine dehydrogenase (photorespiration). Equilibration of the CO_2 imbalance between mitochondria and plastids could be based on diffusion of CO_2 across organellar membranes (slow, dashed line) or by active transport of HCO_3^- by bicarbonate transporters (BCTs) (fast, solid line). The mitochondrial CAs are proposed to play a role in the latter mechanisms.

Outlook

So far, many aspects of γCA/γCAL function are still a mystery. Further experiments on physiological and genetic levels will be required to better understand the biological role of this interesting group of proteins. Because homologues of the plant mitochondrial γCA/γCAL proteins are present in cyanobacteria and other eubacteria, gene knock out approaches using prokaryotes might be the fastest way to obtain new insights. But the biological context of the mitochondrial γCAs/γCALs of green algae and higher plants might be different. Knock out experiments using *Arabidopsis* so far did not allow to clarify the physiological role of these proteins, probably because of functional redundancies within this protein family. Characterization of double and triple knock out mutants is on the way in our laboratories. However, presence of five structurally related γCA or γCAL proteins in *Arabidopsis* probably also reflects distinct functional roles of the individual members of this protein family. Expression analyses of the corresponding genes in different tissues and developmental stages will be important to investigate this aspect. Data already available in *Arabidopsis* microarray databases might be helpful in this respect.

The complex I–integrated γCA/γCAL subunits are another fascinating example underlining the uniqueness of plant mitochondria.

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